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Noninvasive diagnostic tool for inflammation-induced oxidative stress using electron spin resonance spectroscopy and an extracellular cyclic hydroxylamine

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Abstract

Inflammation is one of the leading causes of the many pathological states associated with oxidative stress. A crucial role in the development of inflammation-induced oxidative stress is played by reactive oxidant species (ROS), which are very difficult to detect in vivo. One of the most sensitive and definitive methods in the detection of ROS is electron spin resonance, especially as used in conjunction with spin trapping. Unfortunately, the commonly used nitrone spin traps have a very low efficacy for trapping superoxide radicals, and their radical adducts are not stable. To address this deficiency, we have developed negatively charged cyclic hydroxylamines such as 1-hydroxy-4-phosphonooxy-2,2,6,6-tetramethylpiperidine (PP-H) for the detection of reactive oxidant species as a diagnostic tool for extracellular inflammation-induced oxidative stress. We used inflammation induced by a bacterial endotoxin lipopolysaccharide (LPS) as a model. ROS formation was tested in cultured macrophages, in blood and in vivo. PP-H reacts with reactive oxidant species generating the stable nitroxide radical 4-phosphonooxy-TEMPO. It was shown that a 5-h treatment of macrophages with LPS (1 μg/ml) leads to a threefold increase in superoxide formation as demonstrated using superoxide dismutase. Formation of reactive oxidant species 5 h after LPS (1 mg/kg) treatment of Fischer rats was analyzed in arterial blood; formation of reactive oxidant species in LPS-treated animals increased by a factor of 2.2 and was dependent upon the LPS dose. Diphenyleneiodonium (0.1 mM) inhibited formation of LPS-stimulated reactive oxidant species by 80%. We suggest that this test could be used as a noninvasive diagnostic tool for inflammation-induced oxidative stress.

Keywords: Inflammation; Oxidative stress; Reactive oxygen species; Superoxide; Hydroxylamine; Nitroxide; Lipopolysaccharide; Free radicals

Inflammation is a complex response of the immune system to a pathogen [1,2]. Although it plays an important role in the immune defense, it also contributes to the pathogenesis mediated by oxidative stress [1–6]. Immune injury to the kidney, liver, and lung is well documented [3], and the role of macrophages in the pathology of atherosclerosis is extensively characterized [6].

In part, the inflammatory response activates neutrophils, leading to an increase of the NADPH oxidase activity which generates superoxide radicals and hydrogen peroxide [4]. Hydrogen peroxide can be utilized by myeloperoxidase to form hypochlorous acid, a very powerful oxidant [5]. Thus inflammation leads to oxidative stress associated with an increase in the formation of a number of reactive oxidant species (ROS).[2]

In order to develop optimal anti-inflammatory treatment, it is necessary to monitor oxidative stress.

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[2] *Abbreviations used:* DEPMPO, spin-trap 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline *N*-oxide; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; DTPA, diethylenetriaminepentaacetate; ESR, electron spin resonance; LPS, lipopolysaccharide; PP-H, 1-hydroxy-4-phosphonooxy-2,2,6,6-tetramethylpiperidine; PP•, 4-phosphonooxy-2,2,6,6-tetramethyl-piperidinyloxyl; PMA, phorbol 12-myristate 13-acetate; ROS, reactive oxidant species; SOD, superoxide dismutase.

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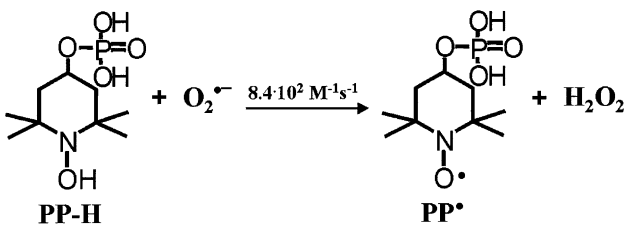
For this purpose the diagnostic tool must be as noninvasive as possible. However, detection of ROS in vivo is very difficult, and unfortunately, most of the available techniques (such as the cytochrome *c* assay) are applicable only in vitro.

One of the most sensitive and definitive methods of oxygen-derived free radical detection is electron spin resonance (ESR). The ESR spin-trapping technique has been used to detect superoxide radicals induced by inflammation via neutrophil NADPH oxidase in cellular systems in vitro [7,8]. However, the commonly used nitrone spin traps have a very low efficacy for trapping of superoxide radicals [9], and the superoxide radical adduct is not stable in the presence of glutathione peroxidase or reducing agents such as ascorbate [10].

Cyclic hydroxylamines were previously found to be effective scavengers of superoxide radicals [11,12]. In particular, the new cyclic hydroxylamine, 1-hydroxy-4-phosphonooxy-2,2,6,6-tetramethylpiperidine (PP-H), reacts with superoxide radical as well as peroxynitrite [13] to produce the stable nitroxide 4-phosphonooxy-2,2,6,6-tetramethyl-piperidinyloxyl (PP$^{\bullet}$). The rate constant for the reaction of PP-H with superoxide is $840 \pm 60\,M^{-1}\,s^{-1}$ (pH 7.4), which is over 100 times greater than the rate constant of nitrone spin traps such as DMPO or 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline *N*-oxide (DEPMPO) [12,14]. The background oxidation of PP-H in blood was minimal. Slow penetration of PP-H into cells limits detection to extracellular superoxide radical. Although PP-H has been successfully used for detection of superoxide radical in vivo [15], it has not been approved for human use. Therefore, we applied PP-H ex vivo in arterial blood in order to assay reactive oxidant species formation in vivo as a noninvasive diagnostic tool. ROS is usually an abbreviation for reactive oxygen species. In this work it stands for reactive oxidant species, because any one-electron oxidant will form PP$^{\bullet}$ from PP-H.

In this work we have tested production of reactive oxidant species in the blood of lipopolysaccharide (LPS)-treated animals using the cyclic hydroxylamine PP-H (Scheme 1) as a potential diagnostic tool for inflammation-induced oxidative stress. Our results show that PP-H was able to quantify at least 10-fold lower superoxide radical formation than the spin trap DEPMPO. This superior sensitivity of the cyclic hydroxylamine PP-H made measurements of inflammation-induced reactive oxidant species possible.



Scheme 1. Chemical reaction of cyclic hydroxylamine PP-H with superoxide radical.

Materials and methods

Alexis Corporation (Switzerland) supplied PP-H and TEMPOL nitroxide. DTPA, LPS, phorbol 12-myristate 13-acetate (PMA), superoxide dismutase from bovine erythrocyte (SOD), superoxide dismutase–polyethylene glycol, diphenyleneiodonium chloride, and xanthine were obtained from Sigma (St. Louis, MO). Xanthine oxidase was purchased from Roche Molecular Biochemicals (Indianapolis, IN). The spin trap DEPMPO was obtained from Oxis (Portland, OR). DEPMPO was stored at −70 °C.

Preparation of PP-H stock solution. PP-H was dissolved in oxygen-free (bubbled for 20 min with argon) 50 mM sodium phosphate buffer (pH 7.4) in the presence of 0.9% NaCl and 1 mM DTPA. DTPA was used to decrease the autooxidation of hydroxylamines catalyzed by trace transition metal ions. A stock solution of PP-H (5 mM) was kept under argon and was prepared daily. The concentration of PP-H was calculated gravimetrically.

ESR experiments. All ESR samples were placed in 50-μl glass capillaries (Corning, Corning, NY) and were prepared using 50 mM sodium phosphate buffer (pH 7.4) with 0.9% NaCl. In order to inhibit iron-catalyzed reactions, DTPA (200 μM) was added to all samples. The ESR spectra were recorded using an Elexsys ESR spectrometer (Bruker) and a super-high Q microwave cavity. The ESR instrumental settings for experiments with xanthine oxidase were as follows: field sweep, 120 G; microwave frequency, 9.78 GHz; microwave power, 20 mW; modulation amplitude, 1 G; conversion time, 83 ms; time constant, 83 ms; receiver gain, 1×10^5 (74 dB); and number of scans, 8. ESR spin-trapping experiments were done at least three times. The ESR instrumental settings for experiments with cells and blood were as follows: field sweep, 50 G; microwave frequency, 9.78 GHz; microwave power, 20 mW; modulation amplitude, 2 G; conversion time, 656 ms; time constant, 656 ms; 512 points resolution and receiver gain, 1×10^5 (74 dB). Kinetics were recorded using a 1312-ms conversion time and a 5248-ms time constant by monitoring the ESR amplitude of the low-field component of the ESR spectrum of PP$^{\bullet}$.

In vivo and in vitro experiments. We used inflammation induced by lipopolysaccharides from *Escherichia coli* 055:B5 in Fischer male rats (300–400 g) as an in vivo model. Nonfasted rats were anesthetized by ip injection of 50 mg/kg body wt Nembutal. Arterial blood was drawn from the carotid artery of the rat into a Vacutainer with heparin.

Mouse macrophages (RAW264.7) were used for in vitro experiments. In ESR experiments, macrophages (10,000 cells/μl) were suspended in 50 mM phosphate buffer with 0.9% NaCl, pH 7.4.

Superoxide radical generation. The xanthine oxidase superoxide generating system [16] contained xanthine oxidase, xanthine (0.5 mM), and DTPA (0.2 mM) in 50 mM sodium phosphate buffer (pH 7.4) in the presence of 0.9% NaCl. The rate of superoxide radical generation was determined by the cytochrome *c* assay [17].

Determination of rates of ROS formation. The rate of superoxide radical formation in a xanthine/xanthine oxidase system was determined in the presence of PP-H by measuring the SOD- (100 U/ml) inhibited formation of nitroxide radicals. The amount of PP$^{\bullet}$ formed was calculated from a calibration curve for the double integral of ESR spectra of the nitroxide TEMPOL. The rate of nitroxide formation was compared with cytochrome *c* reduction.

As formation of reactive oxidant species in vivo and ex vivo is not limited to superoxide, SOD does not always inhibit the formation of PP$^{\bullet}$. Total reactive oxidant species formation in blood was determined from the time-dependent accumulation of the stable nitroxide radical PP$^{\bullet}$ [13,15]. The rate of reactive oxidant species formation ex vivo in arterial blood was determined from the rate of the oxidation of the hydroxylamine PP-H (0.5 mM) to PP$^{\bullet}$. The rate of PP$^{\bullet}$ formation was measured from the ESR kinetics by monitoring the amplitude of the low-field component of the ESR spectrum. The concentration of PP$^{\bullet}$ was determined from a calibration curve for the double integral of ESR spectra of the nitroxide TEMPOL. Because PP$^{\bullet}$ is very resistant to reduction by ascorbate [18], we were able to monitor the accumulation of PP$^{\bullet}$ in whole blood without decay of the ESR signal.

Statistics. All values are expressed as means ± SD. Statistical significance was determined by Student's *t* test for paired data. Two groups of data were considered to be significantly different at $P < 0.05$.

Results

Quantification of superoxide radical

We have shown previously that the cyclic hydroxylamine PP-H reacts with superoxide radical to form the stable nitroxide radical PP$^{\bullet}$ [13], which can be assayed by ESR spectroscopy. Cyclic hydroxylamines are very effective scavengers of superoxide radicals [12,13,15] and can be used to quantify superoxide formation (Scheme 1).

The quantity of trapped superoxide radical was calculated from the ESR spectrum of the probe (Fig. 1). The background ESR signal of PP-H was relatively insignificant (Fig. 1A). A strong ESR signal of PP$^{\bullet}$ formed by superoxide generated by xanthine/xanthine oxidase was detected (Fig. 1B), and its concentration was determined from the intensity of the signal (*I*); the concentration of PP$^{\bullet}$ equals the concentration of the reacted oxidant species. The signal decreased in the presence of SOD, demonstrating that the PP$^{\bullet}$ was formed by superoxide radical (Figs. 1B and C) and not by some other one-electron oxidant.

The formation of superoxide radical was also calculated from the kinetics of nitroxide accumulation (Figs. 1D–F) by monitoring the intensity of the low-field component of the nitroxide ESR spectrum (Fig. 1B). The control sample showed little accumulation of PP$^{\bullet}$ (Fig. 1D), while superoxide generation caused a sharp increase in the rate of PP$^{\bullet}$ generation (Fig. 1E). The accumulation of PP$^{\bullet}$ was inhibited by SOD (Fig. 1F), confirming that this PP$^{\bullet}$ was dependent upon superoxide formation.

Comparison of superoxide trapping by DEPMPO and PP-H

Because the superoxide radical is often detected in vitro using nitrone spin traps such as DEPMPO [11,14], we compared the sensitivity of the spin-trap DEPMPO and the cyclic hydroxylamine PP-H using a xanthine oxidase system (Fig. 2). At a rate of superoxide radical formation of 20 nM/min, the ESR spectrum of DEPMPO/$^{\bullet}$OOH was barely recognizable and could not be quantified (Fig. 2B). At the same rate, incubation of PP-H gave rise to a strong PP$^{\bullet}$ signal (Fig. 2D).

At a superoxide production rate of 200 nM/min, we were able to detect the characteristic ESR spectrum of the DEPMPO superoxide radical adduct (Fig. 2A). However, 3 min after the initiation of superoxide radical formation the concentration of DEPMPO/$^{\bullet}$OOH became constant, reflecting the achievement of the steady-state condition. Thus under these conditions the lifetime of DEPMPO/$^{\bullet}$OOH is much less than 3 min, and the rate of superoxide radical formation could not be directly calculated from the ESR spectrum of DEPMPO/$^{\bullet}$OOH even if the ESR spectrum was readily detected. We concluded that at low rates of superoxide radical formation, the cyclic hydroxylamine PP-H has a superior sensitivity for the detection of superoxide radicals (Figs. 2B and D) and can be used to quantify superoxide radicals (Fig. 2E).

With the same xanthine/xanthine oxidase superoxide generating system, we examined superoxide radical detection with PP-H using both ESR spectra and ESR kinetic measurements (Figs. 2C–E). The stock solution of PP-H contained a weak background signal of PP$^{\bullet}$ (Fig. 2C). The formation of superoxide radical could be readily determined by the rate of accumulation of the PP$^{\bullet}$ monitored by the ESR intensity of the low-field component (Fig. 2E). Under the same conditions the

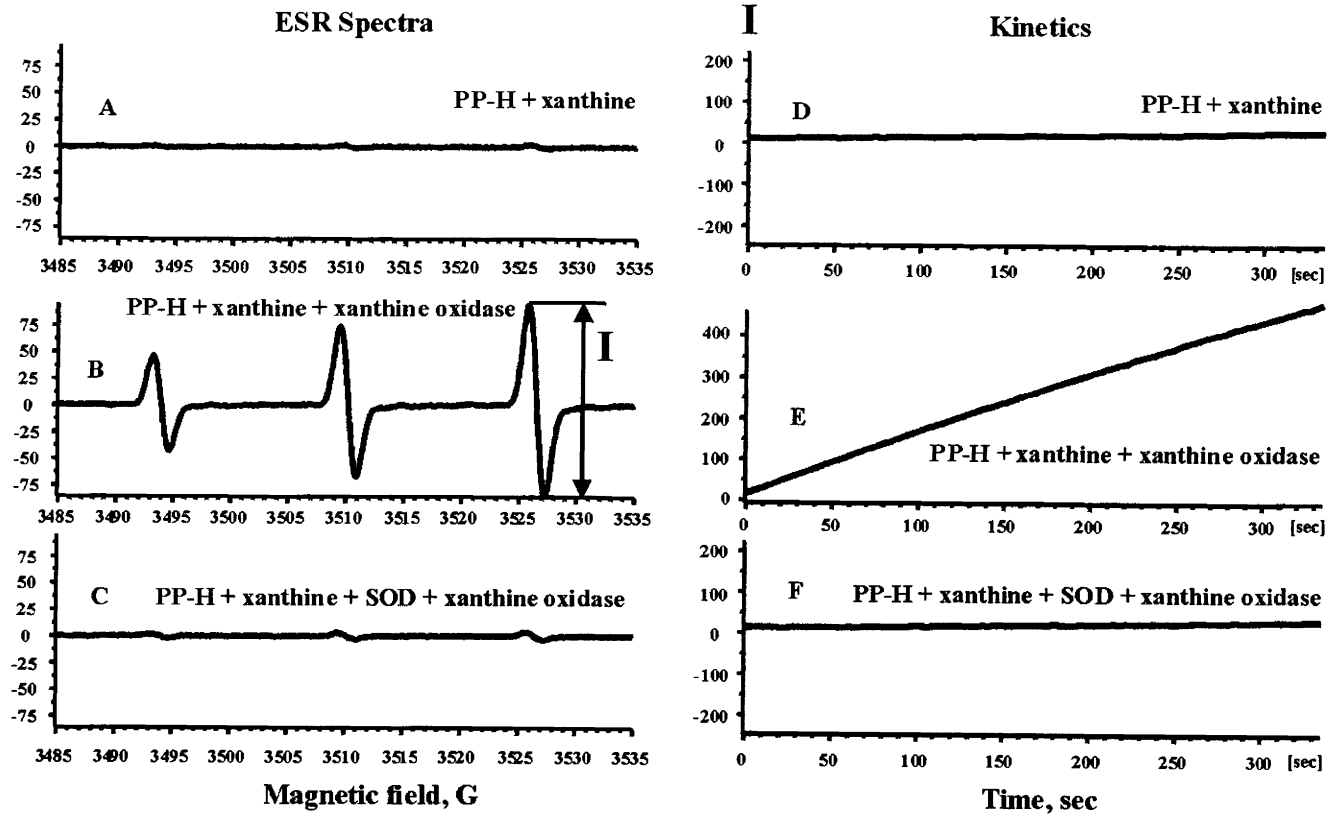


Fig. 1. ESR detection of superoxide radical using the extracellular cyclic hydroxylamine PP-H. (A) ESR spectrum of 0.5 mM PP-H and 0.5 mM xanthine. (B) ESR spectrum of PP$^{\bullet}$ in a xanthine oxidase/xanthine system generating superoxide radical at a rate of 50 nM/min. (C) ESR spectrum of PP$^{\bullet}$ in a xanthine oxidase/xanthine system generating superoxide radical at a rate of 50 nM/min in the presence of 100 U/ml superoxide dismutase. (D) Kinetics of PP$^{\bullet}$ accumulation in sample with 0.5 mM PP-H and 0.5 mM xanthine. (E) Kinetics of PP$^{\bullet}$ accumulation in a xanthine oxidase/xanthine system generating superoxide radical at a rate of 50 nM/min. (F) Kinetics of PP$^{\bullet}$ accumulation in a xanthine oxidase/xanthine system generating superoxide radical at a rate of 50 nM/min in the presence of 100 U/ml superoxide dismutase. ESR spectra were obtained with a single scan of 334 s. "*I*" is the intensity of the ESR spectrum in arbitrary units as seen in WINEPR program (Bruker). ESR settings are described under Materials and methods.

formation of PP$^{\bullet}$ was completely inhibited by SOD (data not shown).

High sensitivity of PP-H for the detection of superoxide radicals is not exclusive to this cyclic hydroxylamine; two other cyclic hydroxylamines, 1-hydroxy-4-oxo-2,2,6,6-tetramethylpiperidine and 1-hydroxy-3-carboxy-2,2,5-tetramethyl-pyrrolidine [12,13], also showed high sensitivity for the detection of superoxide radicals (data not shown). Since PP-H exhibited less background oxidation in blood, it was selected for measurements of reactive oxidant species.

Detection of superoxide radical in cultured macrophages stimulated by PMA and LPS

The cyclic hydroxylamine PP-H has previously been used to assay extracellular ROS in whole blood [13]. We tested our assay in vitro in a suspension of macrophages and in rat blood using inflammation induced by LPS or phorbol PMA.

RAW264.7 macrophages treated with PMA showed extensive superoxide radical formation (Fig. 3). Treatment of macrophages with PMA for 20 min at 37 °C led to a 2.5-fold increase in PP$^{\bullet}$ content (Fig. 3B) compared with untreated macrophages (Fig. 3A). This formation of PP$^{\bullet}$ was inhibited by superoxide dismutase (Fig. 3C). Incubation of macrophages with PMA at room temperature also showed increased superoxide formation (Fig. 3E). Kinetic measurements at room temperature (Fig. 3G) were consistent with ESR spectral results: macrophages stimulated with PMA exhibited strong generation of superoxide radicals, which was inhibited in the presence of extracellular superoxide dismutase.

Acute treatment of macrophages with LPS (1 μg/ml) also strongly increased superoxide formation (Figs. 4A and B), which was inhibited in the presence of superoxide dismutase (Fig. 4C). It was found that a 5-h pretreatment of cultured macrophages with LPS (1 μg/ml) led to a threefold increase in nitroxide formation (data not shown). Kinetic measurements also revealed significant formation of superoxide by macrophages stimulated with LPS (Fig. 4D). It was found that the in vitro treatment of blood from a control animal with 5 μg/mg LPS led to a twofold increase in the formation of PP$^{\bullet}$.

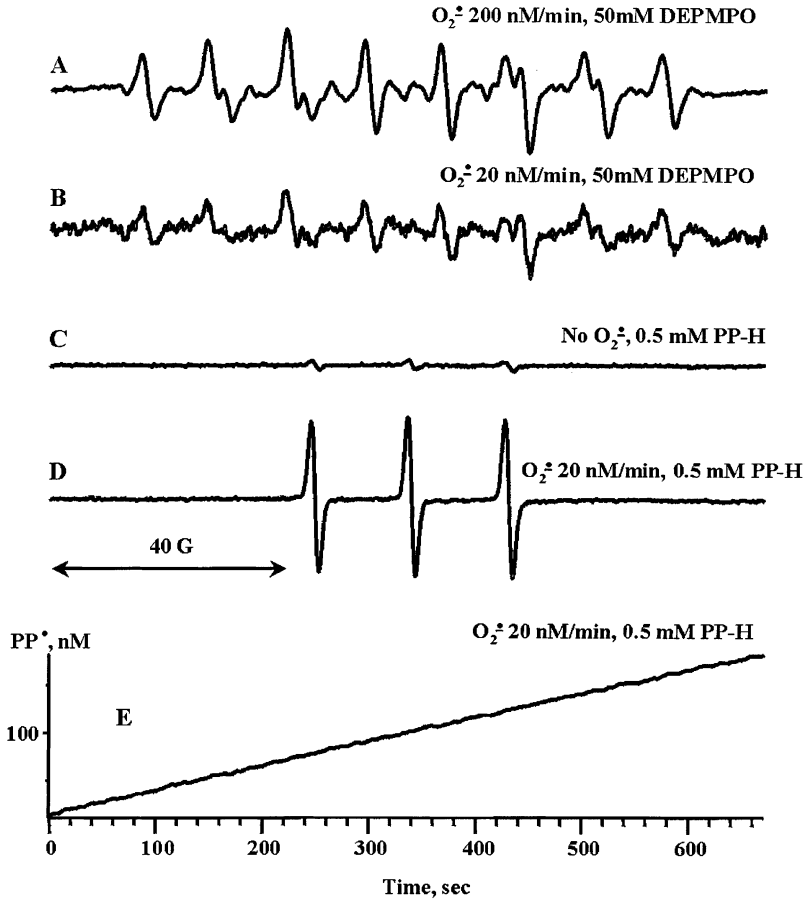


Fig. 2. Detection of superoxide radical generated by xanthine oxidase. (A) ESR spectrum of DEPMPO/·OOH radical adduct in a xanthine oxidase/xanthine system generating superoxide radical at a rate of 200 nM/min. (B) ESR spectrum of DEPMPO/·OOH radical adduct in a xanthine oxidase/xanthine system generating superoxide radical at a rate of 20 nM/min. (C) ESR spectrum of 0.5 mM PP-H. (D) ESR spectrum of PP· in a xanthine oxidase/xanthine system generating superoxide radical at a rate of 20 nM/min. (E) Kinetics of PP· accumulation in a xanthine oxidase/xanthine system generating superoxide radical at a rate of 20 nM/min. ESR spectra A–D were obtained by accumulation of eight scans of 83 s each. The rate of superoxide production was calculated by SOD-inhibited cytochrome *c* reduction at higher activity of xanthine oxidase (10 mU/ml was equivalent to 450 nM/min superoxide production). ESR settings are described under Materials and methods.

Detection of reactive oxidant species in the blood of control or LPS-treated rats

Although in vivo ROS formation has been previously assayed using the cyclic hydroxylamine PP-H [15], PP-H has not been approved for use in humans. Therefore, we explored a noninvasive ex vivo approach for the detection of reactive oxidant species formation in arterial blood. The formation of reactive oxidant species was determined by the kinetics of PP· formation in arterial blood at room temperature expressed as the rate of nitroxide formation. Analysis of control animals revealed slow nitroxide formation of unknown origin (Fig. 5, control). It is important to note that the nitroxide formation in the blood of control animals was extremely reproducible with very minor deviation.

Five hours after administration of LPS (1 mg/kg) to Fischer rats, formation of PP· increased 2.2-fold ($P < 0.05$) over that of blood from control animals (Fig. 5). The formation of reactive oxidant species increased with the dose of injected LPS (Fig. 5) and reached a maximum (4.4-fold higher than in the control) in the animals treated with 20 mg/kg LPS (Fig. 5). An attempt was made to determine the origin of this reactive oxidant species detected by PP-H. Formation of PP· in the blood plasma of LPS-treated rats was not significantly different from that of the control rate. Addition of an inhibitor of flavin-containing enzymes, diphenyleneiodonium (0.1 mM), to the blood inhibited formation of LPS-stimulated reactive oxidant species by 80% (data not shown). Ex vivo addition of polyethylene glycol-conjugated SOD (200 U/ml) inhibited the formation of PP· by 40% (data not shown). These data show that the origin of the LPS-stimulated reactive oxidant species is flavin-containing enzymes, most probably primarily a phagocytic NADPH oxidase. The effect of SOD supports the role of phagocytic NADPH oxidase, which is a major source of superoxide radical in the activated phagocytes.

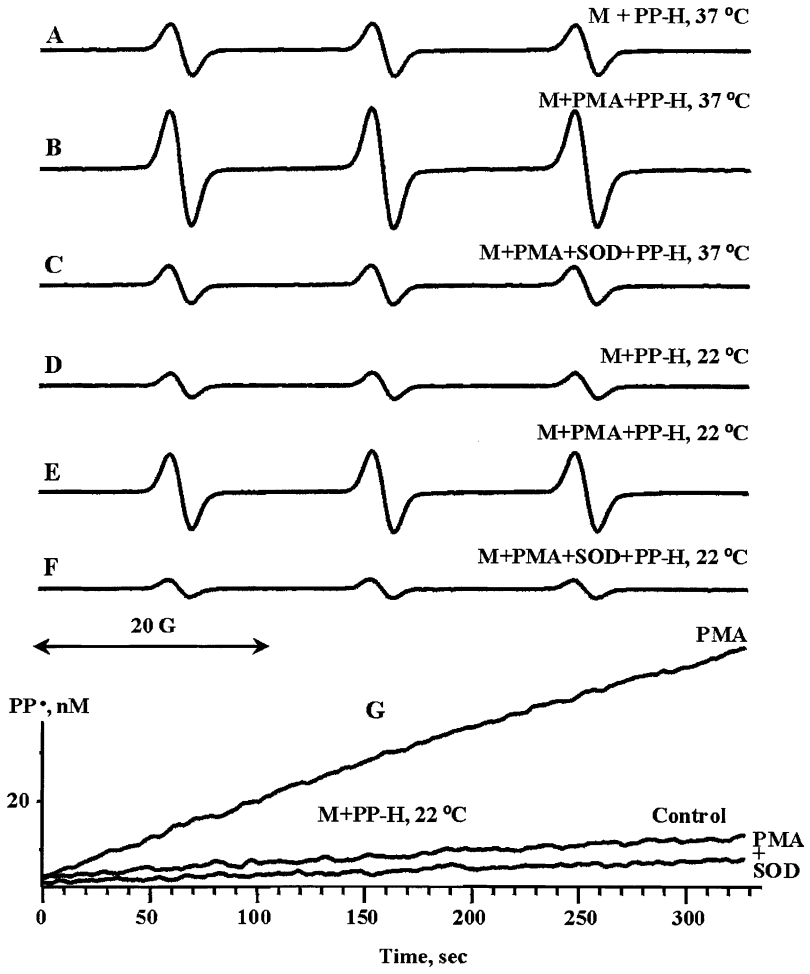


Fig. 3. Detection of superoxide radical in cultured macrophages (M) stimulated with PMA. (A) ESR spectrum of a suspension of macrophages and PP-H incubated for 20 min at 37 °C. (B) ESR spectrum of a suspension of macrophages stimulated with PMA in the presence of PP-H incubated for 20 min at 37 °C. (C) ESR spectrum of a suspension of macrophages stimulated with PMA in the presence of superoxide dismutase and PP-H incubated for 20 min at 37 °C. (D) ESR spectrum of a suspension of macrophages and PP-H incubated for 20 min at 22 °C. (E) ESR spectrum of a suspension of macrophages stimulated with PMA in the presence of PP-H incubated for 20 min at 22 °C. (F) ESR spectrum of a suspension of macrophages stimulated with PMA in the presence of superoxide dismutase and PP-H incubated for 20 min at 22 °C. (G) Kinetics of PP• accumulation at 22 °C in samples with control macrophages and macrophages stimulated with PMA with and without 100 U/ml superoxide dismutase.

It has been previously reported that both cyclic hydroxylamines and their corresponding nitroxides react with superoxide. Moreover, the rate constant of the reaction of superoxide with the corresponding nitroxide is expected to be much higher than that with the parent PP-H, resulting in the catalytic removal of superoxide through the SOD activity of nitroxides [19]. In order to determine the effect of PP• on this assay, we compared the detection of superoxide radical in a xanthine oxidase system at various PP•/PP-H ratios from 0.02 to 2%, but found no effect on the formation of PP•. Only when the PP• was 10% of PP-H did we find a 15% decrease in the amount of detected superoxide. SOD completely inhibited nitroxide formation at any PP•/PP-H ratio (data not shown). In our experiments with blood the content of PP• did not exceed 0.1%. Thus, the competition between PP-H and PP• does not limit the validity of the assay.

The fact that high concentrations of PP• did not affect the detection of superoxide as measured by the accumulation of PP• may be explained by the presence of a very fast reaction between the oxoammonium cation PP^+ and PP-H.

$$PP\text{--}H + O_2^{\cdot-} + H^+ \rightarrow PP^{\cdot} + H_2O_2$$

$$PP^{\cdot} + O_2^{\cdot-} + 2H^+ \rightarrow PP^+ + H_2O_2$$

$$PP^+ + PP\text{--}H \rightarrow 2PP^{\cdot} + H^+.$$

The last reaction should be very fast with a rate constant close to diffusion-limited. This may explain why even at high PP• concentrations we did not find evidence of competition between PP-H and PP• for superoxide radical.

In this work we used PP-H stock solutions with 0.02% of PP•. The maximum PP• content in the blood

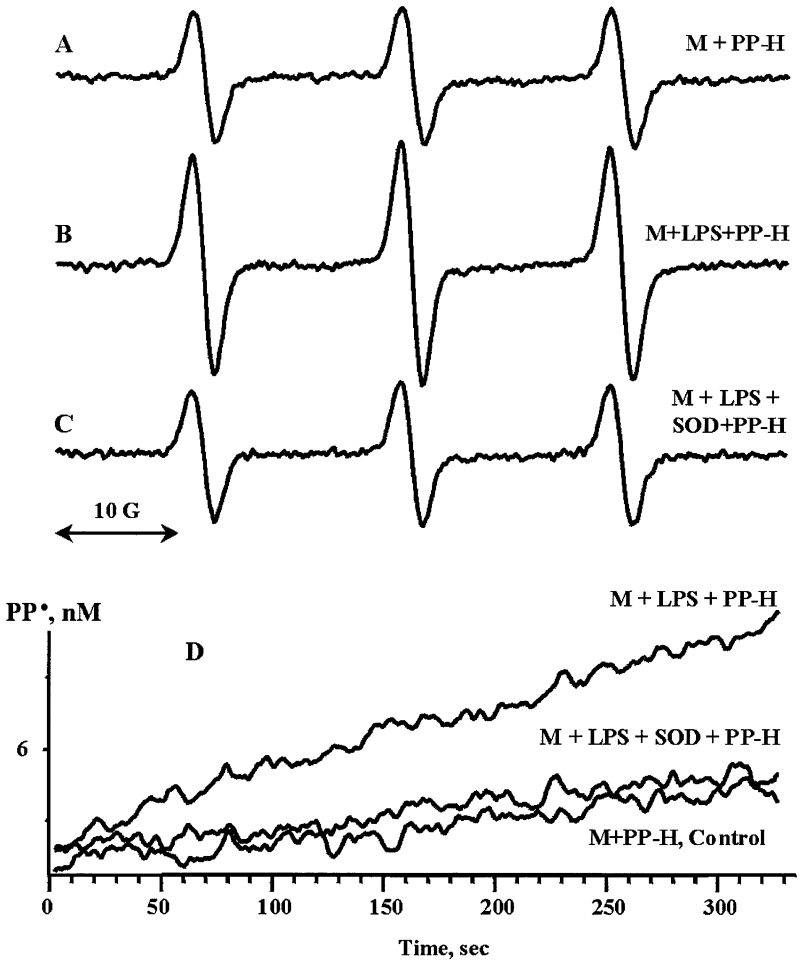


Fig. 4. Detection of superoxide radical in cultured macrophages (M) stimulated with LPS. (A) ESR spectrum of a suspension of macrophages plus PP-H. (B) ESR spectrum of a suspension of macrophages stimulated with LPS in the presence of PP-H. (C) ESR spectrum of a suspension of macrophages stimulated with LPS in the presence of superoxide dismutase and PP-H. (D) Kinetics of PP• accumulation in samples with control macrophages and macrophages stimulated with LPS with and without superoxide dismutase.

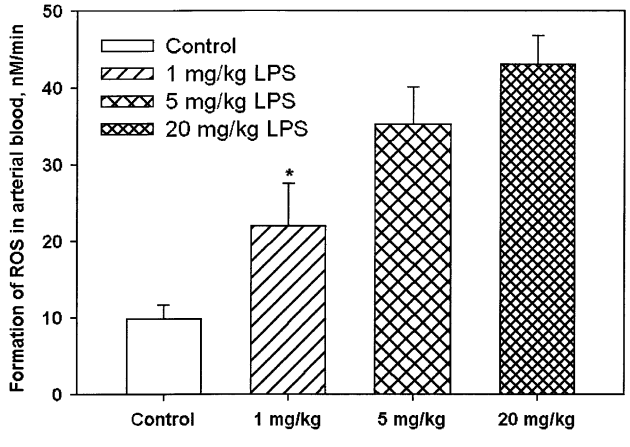


Fig. 5. Detection of reactive oxidant species in the blood of control and LPS-treated rats. Detection of reactive oxidant species in the blood of control or LPS-treated rats measured in whole blood (80%) as the rate of 4-phosphonooxy-TEMPO formation using PP-H. Standard deviations for columns are shown (*$P < 0.05$ vs control).

samples was not higher than 0.2%. The presence of this amount of PP• did not affect the measurement of reactive oxidant species in blood (Figs. 6A and B).

Discussion

Despite the in vitro evidence of the involvement of ROS in inflammatory injury, a noninvasive diagnostic tool for ROS detection in vivo is yet to be developed. An assay for ROS formation is of particular importance because of the paradoxical role of ROS in the inflammation process, exerting either a positive or a negative effect on the inflammatory response with age [20].

Previously, nitrone spin traps have been used to analyze the in vitro formation of oxygen radicals generated by inflammation [21–23]. However, nitrone spin traps have not been used to quantify reactive oxidant species in blood, presumably because of the low efficacy of trapping of superoxide radicals, the reduction of radical

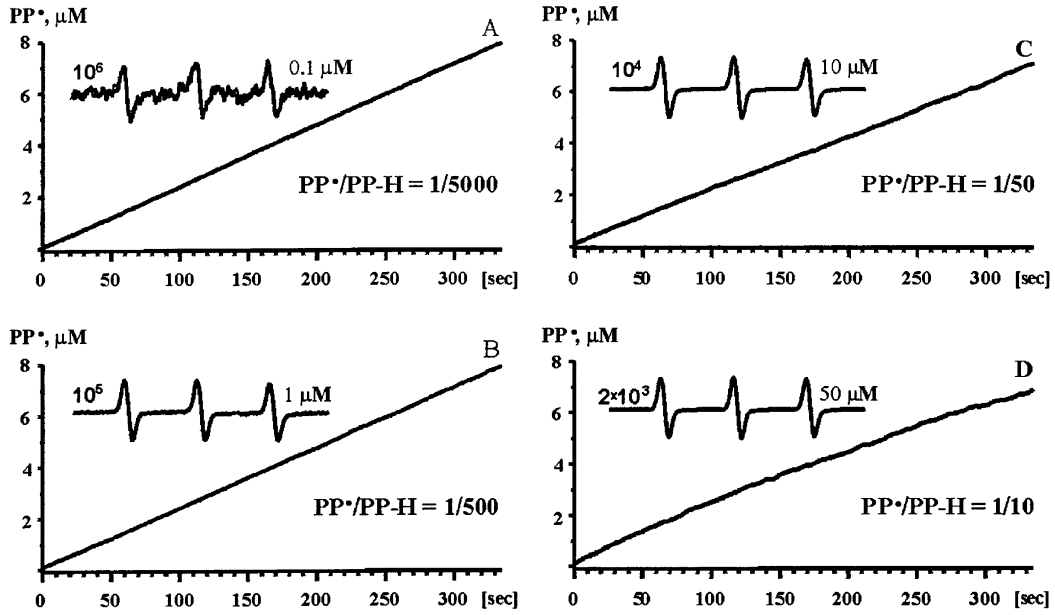


Fig. 6. Quantification of superoxide radical generated by xanthine oxidase at different concentrations of PP•. (A) (Inset) ESR spectrum of the initial level of PP• (0.1 μM). Time scan shows kinetics of PP• accumulation with an initial PP•/PP-H ratio of 1:5000. (B) (Inset) ESR spectrum of the initial level of PP• (1 μM). Time scan shows kinetics of PP• accumulation with an initial PP•/PP-H ratio of 1:500. (C) (Inset) ESR spectrum of the initial level of PP• (10 μM). Time scan shows kinetics of PP• accumulation in a xanthine oxidase/xanthine in probe with an initial PP•/PP-H ratio of 1:50. (D) (Inset) ESR spectrum of the initial level of PP• (50 μM). Time scan shows kinetics of PP• accumulation with an initial PP•/PP-H ratio of 1:10. All experiments (A–D) had the same activity of xanthine oxidase (1 mU/ml) and concentrations of xanthine (0.1 mM) and PP-H (0.5 mM).

adducts, and the presence of extracellular superoxide dismutase. Recently, cyclic hydroxylamine PP-H was found to be an effective scavenger of superoxide radicals both in vitro and in vivo [13,15]. PP-H reacts with reactive oxidant species to form PP•, which is stable in blood [15].

In this work we have shown that the cyclic hydroxylamine PP-H can be used to assay formation of reactive oxidant species resulting from inflammation-induced oxidative stress. Our results show that PP-H could be used to detect a 10-fold lower superoxide radical formation rate than the spin-trap DEPMPO, thereby providing superior sensitivity for quantification of superoxide radical in vitro. Moreover, we found that the use of ESR detection of PP• to measure the inflammation-induced formation of reactive oxidant species in the blood was a very sensitive and reproducible method.

In this work we clearly demonstrated the formation of superoxide radical by macrophages stimulated with PMA or LPS. In LPS-treated animals ROS formation is thought to be catalyzed by NADPH oxidases [4]. The importance of NADPH oxidases as the main source of extracellular ROS in LPS-treated animals was supported by the inhibition of PP• formation by the ex vivo addition of polyethylene-glycol-conjugated superoxide dismutase or diphenyleneiodium, although it is possible that a number of secondary reactive oxidant species are involved in the oxidation of PP-H.

The superior sensitivity of PP-H over DEPMPO for superoxide detection provides an important technique to assay superoxide and other reactive oxidant species under inflammation-induced oxidative stress. The method described can be successfully used both in vitro and in vivo for reactive oxidant species detection. We suggest that this test can be used as a noninvasive diagnostic tool for inflammation-induced oxidative stress.

Acknowledgments

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