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Original Contribution

A NEW APPROACH FOR EXTRACELLULAR SPIN TRAPPING OF NITROGLYCERIN-INDUCED SUPEROXIDE RADICALS BOTH IN VITRO AND IN VIVO

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Abstract—Anti-ischemic therapy with nitrates is complicated by the induction of tolerance that potentially results from an unwanted coproduction of superoxide radicals. Therefore, we analyzed the localization of in vitro and in vivo, glyceryl trinitrate (GTN)–induced formation of superoxide radicals and the effect of the antioxidant vitamin C and of superoxide dismutase (SOD). Sterically hindered hydroxylamines 1-hydroxy-3-carboxy-2,2,5,5-tetramethylpyrrolidine (CP-H) and 1-hydroxy-4-phosphonooxy-2,2,6,6-tetramethylpiperidin (PP-H) can be used for in vitro and in vivo quantification of superoxide radical formation. The penetration/incorporation of CP-H or PP-H and of their corresponding nitroxyl radicals was examined by fractionation of the blood and blood cells during a 1-h incubation. For monitoring in vivo, GTN-induced (130 μg/kg) $O_2^{\bullet -}$ formation CP-H or PP-H were continuously infused (actual concentration, 800 μM) for 90 to 120 min into rabbits. Formation of superoxide was determined by SOD- or vitamin C–inhibited contents of nitroxide radicals in the blood from *A. carotis*. The incubation of whole blood with CP-H, PP-H, or corresponding nitroxyl radicals clearly shows that during a 1-h incubation, as much as 8.3% of CP-H but only 0.9% of PP-H is incorporated in cytoplasm. Acute GTN treatment of whole blood and in vivo bolus infusion significantly increased superoxide radical formation as much as 4-fold. Pretreatment with 20 mg/kg vitamin C or 15,000 U/kg superoxide dismutase prevented GTN-induced nitroxide formation. The decrease of trapped radicals after treatment with extracellularly added superoxide dismutase or vitamin C leads to the conclusion that GTN increases the amount of extracellular superoxide radicals both in vitro and in vivo. © 2000 Elsevier Science Inc.

Keywords—Reactive oxygen species, Electron spin resonance, Superoxide dismutase, Vitamin C, Hydroxylamine penetration, Rabbits, Free radicals

INTRODUCTION

Recent results have shown that development of tolerance to nitrates is induced primarily by the enhanced, nitrate-induced formation of reactive oxygen species and secondarily by the enhanced reduced form of nicotinamide-adenine dinucleotide (NADH) oxidase activity and continuous $O_2^{\bullet -}$ production and release from the blood and the vascular cells [1–3]. At the same time, development of nitrate tolerance is a multifactorial phenomenon that, in addition, includes both neurohormonal counterregulation and enhanced responses to vasoconstrictor responses [3,4].

Despite many hypotheses and investigations, the exact mechanisms of nitrate tolerance have not completely understood [5–10]. It was assumed that release of nitric oxide (NO) during the metabolism of nitrates is affected by a considerable coproduction of superoxide radicals in vessels, thus leading to inactivation of NO. In turn, this may lead to a decrease of intra- and/or extracellular low-molecular-weight thiols [11–13], to diminished cyclic guanosine monophosphate (cGMP) production in smooth muscle cells and in platelets [10,14,15] and to impaired vasomotor responses to the endothelium-derived relaxant factor [16].

Nitrate-induced superoxide radicals react with NO to form peroxynitrite [17,18]. Peroxynitrite affects the activity of important enzymes through irreversible oxidation of SH groups, including soluble guanylyl cyclase. This NO-mediated increase in the activity of soluble

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guanylyl cyclase depends decisively on the redox state of the SH groups [19,20]. Peroxynitrite causes vascular dysfunction in isolated hearts [21,22]. The vasodilator efficacy of organic nitrates in vitro is improved when additional superoxide dismutase (SOD) activity is supplied [23]. Therefore, development of nitrate tolerance can result from both superoxide- and peroxynitrite-mediated oxidative damage. In turn, the efficacy of organic nitrates as exogenous NO donors and promoters of cGMP-dependent vasodilatation depends on the balance between the concentrations of NO formed and the rate of nitrate-induced formation of superoxide radicals in the vasculature.

Recently, using electron-spin resonance (ESR) spectroscopy and cyclic hydroxylamines as spin traps, we have shown that glyceryl trinitrate (GTN) induces reactive oxygen species (e.g.; $O_2^{\bullet -}$ and $ONOO^-$) formation both in vitro and in vivo [1,24,25]. The mechanisms of this increase in superoxide radical formation, however, remain unclear. The aim of this work was to analyze both in vivo and in vitro GTN-induced superoxide radical formation and to study the modulating effect of the antioxidant vitamin C and of superoxide dismutase using ESR spectroscopy.

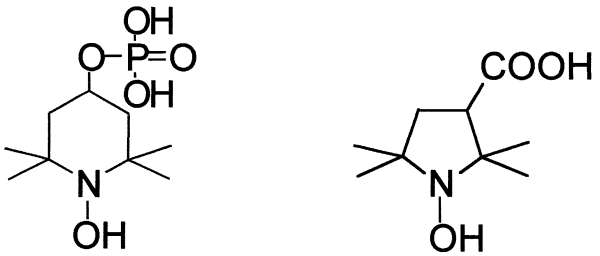
MATERIALS AND METHODS

Spin trapping of superoxide radicals

Using an $O_2^{\bullet -}$-generating system (50 μM xanthine + 0.01 U/ml xanthine oxidase), we examined the capacity of hydroxylamines 1-hydroxy-3-carboxy-2,2,5,5-tetramethylpyrrolidine (CP-H) and 1-hydroxy-4-phosphonooxy-2,2,6,6-tetramethylpiperidin (PP-H) to trap $O_2^{\bullet -}$ along with the formation of 3-carboxy-proxyl (CP) or 4-phosphonooxy-TEMPO-radicals (PP). Analysis of the radical formation was performed using phosphate buffer (50 mM; pH 7.4) both with and without 100 U/ml superoxide dismutase. The transition metal–catalyzed oxidation of CP-H and PP-H was suppressed by the addition of 20 μM deferoxamine as a chelating agent.

Analysis of incorporation or intracellular penetration of CP-H and PP-H

The location of the spin traps was examined after incubation of CP-H or PP-H (800 μM) in whole blood of rabbits with subsequent fractionation. After a 1-h incubation at 37° C with periodic swiveling (5 times/min), blood was centrifuged for 5 min at 500 *g*. The plasma was carefully decanted at a safe distance from the cell sediment. After washing twice, the cell sediment was resuspended in 5 ml of phosphate buffer (50 mM) and centrifuged for 5 min at 500 *g*, after which the supernatant was decanted and discarded. The cells were then ultrasonically destroyed (3 × 5 s at 100 W; Labsonick, B. Braun, Melsungen, Germany). Next, the membranes and cytosolic fractions were separated [26]. Finally, using ESR spectrometry, the fractions (plasma, cell membranes, cytosol) were analyzed after oxidation of CP-H and PP-H with 10 mM KO_2.



$$\text{PP-H} + O_2^{\bullet -} \rightarrow \text{PP}^{\bullet} + H_2O_2 \qquad \text{CP-H} + O_2^{\bullet -} \rightarrow \text{CP}^{\bullet} + H_2O_2$$

Fig. 1. Chemical structures of PP-H and CP-H.

Rabbit experiments

Twelve adult rabbits of either sex (body weight, 1.2–1.5 kg) were anesthetized using sodium pentobarbital (25 mg/kg). A silicone catheter was then implanted into the carotid artery, and 2 h infusions using five different protocols were performed randomly in a consecutive manner: (i) 130 μg/kg GTN; (ii) 15,000 units of SOD per 1 kg and, 20 min later, 130 μg/kg GTN, (iii) 30 μg/kg vitamin C and, 20 min later, 130 μg/kg GTN, (iv) 300 μg/kg adenosine; and (v) saline at identical infusion volumes. In these 2 h infusion during experiments with CP-H, 9 mg/kg CP-H was initially used for bolus infusions, and then the concentrations of spin-trap CP-H in blood were maintained constant using continuous infusion of CP-H (0.225 μg/kg/min). In experiments with PP-H, 12 mg/kg PP-H was initially used for bolus infusions, and then the concentrations of spin trap PP-H in blood were maintained constant using continuous infusions of PP-H (0.30 μg/kg/min). These spin trap amounts guaranteed complete trapping under basal conditions. Five minutes after each specific spin-trap infusion, 200 μl of blood were obtained from *A. carotis* to determine the CP or PP formation. The amount of superoxide radicals trapped in rabbits was quantified as CP or PP formation.

ESR measurements

The cyclic hydroxylamines CP-H and PP-H were used for quantification of superoxide radical formation (Fig. 1). During the reaction of PP-H or CP-H with the super-

oxide radical, stable and reductant-resistant nitroxide radicals [27] were formed (Fig. 1). The amounts of the trapped superoxide radicals were assayed by quantifying the concentrations of the nitroxide radicals CP and PP, respectively. Quantification of superoxide radicals in suspension of blood cells was performed using 0.5 mM CP-H or PP-H monitoring of the low-field component of ESR spectra. Blood samples for ESR measurements were analyzed in 100 μl glass capillaries (NeoLab, Heidelberg, Germany). To inhibit metal-catalyzed oxidation of spin traps, CP-H or PP-H was infused into animals in the presence of deferoxamine (stock solution, 20 mM CP-H and 1 M deferoxamine) [28]. The ESR measurements were performed at room temperature using an EMX-A ESR spectrometer (Bruker, Karlsruhe, Germany). The ESR settings were as follows: field center, 3474 G; field sweep, 60 G; microwave frequency, 9.72 GHz; microwave power, 20 mW; magnetic field modulation, 100 kHz; modulation amplitude, 2.0 G; conversion time, 163 ms; detector time constant, 655 ms; and sweep time, 83 s. The ESR spectra were recorded 1 in after blood sampling.

Determination of superoxide radical formation in vivo

Using ESR and CP-H and PP-H as spin traps [27], the amounts of superoxide radicals formed in vivo can be determined by monitoring accumulation of the corresponding stable nitroxide radicals in the blood. The nitroxide radicals CP and PP are formed in the reaction of CP-H and PP-H with superoxide radicals. The amount of trapped superoxide radicals can be assayed using the SOD- or vitamin C–inhibited formation of the nitroxide radicals CP and PP, respectively. Experimental concentrations of CP and PP were determined from the calibration curve using CP or PP obtained from Sigma (Deisenhofen, Germany). The nitroxide radicals CP and PP were very stable compounds both in vivo and ex vivo (in blood). The lifetime of the nitroxides was more than 4 h, whereas ESR measurements were performed during the first 5 min. Nitroxide formation ex vivo during the first 10 min was negligible.

Measurements of hemodynamic responses

Indwelling cannulas were inserted into the common carotid arteries and the tibial veins of anesthetized rabbits in a prone position. Phasic and mean tracings from the recorded parameters were processed using PO-NE-MAH Digital Acquisition Analyses and Archive Systems (Aquire Plus 1.0; Hugo Sachs Electronic, March-Hugstetten, Germany). All measurements were started after a resting period of 10 min.

Preparation of CP-H and PP-H solutions

The cyclic hydroxylamines CP-H and PP-H were dissolved in oxygen-free (nitrogen bubbled), 0.05 M sodium phosphate buffer (pH 7.4) in the presence of 0.9% NaCl and 1 mM deferoxamine. The concentration of CP-H and PP-H in the stock solutions was 20 mM. Stock solutions were kept either frozen or in a cool place under anaerobic conditions before experiments.

Chemicals and drugs

The GTN used was from Pohl Boskamp (Hohenlockstedt, Germany). The spin traps CP-H and PP-H (purity grade analyzed using infrared spectroscopy) were from Alexis Corporation (Läufelfingen, Switzerland). The CP, PP, and deferoxamine mesylate were obtained from Sigma.

Statistics

Values are expressed as mean $\pm$ SEM. Statistical significance was determined using Student's *t*-test for paired data. Two groups of data were considered to be significantly different at $p < .05$.

RESULTS

Reactions with superoxide radicals

Reactions of PP-H or CP-H with superoxide radicals were studied in a xanthine oxidase superoxide-generating system. Stock solutions of PP-H or CP-H contained trace quantities of PP or CP (Fig. 2, spectra A and 2). A significant amount of the nitroxide PP or CP was formed during the reaction with superoxide radicals (Fig. 2, spectra B and F). The ESR amplitude of PP or CP increased constantly as a result of the continuous formation of superoxide radicals and their reaction with PP-H or CP-H. The addition of SOD (100 U/ml) or vitamin C (20 μg/ml) completely inhibited formation of PP or CP (Fig. 2, spectra C, D, G, and H, respectively). The rate constants for the reaction of $O_2^{\bullet -}$ with PP-H or CP-H are 8.4×10^2 $M^{-1}s^{-1}$ and 3.2×10^3 $M^{-1}s^{-1}$, respectively [27]. The amounts of superoxide radical trapped were almost the same both for 0.5 mM CP-H and 0.5 mM PP-H.

Penetration or incorporation of PP-H or CP-H

A 1 h incubation of whole blood with PP-H was performed with a minimal penetration of 0.9 $\pm$ 0.6% in the cytosol of blood cells (Fig. 3). The CP-H penetrated as much as 8.3 $\pm$ 0.4% in cytosol within

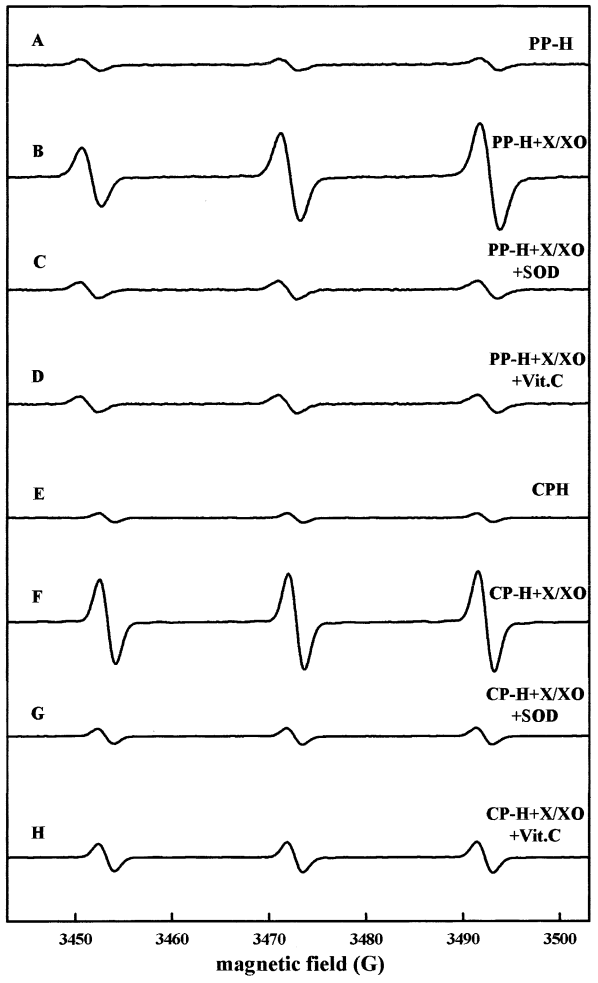


Fig. 2. Detection of superoxide radicals using PP-H or CP-H monitoring PP or CP nitroxide formation. Formation of PP or CP nitroxide in xanthine plus xanthine oxidase (X/XO) superoxide-generating system in the following samples: (spectrum A) 0.5 mM PP-H; (spectrum B) 0.5 mM PP-H + X/XO; (spectrum C) 0.5 mM PP-H + 100 U/ml SOD + X/XO; (spectrum D) 0.5 mM PP-H + 20 μg/ml vitamin C (Vit.C) + X/XO; (spectrum E) 0.5 mM CP-H; (spectrum F) 0.5 mM CP-H + X/XO; (spectrum G) 0.5 mM CP-H + 100 U/ml SOD + X/XO; (H) 0.5 mM CP-H + 20 μg/ml vitamin C + X/XO. ESR settings were as described in the text. The superoxide radical–generating system contained xanthine oxidase (0.01 U/ml), xanthine (50 μM), deferoxamine (20 μM) in 50 mM sodium phosphate buffer (pH 7.4) in the presence of 0.9% NaCl. The hyperfine ESR splitting constant of PP nitroxide was a_N = 17.1 G with a line width of 1.7 G and for CP, a_N=19.2 G with a line width 2.0 G.

1 h. The incorporation of PP-H or CP-H in the membranes of blood cells, however, was barely detectable. The greater part of the 800 μM of the spin trap PP-H (as much as 98.5 ± 0.5%) or CP-H (as much as 91.1 ± 0.6%) remained in the plasma (Fig. 3). The experiments with PP and CP radicals showed almost identical penetration and incorporation in the blood cells. Incubation of blood plasma or of cytosol in erythrocytes with CP-H or PP-H did not produce any signif-

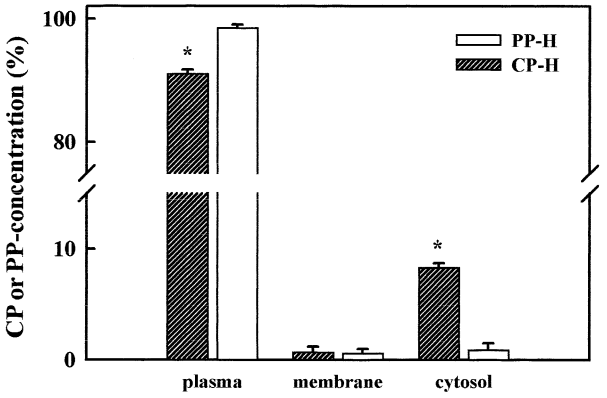


Fig. 3. Analysis of the incorporation or penetration of spin-trap CP-H or PP-H. The quantification of incorporation or penetration was examined after a 1-h incubation of spin traps (800 μm) at 37°C in whole blood of rabbits. Subsequently, the whole blood and blood cells were fractionated to blood plasma and to cell membrane and cytosol, respectively. Data are mean ± SEM, n = 4. *p < .05 vs. PP-H.

icant increase of nitroxide intermediates during the first 60 min.

Efficiency of the reaction of PP-H with superoxide radical

Even in whole blood, CP-H is an effective scavenger of superoxide radicals [2]. The efficacy of the reaction of PP-H with superoxide radical generated extracellularly using xanthine oxidase in the whole blood was compared with that of CP-H. The amount of superoxides trapped by PP-H in the pure xanthine oxidase system was approximately the same as that with CP-H (Fig. 4). The presence of whole blood resulted in the 3-fold decrease in the efficacy of su

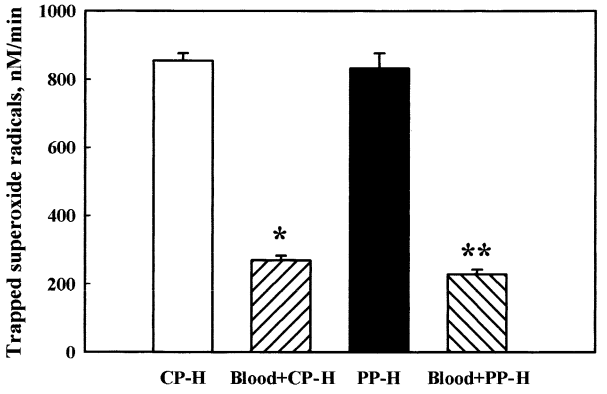


Fig. 4. Quantification of superoxide radical formation in xanthine oxidase plus xanthine (XO/X) superoxide-generating systems in the presence and absence of the whole blood as measured by the formation rate of CP or PP using CP-H and PP-H, respectively. Standard deviations for columns are shown, n = 6. *p < .05 vs. XO/X+CP-H; **p < .01 vs. XO/X+PP-H.

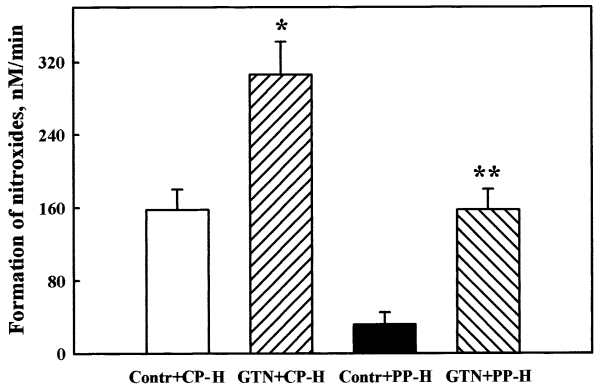


Fig. 5. Quantification of GTN-induced superoxide radical formation in whole blood as measured by the formation rate of CP or PP using CP-H or PP-H in nM/min, respectively. Standard deviations for columns are shown, $n = 6$. *$p < .05$ vs. control (CP-H); **$p < .01$ vs. control (PP-H).

competition between hydroxylamines and blood antioxidant systems (e.g.; vitamin C, low–molecular weight thiols, uric acid). The amount of superoxide radicals trapped by PP-H in whole blood was similar to the amount with CP-H (Fig. 4). Therefore, PP-H is an effective scavenger of superoxide radicals, even in the presence of an extracellular antioxidant system of the blood.

In vitro superoxide formation inducted by GTN

Basal oxidation of CP-H in blood was greater than that of PP-H (Fig. 5). Acute treatment of blood with GTN resulted in a 2-fold increase in nitroxide formation as detected using CP-H and a 5-fold increase as detected using PP-H (Fig. 5). Both PP-H and CP-H showed practically the same rates of nitroglycerin-induced superoxide radical formation (Fig. 5, difference between control and nitroglycerin columns).

In vivo detection of GTN-induced superoxide formation

For in vivo detection of superoxide radicals, continuous infusions of CP-H or PP-H were used to provide constant spin trap concentrations in the blood. Using an initial bolus and continuous infusions of CP-H or PP-H, appropriate conditions for in vivo experiments were found that caused very small changes in the ESR amplitudes of the controls (Figs. 6A and 6E). Twenty minutes after spin trap infusions, the amplitude of the ESR spectra of CP or PP was stabilized, and only a slight time-dependent increase was observed. After injection of 130 μg/kg GTN, the amplitude of the ESR spectra of the blood samples rapidly increased and, in 10 to 20 min,

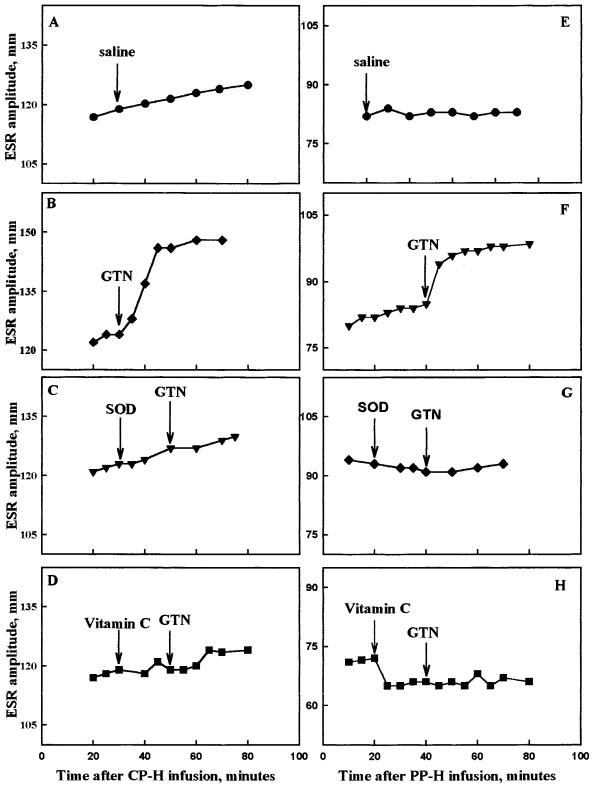


Fig. 6. In vivo formation of CP or of PP in control rabbits (A, E), after injection of 130 μg/kg GTN (B, F), after injection of 15,000 U/ml SOD and 130 μg/kg GTN (C, G), and after injection of 20 mg/kg vitamin C and 130 μg/kg GTN (D, H). Superoxide radical formation was determined by the oxidation of CP-H or PP-H yielding CP or PP, respectively. The administration of GTN leads to a 2.0 ± 0.4 μM increase in GTN-induced superoxide formation. Data from typical experiments are presented at an identical scale.

reached the maximum (Figs. 6B and 6F). Injection of SOD (15,000 U/kg) 20-min before infusion of 130 μg/kg GTN prevented formation of nitroxide radicals (Figs. 6C and 6G).

The effect of vitamin C on GTN-induced superoxide radical formation was also tested. Initially, 20 mg/kg vitamin C was injected, and 20-min later, 130 μg/kg GTN was infused (Figs. 6D and 6H). Infusion of GTN after pretreatment with vitamin C did not cause any change in the amplitude of the ESR signals of CP or PP in the blood samples (Figs. 6D and 6H). The GTN-induced formation of reactive oxygen species in vivo was estimated as an increase in nitroxide contents (Figs. 6B and 6F, arrows). In vivo, GTN-induced superoxide formation followed by injection of GTN (130 μg/kg) was 2.0 ± 0.4 μM. Infusion of vitamin C or SOD before injection of GTN led to complete scavenging of superoxide radicals. The injection of the NO-independent vasodilator adenosine led to an insignificant increase in nitroxide formation.

Changes in hemodynamic parameters

During the in vivo experiments, changes were observed in systolic and diastolic blood pressure and in heart rate as an expression of the effect of GTN and as a control of the hemodynamic changes after infusions of spin traps, vitamin C, and SOD. A GTN bolus infusion produced a drastic drop in systolic (drop, 12 ± 1 mm Hg) and diastolic (drop, 9 ± 1 mm Hg) blood pressures. At the same time, the heart rate increased to 17 ± 2 bpm. Pretreatment of rabbits with vitamin C or SOD followed by bolus infusion of GTN produced a significant drop in systolic (14 ± 1 mm Hg) and diastolic (11 ± 1 mm Hg) blood pressure. A bolus or continuous infusion of spin traps, vitamin C, or SOD produced no hemodynamic changes.

DISCUSSION

Our results have shown that the inability of the spin trap PP-H to penetrate the blood cells permits analysis of the extracellular formation of reactive oxygen species. The advantages of using PP-H (low rate of background oxidation compared with that using CP-H) were evident in our analysis of GTN-induced formation of reactive oxygen species both in blood (Fig. 5) and in vivo (Fig. 6). We assume that GTN-induced formation of reactive oxygen species may result from $O_2^{\bullet -}$ generation, because it could be completely inhibited by SOD (extracellular applied and acting) and by the antioxidant vitamin C.

The release of $O_2^{\bullet -}$ radicals may be caused by NO-induced vasodilation, and this effect was tested using NO/cGMP-independent vasodilation. For this purpose, adenosine was used as a vasodilator. Adenosine activates adenylate cyclase of the endothelial and smooth muscle cells, thus producing increased synthesis of cAMP. In addition, it activates the ATP-sensitive K^+ channels [29, 30]. It was found that NO-independent vasodilation by adenosine did not affect radical formation in vivo (data not shown).

Until recently, an adequate analysis of the release of reactive oxygen species either in vivo or in vitro was difficult to perform, because lucigenin was known to enhance $O_2^{\bullet -}$ radical formation [31]. The present analysis was possible only through the synthesis of new, stable spin traps such as CP-H and PP-H, which were not so sensitive to reduction by thiols and vitamin C in plasma [2,24,32]. Results of various studies of our own and other working groups have shown a greatly enhanced release of the $O_2^{\bullet -}$ radical after both GTN metabolism (in vitro or in vivo) [1,2,25,33] and the development of nitrate tolerance. It remains to be clarified, however, whether tolerance to nitrates results from GTN-induced $O_2^{\bullet -}$ radical release or whether other mechanisms, such as reduced bioconversion of GTN, imbalance in the neurohumoral system, or reduced activity of guanylyl cyclase, are mainly responsible [15,16,34–38].

To a great extent, GTN-induced superoxide radical formation results from the simultaneously induced, enhanced activity of NADH phosphatase oxidoreductases [2,39]. Hydralazine suppresses a GTN-induced, elevated formation of $O_2^{\bullet -}$ in the endothelial and smooth muscle cells, probably by decreasing the activity of NADH oxidases that are immobilized in plasma membranes [35,40]. By increasing the activity of NADH oxidases, formation of $O_2^{\bullet -}$ in platelets can be augmented [2]. Our results, which were obtained with GTN-treated platelets that were preincubated with hydralazine, point out that only some of the enzymes involved in a GTN-induced, elevated formation of superoxide in platelets are inhibited by the action (or actions) of hydralazine. The results of Parker et al. [41] confirm that in therapeutic dosages, hydralazine only partially suppresses tolerance to nitrates.

An incomplete suppression of nitrate tolerance by the hyperpolarizing agent hydralazine (activity of oxidases depends on the membrane potential [40]) makes it likely that additional oxidases participate in generation of $O_2^{\bullet -}$ radicals. Xanthine oxidase generates $O_2^{\bullet -}$ radicals both intra- and extracellularly [42–45], and it participates in the metabolism of organic nitrates, especially under ischemic conditions [46–49]. It should be emphasized, however, that intracellularly generated reactive oxygen species ($O_2^{\bullet -}$, $ONOO^-$, H_2O_2) can penetrate the cell membranes [50,51] to affect the extracellular milieu. Thus, our new spin trap PP-H (Fig. 4), which cannot penetrate the intracellular compartment, does not exclusively trap extracellularly generated, oxygen-derived radicals. Our results with extracellular SOD (Fig. 6) lead to the conclusion that GTN primarily induces an extracellular generation of $O_2^{\bullet -}$ radicals.

Treatment with vitamin C prevents development of nitrate tolerance [10,14,52–54]; however, the mechanisms of this phenomenon remain unclear. Pretreatment with vitamin C led to a substantial decrease in detectable superoxide radicals. Moreover, the amount of GTN-induced superoxide radicals detected after pretreatment with vitamin C was very close to the ESR detection limits. Therefore, vitamin C effectively scavenges almost all GTN-induced superoxide radicals, which can also prevent formation of peroxynitrite. The tolerance-suppressing effect of vitamin C, which has been reproduced in numerous studies involving dogs, volunteers, and patients with congestive heart failure [10], supports the conclusion that vitamin C can inhibit development of nitrate tolerance through scavenging of superoxide radicals [14,52,55].

Our data support the key role of the increased formation of superoxide radicals in development of nitrate tolerance. Our data show the GTN-induced, extracellular formation of superoxide radicals.

CONCLUSION

Our results show that PP-H is an effective scavenger of superoxide radicals and can be used in the study of extracellular formation of superoxide radicals. Both acute and chronic treatments with nitroglycerin were found to induce extracellular formation of superoxide radicals. The data obtained show that GTN induces superoxide radical formation both in vitro and in vivo. Our experiments with vitamin C support the conclusion that it is an effective antioxidant for protection against GTN-induced superoxide formation in vivo.

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