Production of superoxide in plasma, endothelial cells, and isolated heart studied with new cell permeable cyclic hydroxylamine.

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Abstract: In this work, we studied membrane permeability and reaction with $O_2^{\bullet-}$ of a new spin probe, 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH), for analysis of $O_2^{\bullet-}$ production in blood, endothelial cells and isolated guinea pig hearts. After 10 min incubation, the intracellular concentration of CMH in erythrocytes reached 18.1 %, while the content of 1-hydroxy-3-carboxy-2,2,5,5,-tetramethylpyrrolidine (CPH) was only 9.2%. Rate constant of CMH reaction with $O_2^{\bullet-}$ ($1.2 \times 10^4\ M^{-1}s^{-1}$) was 3.7-times higher than that of CPH**,** which is consistent with scavenging 2-times more $O_2^{\bullet-}$ in blood plasma by CMH than CPH. Intracellular $O_2^{\bullet-}$ production was measured from PEG-SOD inhibited formation of 3-methoxy-carbonyl radical ($CM^{\bullet}$). Treatment of endothelial cells with peroxynitrite lead to 3-fold increase in $O_2^{\bullet-}$ production, while lactate (10 mM) caused a 2.2-fold increase in $O_2^{\bullet-}$ production in isolated guinea pig hearts. Therefore, using CMH we detected intracellular $O_2^{\bullet-}$ production from uncoupled eNOS in endothelial cells and lactate-induced $O_2^{\bullet-}$ production in isolated hearts. High cell permeability and high reactivity with $O_2^{\bullet-}$ of CMH allow effective detection of low amounts of intra- and extracellular $O_2^{\bullet-}$.

Keywords: reactive oxygen species, superoxide, endothelial nitric oxide synthase, uncoupling, lactate, intracellular, spin probe

INTRODUCTION

A number of physiological conditions such as exercise [1], aging [2], oxidative phosphorylation in mitochondria [3], and activation of the adrenergic system [4], as well as a number of pathological conditions such as cardiovascular diseases (5), diabetes (6), male infertility [7], lung diseases [8], hypercholesterolemia [9], and nitrate tolerance [10] are associated with increased production of superoxide ($O_2^{\bullet-}$). $O_2^{\bullet-}$ forms reactive oxygen species (ROS), which modulate gene transcription [11], affect tyrosine phosphorylation [12], cause DNA damage [13], induce lipid oxidation [9], and modulate activity of matrix metalloproteinases [14]. The dismutation product of $O_2^{\bullet-}$, H_2O_2, is involved in vasoconstriction [15], in defense from infection [16], and in expression of enzymes like extracellular superoxide dismutase [17] or endothelial nitric oxide synthase (eNOS) [18]. The $O_2^{\bullet-}$ also reacts rapidly with nitric oxide, resulting in loss of nitric oxide's vasodilative and antiatherogenic properties and formation of peroxynitrite, a strong oxidant [19]. Many of these redox events occur in either the cell cytoplasm or cell organelles and thus may not be affected by interventions targeting extracellular ROS.

Recent studies have shown that chronic enhanced $O_2^{\bullet-}$ production from phagocytic and nonphagocytic NAD(P)H oxidases contribute to development and progression of atherosclerosis [20] and cardiomyopathy [21]. The activity of NAD(P)H oxidases is regulated by hormones, growth factors and physical forces [22-24]. Experimental data suggest that the production of $O_2^{\bullet-}$ is required for the normal, physiologic activity of cardiac cells, but abnormal activation of the nonphagocytic NAD(P)H oxidases in response to neurohormones (angiotensin II, norepinephrine, tumor necrosis factor-α) has been shown to contribute to cardiac myocyte hypertrophy [21,25]. Activation of NAD(P)H oxidases closely correlates with the tissue pyruvate to L-lactate concentration ratio [26]. Sustained changes in redox reactants such as pyruvate or L-lactate are associated with altered activity of lactate dehydrogenase (LDH) and with an excessive increase of mitochondrial NAD(P)H oxidase activity in cardiac cells [27].

Another very important source of chronic $O_2^{\bullet-}$ production, which may contribute to the genesis and progression of atherosclerosis and diabetes, is uncoupled endothelial nitric oxide synthase (eNOS) [28-30]. It was found that under conditions of tetrahydrobiopterin (BH_4) deficiency, eNOS is in an uncoupled state, which means that electrons flowing from the eNOS reductase domain to the oxygenase domain are diverted to molecular oxygen rather than to L-arginine, resulting in production of $O_2^{\bullet-}$. Indeed, experimental [30] and clinical studies [31] have shown that the administration of the eNOS cofactor BH_4 improves endothelial dysfunction in humans with diabetes mellitus [32] and with hypercholesterolemia [33]. Furthermore, $O_2^{\bullet-}$ released from uncoupled eNOS enhances degradation of $NO^{\bullet}$ with formation of the strong oxidant peroxynitrite, which alters residual eNOS function by oxidation of BH_4 and by oxidation of intracellular glutathione [30,34].

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Recently we have described a new electron spin resonance (ESR) technique for detection of $O_2^{\bullet-}$ in vitro and in vivo, which was more sensitive and quantitative [35–37] in comparison to commonly used nitrone spin traps DMPO and DEPMPO. A strength of this new technique is that cyclic hydroxylamines such as 1-hydroxy-3-carboxy-pyrrolidine (CPH), are very effective scavengers of $O_2^{\bullet-}$ radicals [35–37]. The long half-life of the nitroxide radical produced in the reaction with reactive oxygen species (ROS) is a distinct advantage over nitrone spin traps, which form unstable $O_2^{\bullet-}$ radical adducts in biological samples, while nitroxides similar to 3-carboxy-proxyl are stable in the presence of reducing agents [38]. Therefore, cyclic hydroxylamines provide quantitative measurements of $O_2^{\bullet-}$ radicals with higher sensitivity than the nitrone spin traps [37].

A weakness of the cyclic hydroxylamines is that the product formed upon reaction with different reactive oxygen species yields the same nitroxide regardless of the ROS trapped [38] and the resultant ESR spectra are therefore identical for several different ROS. In order to overcome this limitation, it is necessary to perform additional studies using various antioxidants and inhibitors specific for the various ROS. For example, $O_2^{\bullet-}$ radicals can be determined as SOD-inhibitable CP-nitroxide formation.

CPH was successfully used for detection of $O_2^{\bullet-}$ in vascular cell suspension [35] and in vivo [36]. Nevertheless, despite the higher reactivity of CPH with $O_2^{\bullet-}$ compared to the nitrone spin trap DMPO, penetration of CPH is only 10% of the concentration applied to cells. Therefore, this makes it inefficient to investigate slow intracellular $O_2^{\bullet-}$ production. This inspired us to test a new cell permeable cyclic hydroxylamine, 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH), for detection of slow production of $O_2^{\bullet-}$ in intracellular and extracellular compartments, which may help to define the contribution of different sources of $O_2^{\bullet-}$ in the genesis and progression of vascular diseases.

MATERIALS AND METHODS

Chemicals and drugs

The spin probes 1-hydroxy-3-carboxy-pyrrolidine (CPH) and 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH) and peroxynitrite were obtained from Alexis Corporation (San Diego, CA, USA). The chemicals 3-carboxy-proxyl (CP), dimethylsulfoxide (DMSO), diethylenetriaminepentaacetic acid (DTPA), bovine erythrocyte superoxide dismutase (SOD), catalase and xanthine were obtained from Sigma (St. Louis, MO, USA). Xanthine oxidase was supplied by Roche (Manheim, Germany). 2-Methylbutane was purchased from Acros Organics (NJ, USA). All other chemicals were of analytical grade, purchased from Sigma (St. Louis, MO, USA).

Detection of $O_2^{\bullet-}$ using xanthine/xanthine oxidase $O_2^{\bullet-}$ generating system

Using a xanthine/xanthine oxidase $O_2^{\bullet-}$ generating system (50 µM xanthine + 10 mU/ml xanthine oxidase), we examined the reaction of CPH and CMH with $O_2^{\bullet-}$ by following formation of 3-carboxy-proxyl radical ($CP^{\bullet}$) or 3-methoxy-carbonyl radical ($CM^{\bullet}$). The analysis of efficacy of $O_2^{\bullet-}$ scavenging was carried out in 50 mM sodium phosphate buffer (pH 7.4) in the presence of 0.9% NaCl and with various concentrations (1.5 or 60 U/ml) of superoxide dismutase. The transition metal catalyzed oxidation of CPH and CMH was suppressed by the addition of 50µM deferoxamine as a chelating agent. The amount of $CP^{\bullet}$ or $CM^{\bullet}$ formed was calculated from a calibration curve for the double integral of ESR spectra and compared with the rate of cytochrome c reduction [37,39].

Oxidation of spin probes by myeloperoxidase and H_2O_2

In order to study possible interference of the $O_2^{\bullet-}$ measurement with the presence of H_2O_2, we tested direct oxidation of CMH and CPH by 10 µM H_2O_2 alone and in the presence of myeloperoxidase (MPO, 1 mU/ml) in 50 mM sodium phosphate buffer (pH 7.4) in the presence of 0.9% NaCl and 0.1 mM DTPA. The amount of formed $CP^{\bullet}$ or $CM^{\bullet}$ was calculated from a calibration curve for the double integral of ESR spectra.

Detection of $O_2^{\bullet-}$ formation in plasma

Total $O_2^{\bullet-}$ formation in plasma was determined from the time-dependent accumulation of the stable nitroxide radical [37]. Because $CP^{\bullet}$ is very resistant to reduction by ascorbate and low molecular thiols [38], we were able to monitor the accumulation of $CP^{\bullet}$ in blood plasma without decay of the ESR signal. We compared the scavenging efficacy of $O_2^{\bullet-}$ production in plasma using spin probes CPH and CMH (1 mM) and xanthine/xanthine oxidase $O_2^{\bullet-}$ generating system.

Heart preparation

The hearts of ketamine (100 mg/kg im)/xylazine (4 mg/kg i.m.) anesthetized guinea pigs were quickly isolated and perfused at constant flow with a Krebs-Henseleit solution containing 5.5 mM glucose, 1.25 mM $CaCl_2$, 120 mM NaCl, 25 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 1.2 mM NaH_2PO_4, and 4 mM KCl, equilibrated with 95% O_2-5% CO_2 (pH 7.4) at 37°C. Initial perfusion rate was set to

perfusion pressure of 60 mmHg. During the course of the experiments, perfusion rate was held constant at 11 ml/min, and pressures varied between 55 and 65 mmHg, well within the coronary flow autoregulatory range of the guinea pig heart. After an initial stabilization period of 20 min, hearts were electrically paced at 250 beats/min. The protocol conformed to the National Research Council's *Guide for the Care and Use of Laboratory Animals*.

Detection of $O_2^{\bullet-}$ in perfused hearts

Cardiac ROS were measured in the presence or absence of L-lactate (10 mM) as been previously described for CPH in Bünger et al. [27]. The spin probe CMH (0.8 mM) was added in perfusion buffer, along with 50 µM deferoxamine to minimize the transition metal oxidation of CMH. The stable paramagnetic reaction product $CM^{\bullet}$ was measured in perfusate and quantified by ESR. Production of superoxide radicals was confirmed by inhibition of $CM^{\bullet}$ nitroxide formation in the perfusion buffer contained Cu,Zn-SOD (60 U/ml).

Analysis of cell membrane permeability of CPH and CMH

The rate of permeability of the spin probes was examined following the incubation of CPH or CMH (500 µM) in whole blood of mice with subsequent fractionation. After periods of 5, 10, 60 min and periodic swiveling (5 times/min) at 37°C, blood was centrifuged for 2 min at 3000 g. The plasma was carefully decanted. The cell sediment was resuspended in an equal volume of 0.9% NaCl solution. Finally, amounts of CPH and CMH in the fractions of plasma and red blood cells were measured by oxidation with 10 mM KO_2 and analysis of nitroxide content using ESR.

Processing of endothelial cells

Endothelial cells (EC) were washed from bovine aortas as described by Drummond et. al. [18] and grown in culture (M 199; Gibco BRL cat N° 31095-029, Life Technologies, USA) as previously described. EC were resuspended in 50 mM phosphate buffer saline, pH 7.4, at on 4000 cells/µl. Suspensions of EC were treated with 500 µM peroxynitrite before addition of the spin probe CMH (1 mM). $O_2^{\bullet-}$ production was measured as SOD-inhibited accumulation. L-NAME (1mM) was added to suspension of cells after peroxynitrite treatment to determine $O_2^{\bullet-}$ production by uncoupled eNOS.

ESR analysis

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. The ESR spectra were recorded using an EMX ESR spectrometer (Bruker, Germany) and a super-high Q microwave cavity. The ESR instrumental settings for field scan were as follows: field sweep, 50 G; microwave frequency, 9.78 GHz; microwave power, 20mW; modulation amplitude, 2 G; conversion time, 327 ms; time constant, 655 ms; receiver gain, 1×10^5. The ESR kinetic experiments with cells and blood 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 $CP^{\bullet}$ or $CM^{\bullet}$. Concentration of nitroxides in the time scan has been determined from the ESR amplitude according to calibration curve using standard solutions of the 3-carboxyproxyl radical and measurements of the amplitude of top half of the low-field component of the nitroxide ESR spectra.

Statistics

All values are expressed as mean ± standard deviation. Statistical significance was determined by Student's *t* test for paired data. Two groups of data were considered to be significantly different at a p value <0.05.

RESULTS

Comparison of $O_2^{\bullet-}$ scavenging by CPH and CMH

Previously, we studied the reaction of the spin probe CPH with $O_2^{\bullet-}$ generated by xanthine/xanthine oxidase [38]. The rate constant was calculated as 3.2×10^3 $M^{-1}s^{-1}$, which is 100-times greater than that of nitrone spin traps such as DMPO [40]. Using a previously described method [38], we studied the reaction of CMH with $O_2^{\bullet-}$. Background oxidation of CPH and CMH produced very weak ESR signals (Fig. 1 A and E). A strong ESR signal of $CP^{\bullet}$ and $CM^{\bullet}$ was detected in the presence of the $O_2^{\bullet-}$ generating system, where nitroxide concentrations were equal to the amount of detected $O_2^{\bullet-}$ (Fig. 1B and F). Addition of superoxide dismutase diminished $O_2^{\bullet-}$ dependent oxidation of both cyclic hydroxylamines (Fig. 1C and G). This confirms nitroxide formation via reaction of CPH and CMH with $O_2^{\bullet-}$. Interestingly, addition of 1.5 mU Cu,Zn-SOD decreased CPH oxidation to 10% and CMH oxidation to 39 % (Fig. 1C and G), which implies a higher rate constant of CMH compared to CPH. Only a 40-times higher Cu,Zn-SOD concentration (60 U/ml) completely inhibited the reaction of the spin probe CMH with $O_2^{\bullet-}$ (Fig. 1D and H). The rate constant of CMH reaction with $O_2^{\bullet-}$ was calculated as $1.2 \pm 0.2 \times 10^4$ $M^{-1}s^{-1}$ from the inhibition of CMH oxidation by Cu,Zn-SOD using previously described method with Cu,Zn-SOD as the competitive inhibitor [38].

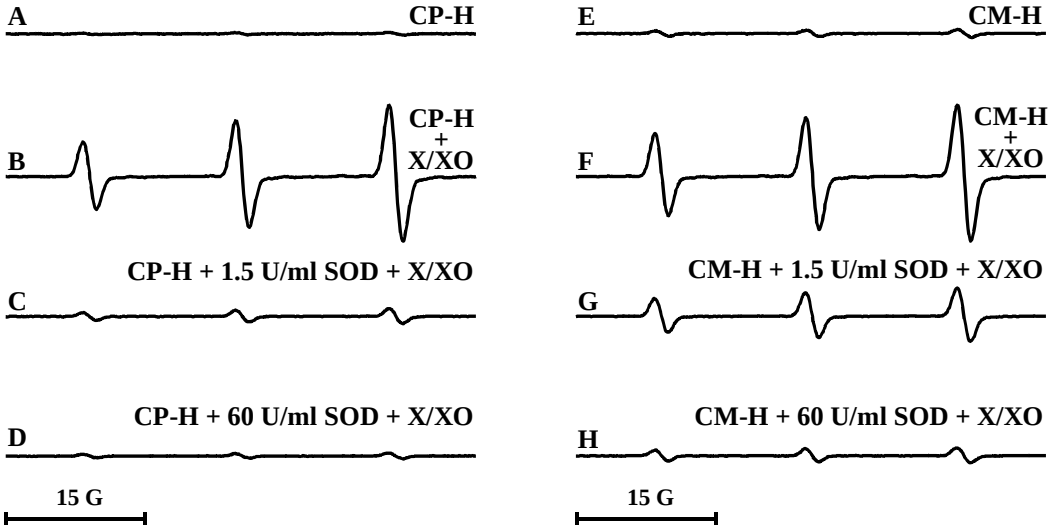


Figure 1. Detection of $O_2^{\bullet -}$ in xanthine/xanthine oxidase system using cyclic hydroxylamines CPH and CMH and effect of superoxide dismutase. ESR spectra of 0.5 mM CPH (A) and 0.5 mM CMH (E) in 50 mM phosphate buffer; nitroxide accumulation in xanthine/xanthine oxidase $O_2^{\bullet -}$ generating system (50 μM xanthine and 10 mU/ml xanthine oxidase) with CPH (B) and CMH (F); inhibition of nitroxide formation with 1.5 and 60 U/ml Cu,Zn-SOD in samples with CPH (C, D) and CMH (G, H). ESR spectra were obtained with a single scan of 167 seconds.

Detection of $O_2^{\bullet -}$ in presence of antioxidants

The presence of antioxidants such as vitamin C and thiols complicates the detection of $O_2^{\bullet -}$ in blood plasma with nitrone spin traps such as DMPO or DEPMPO. Previously, it was reported that $CP^{\bullet}$ is very resistant to reduction by ascorbate and low molecular thiols [38]. The rate constant of reduction by ascorbate was found as low as 0.1 $M^{-1}s^{-1}$, which was 66-times less than the rate constant of TEMONE [38]. Using cyclic hydroxylamines, which form stable products, detection of $O_2^{\bullet -}$ release at a rate of 9.6 nM/min in rat blood, was possible [37].

In order to make sure that detection of will be not affected by the decay of the $CM^{\bullet}$ ESR signal we studied resistance of $CM^{\bullet}$-nitroxide to reduction by ascorbate and low molecular thiols. We did not found significant reduction of 10 μM $CM^{\bullet}$ by glutathione (10 mM) or endothelial cells (data not shown). Ascorbate reduced $CM^{\bullet}$ in a concentration dependent manner (0.11 μM/min at 1mM ascorbate and 10 μM $CM^{\bullet}$). The rate constant of $CM^{\bullet}$ reduction by ascorbate was calculated as low as 0.2±0.02 $M^{-1}s^{-1}$, which is 30-times less than the rate constant of TEMONE. Therefore, reduction of $CM^{\bullet}$ by ascorbate is very slow and we can monitor the accumulation of $CM^{\bullet}$ in blood plasma without significant decay of the ESR signal.

We compared efficiencies of spin probes CMH and CPH to detect plasma $O_2^{\bullet -}$ generated by xanthine/xanthine oxidase (Fig. 2). Scavenging of $O_2^{\bullet -}$ was measured by the kinetics of $CP^{\bullet}$ and $CM^{\bullet}$ accumulation in blood plasma at room temperature. Background oxidation of CPH and CMH was much lower than nitroxide accumulation after addition of xanthine/xanthine oxidase (Fig. 2). The Amount of $O_2^{\bullet -}$ scavenged by CMH (71.4 nM/min, Fig. 2D) was 2.1-fold higher than $O_2^{\bullet -}$ detected by CPH (34.2 nM/min, Fig. 2C). Thus, detection of $O_2^{\bullet -}$ in plasma with CMH was twice as effective as with CPH, which is likely associated with the higher rate constant of the reaction of CMH with $O_2^{\bullet -}$. The difference between the amount of the superoxide detected by CPH and CMH can be calculated from the following equation:

$V_{CMH}/V_{CPH}=\{k_{CMH}\bullet[CMH]/k_{CMH}\bullet[CMH]+k_{Antiox}\bullet[Antiox]\}/\{k_{CPH}\bullet[CPH]/k_{CPH}\bullet[CPH]+k_{Antiox}\bullet[Antiox]\}$
Taking into account that [CMH]=[CPH] and $k_{CMH}/k_{CPH} = 3.7$ we can simplify this equation:
$V_{CMH}/V_{CPH} = (3.7+3.7K) / (3.7+K)$,

where K is the ratio of CPH and antioxidant reaction rates $k_{CPH}\bullet[CPH]/k_{Antiox}\bullet[Antiox]$. In the absence of the antioxidants (Antiox) the V_{CMH}/V_{CPH} ratio will be equal to one. At high concentrations of antioxidants the V_{CMH}/V_{CPH} ratio of the detected superoxide is equal to the ratio of the rate constants (3.7). The plasma V_{CMH}/V_{CPH} ratio was 2.1, implying that K= 2.5.

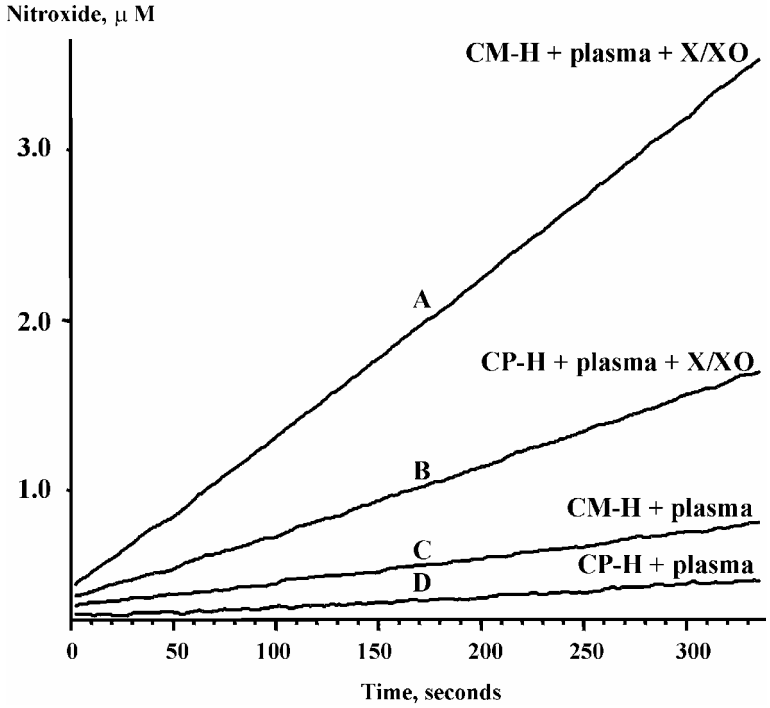


Figure 2. Measurements of $O_2^{\bullet-}$ in presence of blood plasma antioxidants using CMH and CPH. Kinetics of $CP^{\bullet}$ and $CM^{\bullet}$ accumulation in blood plasma with 0.5 mM CPH (A) and 0.5 mM CMH (B). Kinetics of $CP^{\bullet}$ and $CM^{\bullet}$ accumulation in a blood plasma with xanthine/xanthine oxidase $O_2^{\bullet-}$ generating system (50 μM xanthine and 10 mU/ml xanthine oxidase) after addition of CPH (C) or CMH (D). All samples contained 50 μM deferoxamine to inhibit metal catalyzed oxidation of cyclic hydroxylamines. Figure represents original time scans from typical experiments.

Peroxidase mediated cyclic hydroxylamines oxidation

We studied possible interference of the $O_2^{\bullet-}$ measurement by the presence of H_2O_2, which is a product of dismutation of $O_2^{\bullet-}$ and a byproduct of reaction with cyclic hydroxylamines. We tested the direct reaction of H_2O_2 with CMH as well as peroxidase- mediated oxidation.

In the presence of xanthine/xanthine oxidase $O_2^{\bullet-}$ generating system, we observed a fast production of $CM^{\bullet}$ (732 nM/min) (Fig. 3 A) and $CP^{\bullet}$ (558 nM/min) (Fig. 3 B). Addition of 10 μM H_2O_2 to CMH or CPH did not cause significant oxidation (Fig. 3 E, F). However, the presence of both H_2O_2 and myeloperoxidase strongly stimulated oxidation of CPH (Fig. 3C), while causing only a minor increase in oxidation of CMH (Fig. 3D). Flattening of the kinetics of CP accumulation (Fig. 3C) was dealing with the fast consumption of H_2O_2, because secondary addition of H_2O_2 caused further rapid increase in ESR amplitude (data not shown). Faster oxidation of CPH than CMH by myeloperoxidase is likely due to the fact that negatively charged CPH is better substrate of myeloperoxidase than neutral CMH. Addition of catalase (0.1mg/ml) to the xanthine/xanthine oxidase with CMH (Fig. 3A) did not affect nitroxide accumulation (data not shown). Therefore, accumulation of H_2O_2 does not interfere with detection of $O_2^{\bullet-}$ using CMH.

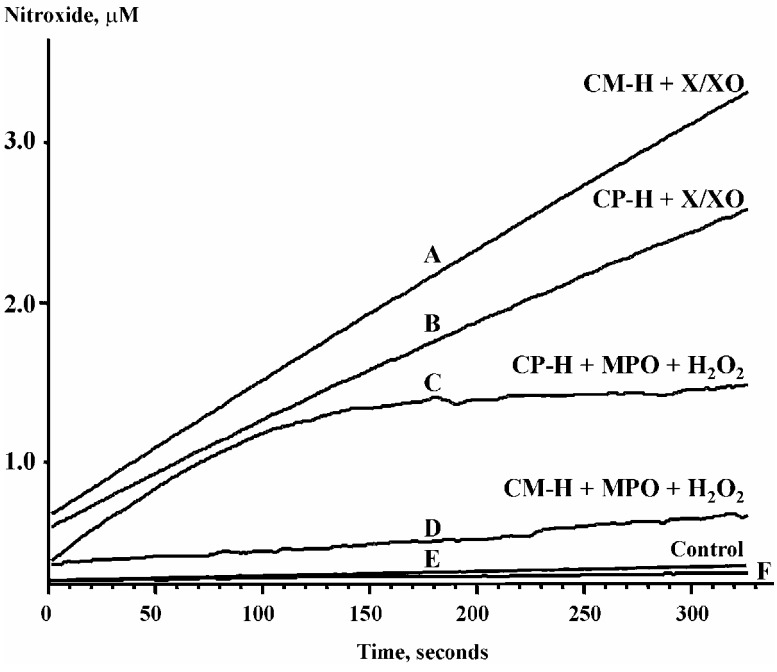


Figure 3. Detection of $O_2^{\bullet-}$ in xanthine/xanthine oxidase $O_2^{\bullet-}$ generating system and effect of hydrogen peroxide. Kinetics of nitroxide accumulation with 0.5 mM CMH (A) and 0.5 mM CPH (B) with xanthine/xanthine oxidase $O_2^{\bullet-}$ generating system (100 μM xanthine and 10 mU/ml xanthine oxidase). Kinetics of nitroxide accumulation in the presence of 10 μM H_2O_2 and 1 U/ml myeloperoxidase with 0.5 mM CPH (C) and 0.5 mM CMH (D). Kinetics of nitroxide accumulation with 0.5 mM CMH plus 10 μM H_2O_2 (E). Kinetics of nitroxide accumulation with 0.5 mM CPH plus 10 μM H_2O_2 (F). All samples contained 0.1 mM DTPA to inhibit metal catalyzed oxidation of cyclic hydroxylamines. Figure represents original time scans from typical experiments.

Analysis of cell permeability by CPH and CMH

It is known that neutral molecules have higher cell permeability than anions. Therefore, we assumed that modification of the CPH chemical structure to the neutral methyl ester might result in an increase in cell permeability. Cell permeability was determined by following the intracellular content of CMH or CPH measured with ESR as described in Materials and Methods. Incubation of CPH and CMH for 10 min resulted in 9.2 % and 18.1 % penetration of spin probes into the blood cells, respectively (Fig. 4). This confirms that CMH is 2-times more cell permeable than the spin probe CPH.

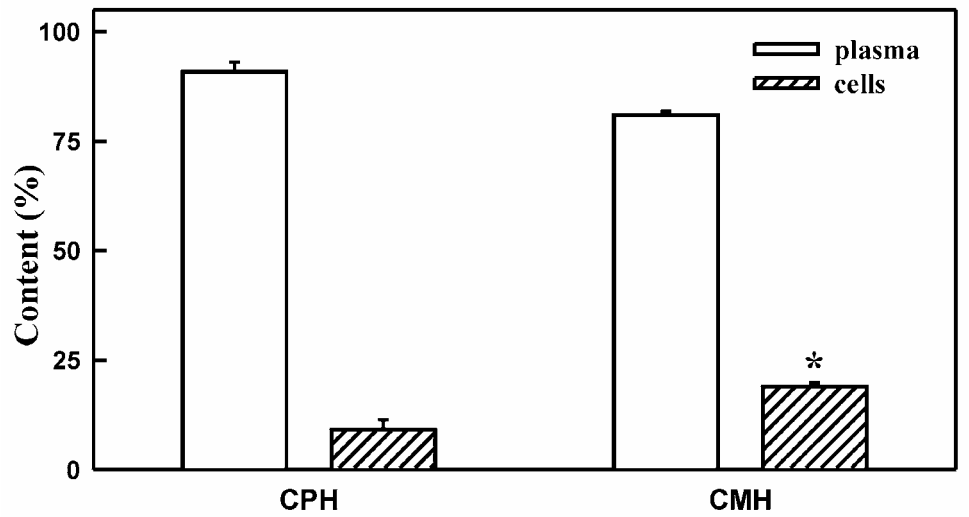


Figure 4. Analysis of cell permeability by CPH and CMH. Cell permeability was determined by following the plasma and intracellular content of CPH and CMH in blood cells measured as described in material and methods. Penetration of CPH and CMH was analyzed after 10 minutes incubation of each spin probe (500 μM) at 37°C in whole blood and followed by blood plasma and blood cell preparation. Data are mean ± SEM (n = 4). * $p < 0.05$ vs. CPH

Hydrolysis of methoxycarbonyl group of CMH: possible conversion of CMH into CPH

Hydrolysis of the methyl ester may result in conversion of CMH into CPH. The stability of methyl ester was studied by the analysis of the CMH/CPH ratio after the mild oxidation with ferric cyanide and extraction of $CM^{\bullet}$ nitroxide by 2-methylbutane. The coefficient of distribution between 2-methylbutane phase and water of $CM^{\bullet}$ was calculated as 3.8±0.1, while no $CP^{\bullet}$ was extracted by 2-methylbutane. Thus, distribution coefficient of $CP^{\bullet}$ is 0.

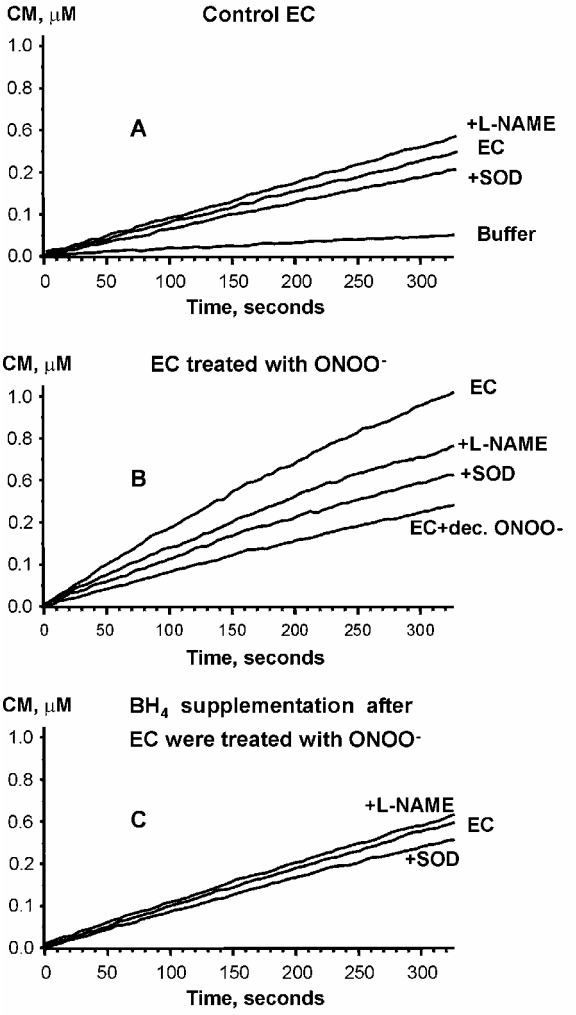


Figure 5. Formation of superoxide in endothelial cells (EC) after uncoupling of eNOS by peroxynitrite ($ONOO^-$). Kinetics of $CM^{\bullet}$ accumulation was followed in endothelial cells after acute

It was found that CMH oxidized by KO_2 was fully converted into $CP^{\bullet}$ (no nitroxide was detected in 2-methylbutane phase) due to hydrolysis in alkaline solution formed after decomposition of KO_2. Oxidation of CMH by ferric cyanide or xanthine/xanthine oxidase produced nitroxide with distribution coefficient of 3.8, implying that mild oxidation of CMH produced $CM^{\bullet}$ only.

Analysis of endothelial cells (1mln/ml) incubated with 1mM CMH for 15-minutes showed that both extracellular and intracellular nitroxides have distribution coefficient of 3.8, implying that incubation of CMH with endothelial cells did not cause significant hydrolysis of the methyl ester. Analysis of cardiac tissue sample produced similar results. The inaccuracy of the measurements of the distribution coefficient was 0.1, which suggests less than 2.6% of the CMH hydrolysis.

Detection of $O_2^{\bullet-}$ in endothelial cells released from uncoupled eNOS

Uncoupled eNOS is an important source of $O_2^{\bullet-}$ under pathophysiological conditions [33]. Recently, it was shown that treatment of cells with peroxynitrite might cause uncoupling of eNOS in endothelial cells [30]. Indeed, treatment of bovine aorta endothelial cells (BAECs) with peroxynitrite strongly stimulated superoxide production (Fig. 5A,B), which was significantly inhibited by L-NAME (Fig. 5B), while addition of L-NAME to untreated BAECs did not inhibit nitroxide accumulation (Fig. 5A). Addition of cell permeable PEG-SOD to a suspension of peroxynitrite treated endothelial cells, diminished oxidation of CMH to the basal level of $O_2^{\bullet-}$ production of non-treated endothelial cells (Fig. 5A,B): coincubation of untreated BAECs with PEG-SOD caused minor inhibition of nitroxide formation (Fig. 5A). The effect of tetrahydrobiopterin (BH_4) supplementation of peroxynitrite treated endothelial cells strongly support the role of BH_4 oxidation in the uncoupling of eNOS (Fig. 5C). This

treatment of endothelial cells (A) with 500 μM peroxynitrite (B). Superoxide production by uncoupled eNOS was analyzed by incubation with L-NAME (1 mM). Amount of superoxide production was assayed by 50 U/ml polyethylene glycol-conjugated superoxide dismutase (SOD). The role of tetrahydrobiopterin (BH_4) oxidation was analyzed by supplementation of 10μM BH_4 after endothelial cells were treated with peroxynitrite (C). Figure shows typical time scans from three original experiments.

experiment confirms that treatment of endothelial cells with peroxynitrite results in uncoupling of eNOS causing a 3-fold increase in intracellular $O_2^{\bullet-}$ production, which can be detected by the new spin probe CMH.

Detection of $O_2^{\bullet-}$ production in isolated heart

Different sources of superoxide can be involved in genesis of cardiovascular diseases. We tested the suitability of CMH for detection of $O_2^{\bullet-}$ production induced by lactate (Fig. 6). Using CPH, we detected formation of $CP^{\bullet}$ up to 2.23 ± 0.3 μM under non-stimulated conditions and up to 4.32 ± 0.48 μM after stimulation of $O_2^{\bullet-}$ production by 10 mM lactate. Perfusion of isolated guinea pig hearts with 500 μM CMH showed up to 8.25 ± 0.48 μM $CM^{\bullet}$ formation under non-stimulated conditions and up to 17.3 ± 1.2 μM after stimulation with lactate (Fig. 6). Analysis of nitroxide extraction in tissue and perfusate (as was described in the previous section) did not show significant hydrolysis of the CMH methyl ester. These results indicate the 4-times higher efficacy of the new spin probe CMH in comparison with CPH for detection of $O_2^{\bullet-}$ production stimulated by lactate in isolated guinea pig hearts.

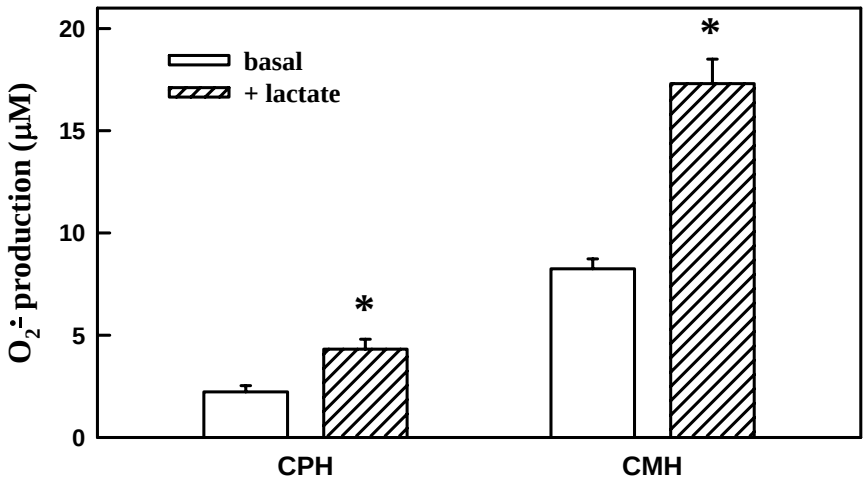


Figure 6. Detection of $O_2^{\bullet-}$ release in Langendorff guinea pig heart preparation under nonstimulated conditions and after induction of $O_2^{\bullet-}$ release with lactate using spin probes CPH and CMH. Production of $O_2^{\bullet-}$ was determined in the presence or absence of L-lactate (10 mM) during perfusion with a Krebs-Henseleit solution at constant flow (11 ml/min) and at 37°C. Air free solutions of CPH or CMH were infused in perfusion solutions via additional pipe connections at a constant rate to reach 800 μM final concentration of each spin probe. Samples were collected into Eppendorf tubes every 2-min and immediately analyzed by ESR. Data are mean ± SEM (n = 4). * $p < 0.05$ vs. basal value.

DISCUSSION

This study describes for the first time the properties and applications of a new cell permeable cyclic hydroxylamine, CMH. CMH has been successfully applied for detection of slow extra- and intracellular $O_2^{\bullet-}$ production, from xanthine oxidase in blood plasma, uncoupled eNOS and in isolated hearts.

In a cell free system, we clearly show that $O_2^{\bullet-}$ released from a xanthine/xanthine oxidase generating system efficiently oxidizes CMH with formation of the nitroxide radical $CM^{\bullet}$. This was confirmed using a previously described method with Cu,Zn-SOD as the competitive inhibitor [38]. Interestingly, even in presence of physiological concentration (1.5 U/ml) of the Cu,Zn-SOD ($k_{O_2^{\bullet-}} = 1.8 \times 10^9\ M^{-1}s^{-1}$) [41], it was possible to detect slow $O_2^{\bullet-}$ production from xanthine oxidase using CMH (300 nM/min, Fig. 2G) but not CPH (100 nM/min, Fig. 1C). Only a high concentration (60 U/ml) of Cu,Zn-SOD completely prevented inhibited reaction of $O_2^{\bullet-}$ with CMH (Fig. 1H). Therefore, the higher scavenging efficacy of $O_2^{\bullet-}$ by CMH ($1.2 \times 10^4\ M^{-1}\ s^{-1}$, scheme 1) represents an important advantage compared to commonly applied nitrone spin traps such as DMPO or DEMPO ($30\ M^{-1}s^{-1}$).

The properties of CMH allow detection of slow $O_2^{\bullet-}$ production in blood plasma with xanthine oxidase and in presence of not only physiological concentrations of Cu,Zn-SOD [41], but also in the presence of physiological concentrations of competitive antioxidants such as vitamin C (2 – 20 μg/ml) [43], SH-group-containing proteins and low molecular thiols (200 – 250 μM) [44]. Of note, not the only higher scavenging efficacy of $O_2^{\bullet-}$, but also high stability of $CM^{\bullet}$ nitroxide in the presence of reducing agents such

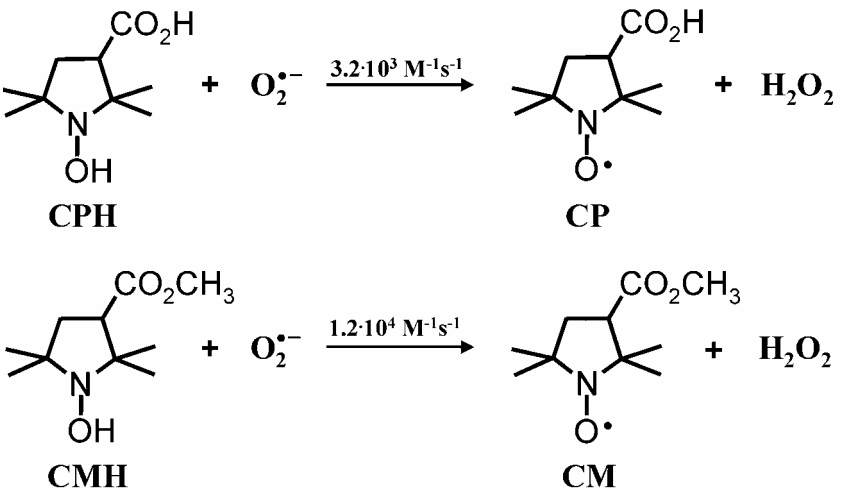
as ascorbate or glutathione [38], permit detection of $O_2^{\bullet-}$ using CMH (530 nM/min, Fig. 2) more efficiently than CPH (220 nM/min, Fig. 2).

Detection of $O_2^{\bullet-}$ can potentially interfere with H_2O_2 in the presence of neutrophils or monocytes releasing MPO. Our data show that neither CMH nor CPH directly react with H_2O_2. Interestingly, in contrast to CPH, the presence of MPO did not stimulate oxidation of CMH, underlining the advantage of CMH for quantification of $O_2^{\bullet-}$ production (Fig. 3) during inflammatory processes.

Previously, it has been shown that $ONOO^-$ causes uncoupling of eNOS in aortas, which is likely due to the oxidation of 5,6,7,8-tetrahydrobiopterin (BH_4) to 7,8-dihydrobiopterin [28-30]. Uncoupled eNOS is an important source for $O_2^{\bullet-}$ production in endothelial cells. We studied $O_2^{\bullet-}$ production in BAECs treated with peroxynitrite. The high reactivity of CMH with $O_2^{\bullet-}$ and the higher penetration rate across the cell membrane (CMH reached 18.1 % steady state concentration after incubation for 2 min (Fig. 4)), allowed us to detect a 3-fold increase in intracellular $O_2^{\bullet-}$ production from uncoupled eNOS after treatment of endothelial cells with peroxynitrite (Fig. 5).

The chronically increased $O_2^{\bullet-}$ production from the vascular NAD(P)H oxidases by increased intracellular $NADH/NAD^+$ ratio, may contribute to increased intracellular oxidative stress in coronary diseases [26]. Bunger et al. demonstrated that lactate increased the intracellular $NADH/NAD^+$ ratio with a subsequent increase in NAD(P)H-oxidase activity in isolated hearts [27]. Excessive exercise may increase local concentration of lactate reached in perfused guinea pig hearts up to 10 mM [45]. This work demonstrates increases in $O_2^{\bullet-}$ production induced by 10 mM lactate up to 9 μM detected by CMH, while only 2 μM was detected by CPH in hearts (Fig. 6). This 4-fold higher efficiency to scavenge $O_2^{\bullet-}$ using CMH demonstrated the higher cell permeability and efficacy of CMH to scavenge slow intracellular $O_2^{\bullet-}$ production.

In summary, our study is the first to show that $O_2^{\bullet-}$ production in blood plasma, endothelial cells with uncoupled eNOS and lactate-treated isolated guinea pig hearts using the new cell permeable cyclic hydroxylamine CMH, which allows effective detection of low amounts of intra- and extracellular $O_2^{\bullet-}$ due to high cell permeability and reactivity with $O_2^{\bullet-}$. Previously, we described cyclic hydroxylamines such as 1-hydroxy-3-carboxy-2,2,5,5,-tetramethylpyrrolidine (CPH) and 1-hydroxy-4-phosphonooxy-2,2,6,6-tetramethylpiperidine (PPH), which were used for detection of extracellular $O_2^{\bullet-}$ and reactive oxygen species production in vitro and in vivo [35–37]. In order to overcome non-specific oxidation of cyclic hydroxylamines, it is necessary to use chelating agents, such as deferoxamine or DTPA, and perform additional experiments using various antioxidants and inhibitors specific for the various ROS. For example, $O_2^{\bullet-}$ radicals can be determined as SOD-inhibitable CM-nitroxide formation. The important advantages of all these cyclic hydroxylamines compared to commonly applied nitrone spin traps such as DMPO or DEPMPO is higher efficacy for scavenging of $O_2^{\bullet-}$ ($1.2 \times 10^4\ M^{-1}\ s^{-1}$ for CMH, scheme 1) and higher stability of reaction products in the presence of reducing agents such as ascorbate or glutathione [37,38], which makes cyclic hydroxylamines useful for detection of superoxide in cells, tissue and blood.



Scheme 1. Chemical structure and reaction of spin probes 1-hydroxy-3-carboxy-2,2,5,5,-tetramethylpyrrolidine (CPH) and 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH) with $O_2^{\bullet-}$ and formation of $CP^{\bullet}$ and $CM^{\bullet}$ nitroxides.

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