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Comparison of different methods for measuring the superoxide radical by EPR spectroscopy in buffer, cell lysates and cells.

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Abstract

As superoxide anion is of keen interest in biomedical research, it is highly desirable to have a technique allowing its detection sensitively and specifically in biological media. If electron paramagnetic resonance (EPR) techniques and probes have been individually described in the literature, there is actually no comparison of these techniques in the same conditions that may help guiding researchers for selecting the most appropriate approach. The aim of the present study was to compare different EPR strategies in terms of sensitivity and specificity to detect superoxide (*versus* hydroxyl radical). Three main classes of EPR probes were used, including paramagnetic superoxide scavengers (such as nitroxides TEMPOL and mitoTEMPO as well as trityl CT-03), a spin trap (DIPPMPO), and diamagnetic superoxide scavengers (such as cyclic hydroxylamines CMH and mitoTEMPO-H). We analyzed the reactivity of the different probes in the presence of a constant production of superoxide or hydroxyl radical in buffers and in cell lysates. We also assessed the performances of the different probes to detect superoxide produced by RAW264.7 macrophages stimulated by phorbol 12-myristate 13-acetate (PMA). In our conditions and models, we found that nitroxides were not specific for superoxide. CT-03 was specific, but the sensitivity of detection was low. Comparatively, we found that nitron DIPPMPO and cyclic hydroxylamine CMH were good candidates to sensitively and specifically detect superoxide in complex biological media, CMH offering the best sensitivity.

Key words: superoxide; EPR ; ESR ; hydroxylamines; trityls; nitroxides; nitrones; spin-trapping

Introduction

Reactive oxygen species (ROS) including superoxide are produced during normal aerobic metabolism in cells and are known to play a key role in the regulation of physiological processes such as cell signaling and cellular defense against pathogens. Physiologically, ROS production is tightly regulated to low/moderate levels by enzymes such as superoxide dismutase (SOD), catalase and by the antioxidant system [1,2]. However, under pathological circumstances, regulatory systems can become deficient and/or ROS production is exacerbated [3-7]. The superoxide radical is a reactive species formed by the one-electron reduction of oxygen. It is mainly produced when electrons leak from complexes I and III of the mitochondrial electron transport chain and by NADPH oxidases. Roles of superoxide are presently investigated in pathologies such as hypertension and cancer metastasis [3,8-10]. Since superoxide and its production are potential pharmacological targets, it is crucial to validate analytical tools that allow specific and sensitive detection of the radical. Nowadays, the most common techniques used for this purpose are fluorescence and electron paramagnetic resonance (EPR).

Fluorescent techniques are inexpensive, fast, easy and widely used in biomedical research. However, the value of fluorescent probes to measure superoxide is controversial [11-13]. Most commonly used probes are dihydroethidium (HE) and its mito-derivatives mito-HE and mito-SOX, dichlorodihydrofluorescein diacetate (DCFH-DA) and ferricytochrome c. The reaction between superoxide and HE generates fluorescent product 2-OH-E⁺. However, other oxidation products are generated in cellular and biological systems. Therefore, superoxide-specific products can only be reliably identified by HPLC [11,12,14,15]. As the chemical reactivity of mito-HE and mito-SOX is similar to the reactivity of HE, all limitations and recommendations are applicable to these mito-derivatives as well [11,14,15]. DCFH-DA is a cell-permeable probe that is hydrolyzed intracellularly, and the DCFH carboxylate anion is trapped inside cells. DCFH reacts with many oxidants to form an intermediate DCF[•] radical that then reacts with oxygen to form superoxide. The dismutation of superoxide leads to an artefactual amplification of the fluorescence [11,16]. In addition, the release of cytochrome c during apoptosis and the presence of redox-active metals may also promote DCFH oxidation [11]. The reduction of ferricytochrome c is another widely used method to detect extracellular superoxide production. However, it is not highly sensitive, it cannot detect intracellular superoxide production and cytochrome c could be oxidized by H₂O₂ and other compounds [12].

EPR is an alternative of choice because it allows a sensitive detection of free radicals in biological media. The superoxide radical is a short-lived species that cannot be directly detected by EPR. In order to detect superoxide, several EPR methods have been developed. They are inherently linked to the nature of the probe that is used (**Fig.1**). Currently, three main classes of EPR probes are used to detect superoxide: spin traps, paramagnetic superoxide scavengers (such as nitroxides and trityls) and diamagnetic superoxide scavengers (such as cyclic hydroxylamines).

Spin traps (nitrones and nitroso compounds) are stable diamagnetic species that produce paramagnetic compounds after their reaction with short-lived radicals (**Fig.1**). Nitron spin traps such as 5-diisopropoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide (DIPPMPO) allow the detection of oxygenated radicals such as the hydroxyl radical and superoxide. The stable spin adduct presents an EPR spectrum that is a fingerprint of the trapped free radical. Despite several drawbacks, it is often recognized as a "gold standard" to measure the type of ROS produced [17]. Spin traps have already been tested to measure superoxide in cells [18] and in intact mitochondria [19].

Paramagnetic superoxide scavengers are molecules that react with superoxide to form diamagnetic species. Nitroxides such as 4-Hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (TEMPOL) and (2-(2,2,6,6-tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl)triphenylphosphonium chloride (MitoTEMPO) provide a

three-lines spectrum. The reaction of nitroxides with superoxide leads to a decrease in their EPR signal (**Fig.1**) because of the formation of an oxoammonium transient species that is further reduced in hydroxylamine in the presence of other reductants, such as NADH [20]. MitoTEMPO is reported to be a specific mitochondrial superoxide scavenger and is often used to target mitochondrial superoxide production [3,9,21]. Tetrathiatriarylmethyl (or TAM or trityl) radicals belong to another class of paramagnetic superoxide scavengers. They are characterized by a single EPR line with a narrow linewidth (**Fig.1**). It has been reported that they react with superoxide and peroxy radicals [22]. As an example, TAM OX063 has been used to detect superoxide in stimulated polymorphonuclear neutrophils [23].

Diamagnetic scavengers (such as cyclic hydroxylamines) share with spin traps their ability to form paramagnetic species after their reaction with ROS (**Fig.1**). In the presence of free radicals, they undergo a non-specific oxidation process to form their corresponding nitroxides. A three-lines EPR spectrum is produced after reaction with several free radicals and oxidative species but not with H_2O_2 . Many studies have been performed using this class of molecules to detect free radicals *in vitro*, mainly superoxide [24-26].

As there exists a high interest in superoxide anion and its implications in pathogenesis, it becomes mandatory to have a technique allowing its detection sensitively and specifically in biological media. While the EPR techniques and probes have been described individually in the literature, no systematic comparison was realized to highlight which EPR probes ones should use for a define purpose. The aim of the present study was to compare the different EPR strategies in terms of sensitivity and specificity to detect superoxide (*versus* hydroxyl radical). For that purpose, we first incubated the different probes in buffer in the presence of different fluxes of superoxide (using variable concentration of xanthine oxidase (XO) 5, 2.5 or 1 mU/mL) or hydroxyl radical. We then incubated the probes in cell lysates while producing superoxide (XO, 5 mU/mL) or hydroxyl radical to compare their performances in media containing reductants and enzymes. Finally, we assessed the performances of the different probes to detect superoxide produced by RAW264.7 macrophages stimulated by phorbol 12-myristate 13-acetate (PMA), a NOX2 activator [27].

Material and methods

Reagents

DIPPMPO (5-diisopropoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide) was purchased from Radical Vision (Marseille, France) and from Enzo Life Science (Antwerpen, Belgium). CMH (1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine), TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl), mitoTEMPO ((2-(2,2,6,6-tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl)triphenylphosphonium chloride) and mitoTEMPO-H (1-hydroxy-4-[2-(triphenylphosphonio)-acetamido]-2,2,6,6-tetramethylpiperidine) were from Enzo Life Science. CT-03 (Finland radical) was synthesized as previously described [28,29]. Catalase conjugated with polyethylene glycol (PEG-CAT), superoxide dismutase conjugated with polyethylene glycol (PEG-SOD), superoxide dismutase (SOD), catalase (CAT), xanthine oxidase (XO), hypoxanthine (HX), hydrogen peroxide, ferric sulfate, diethylene triamine pentaacetic acid (DTPA), cytochrome c oxidase and phorbol 12-myristate 13-acetate (PMA) were from Sigma-Aldrich (Overijse, Belgium). Dulbecco's modified Eagle medium (DMEM), (catalogue number: 41966029) and phosphate buffered saline solution (PBS), (catalogue number: 10010023) were from ThermoFisher.

Superoxide and hydroxyl production in PBS and in lysates

The concentrations of the different reagents and probes were selected according to literature. PBS concentration was 0.01 M, pH 7.4. SiHa cells were cultured in T-75 flask up to confluence and were then lysed by sonication (20 s, Labsonic U, B. Braun). Superoxide was produced using a XO/HX system: XO (final concentration: 5 mU/mL) was added to a PBS solution (or lysate) containing HX (1 mM) and DTPA (1 mM). Other superoxide fluxes were also tested in PBS (XO: 2.5 and 1 mU/mL) to compare the sensitivity of the different EPR probes. Superoxide production rate was measured by the cytochrome c assay. Briefly, the reduction of 50 μ M ferricytochrome c to ferrocytochrome c with various enzyme concentrations was monitored by measuring the absorption at 550 nm using a SpectraMax M2 spectrophotometer (Molecular Devices, Wokingham, UK): the slope of the concentration of cytochrome c oxidized as function of time provides the production rate of superoxide. In our experimental conditions, the measured rates of superoxide production were 1.97, 1.01 and 0.44 μ M/min using 5, 2.5 and 1 mU/ml XO, respectively. We selected 10 minutes as end point to quantify the performances of the probes in PBS and lysates experiments. The hydroxyl radical was produced with the Fenton reaction: hydrogen peroxide (1 mM) was added to a PBS solution (or lysate) containing DTPA (0.1 mM) and FeSO_4 (0.1 mM). Concentrations of the probes were selected according to literature and absence of cell toxicity: spin trap DIPPMPO (50 or 10 mM), diamagnetic scavengers mitoTEMPO-H (0.5 mM) and CMH (0.5 mM), paramagnetic scavengers trityl CT-03 (30 μ M), TEMPOL (30 μ M) and mitoTEMPO (30 μ M) [9,18,22,24,26,30]. When nitroxides were used as superoxide scavenger in PBS buffer, NADH 1 mM was added to media. To assess the specificity of the reactions, SOD (300 U/mL) or catalase (300 U/mL) were added to experimental media. The pH of the different media were checked (mean pH measured: 7.4).

Culture and stimulation of RAW 264.7 macrophages

RAW 264.7 were purchased from the American Type Culture Collection (ATCC) and cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin under 5% CO_2 at 37°C. Confluent cells were stimulated with 5 μ M of PMA during 20 min at 37°C. Cells were then washed with PBS, harvested and resuspended in PBS to perform EPR experiments. RAW264.7 macrophages (20×10^6 cells/ml) were incubated with EPR probes for the EPR measurement. Control experiments were carried out in the presence of PEG-SOD (200 U/mL), PEG-CAT (100 U/mL) or SOD (100 U/mL).

EPR settings

EPR measurements were performed using a Bruker EMX-Plus spectrometer (Bruker, Rheinstetten, Germany), operating in X-band (9.85 GHz) and equipped with a PremiumX ultra low noise microwave bridge and a SHQ high sensitivity resonator.

Typical settings were as follows: microwave power: 2.518 mW (for experiments in PBS and lysates) and 20 mW for cells (to provide a higher signal); modulation frequency: 100 kHz; modulation amplitude: 0.1 mT (for all probes) or 0.03 mT (for CT-03); time constant: 20.48 ms; conversion time: 23.34 ms; sweep width: 15 mT for DIPPMPO, 5.3 mT for nitroxides and cyclic hydroxylamines, 2.5 mT for CT-03.

EPR experiments

Superoxide or hydroxyl were produced in PBS or lysates as described above then aspirated into a gas-permeable polytetrafluoroethylene (PTFE) tubing that was placed in a quartz tube opened at both ends. The tube was directly inserted into the cavity. The EPR cavity was heated at 310 K with air during all experiments. A first EPR measurement was performed 2 min after the reaction has started. Cell viability was verified after all experiments (trypan blue assay). All experiments were done in triplicates except the controls on cells. Computer simulations were performed using the Bruker Xenon Spin fit program.

Presentation of the data

Data are represented as means $\pm$ SEM. The results are expressed in μM thanks to calibration curves. All experiments were performed in triplicates, except controls on cells. One-way ANOVA test was performed to compare the yield of the superoxide trapped.

JUST ACCEPTED

Results

Paramagnetic scavenger: nitroxides

We first tested the reactivity of two nitroxides (mitoTEMPO and TEMPOL) towards the superoxide anion (XO: 5 mU/mL) and the hydroxyl radical in PBS buffer. As described in the literature, addition of NADH to the media was required to promote hydroxylamine formation in the presence of superoxide [20,30,31] (**Fig.S1**). The concentration of TEMPOL significantly decreased by 16.4 μ M after 10 min ($p < 0.001$) in the presence of superoxide production (XO: 5 mU/mL). In the presence of SOD, no decrease of nitroxide concentration was observed (**Fig. 2A**). We also tested our molecules in the presence of two other superoxide fluxes to test their sensitivity (**Fig. S2A and S2B**). A decrease by 3.7 μ M and 0.5 μ M TEMPOL concentration (6.46 μ M and 1.16 μ M for mitoTEMPO) was observed after 10 min when using 2.5 and 1 mU/ml of XO, respectively (**Fig. S2A and S2B**). In the presence of the hydroxyl radical, the initial concentration of TEMPOL remained unchanged with or without NADH (**Fig. 2A**). Similar results were obtained for mitoTEMPO (**Fig. S3A**).

In cell lysates, the reactivity of the probes towards superoxide and the hydroxyl radical was monitored without addition of NADH. When superoxide was produced (XO 5 mU/mL), the concentration of TEMPOL and mitoTEMPO decreased by 9.7 μ M and 6.88 μ M after 10 min, respectively. More surprisingly, they also reacted with the hydroxyl radical and lost about 3 μ M of their initial concentrations after 10 min of incubation. This decrease was significant compared to catalase ($p < 0.001$) (**Fig. 2B and Fig. S3B**). SOD and catalase inhibited the hydroxylamines formation induced by the production of superoxide and hydroxyl, respectively ($p < 0.001$) (**Fig. 2B**).

In macrophages stimulated by PMA, the initial concentration of TEMPOL decreased by 7.87 μ M whereas in the presence of unstimulated cells only 1.75 μ M was lost after 10 min (**Fig. 2C**). In the presence of SOD or PEG-SOD, the nitroxide concentration decrease was 3.2 μ M and 3 μ M after 10 min, respectively, meaning that the main part of the signal decrease was caused by superoxide. We also observed that PEG-CAT partially reduced the concentration decrease observed with PMA (only 6 μ M after 10 min), suggesting that superoxide was not the sole factor responsible for the loss of paramagnetic species (**Fig. 2C**). Similar results were obtained with mitoTEMPO, except that the nitroxide concentration decrease induced by PMA-stimulated cells was not inhibited by PEG-CAT, thus suggesting that superoxide was the only cause of the signal decrease for this compound (**Fig. S3C**).

Paramagnetic scavenger: trityl

In PBS buffer, CT-03 decreased by 7.6 μ M after 10 min when superoxide was produced (XO 5 mU/mL) (**Fig. 3A**). When incubated in the presence of SOD, no significant decrease of trityl concentration was observed (**Fig. 3A**). CT-03 concentration decreased by 3.4 μ M and 2.73 μ M after 10 min using 2.5 and 1 mU/mL, respectively (**Fig. S2C**). In the presence of the hydroxyl radical, the initial trityl concentration remained stable (**Fig. 3A**).

In cell lysates, CT-03 concentration also decreased in the presence of superoxide but to a much lower extent than in PBS. This decrease was significant compared to SOD ($p < 0.001$). As expected, the concentration of CT-03 remained stable in the presence of the hydroxyl radical and did not show any decrease (**Fig3B**).

CT-03 did almost not react with superoxide in the presence of unstimulated macrophages. When macrophages were stimulated by PMA, the concentration decreased by 3 μ M of its initial signal intensity compared to 0.35 μ M in unstimulated cells after 10 min. PEG-SOD inhibited this decay (decrease of trityl concentration by 0.44 μ M after 10 min) whereas PEG-CAT had almost no impact. This suggests that the trityl concentration decrease was caused only by superoxide (**Fig. 3C**).

Spin trap: DIPPMPO

Among potential spin traps, we decided to investigate DIPPMPO because this compound presents a higher membrane permeability compared to other nitrones and because its adduct with superoxide has been shown

to be more stable than other spin traps [32]. In the presence of superoxide, an EPR spectrum corresponding to the DIPPMPO-OOH adduct rapidly appeared in PBS buffer (**Fig. 4A**), and the superoxide adduct concentration increased until it reached a steady-state level (**Fig. 4A**). Indeed 12.6 μM of DIPPMPO superoxide adduct was formed in 10 minutes. Simulation of the spectrum confirmed its correspondence with the DIPPMPO-superoxide adduct (**Fig. S4A**). Same results were obtained with the two other superoxide fluxes but to a lower extent: 8.07 μM and 3.6 μM after 10 min at 2.5 and 1 mU/mL XO respectively (**Fig. S2D**). In the presence of SOD, no EPR signal appeared (**Fig. 4A**). When the hydroxyl radical was produced in PBS buffer, the EPR signal obtained was characteristic of the DIPPMPO-OH adduct, as confirmed by our simulation (**Fig. 4A and S4B**).

When superoxide was produced in cell lysates, the observed EPR spectrum was a mix between DIPPMPO-OH and DIPPMPO-OOH adducts, according to our simulations (**Fig. 4B**). Of note, this EPR signal changed over time. It was completely inhibited by SOD but not by catalase, suggesting that both adducts originated from superoxide production. Concentration of superoxide adduct raised during about one hour and reached 5.55 μM after 10 min incubation. When the hydroxyl radical was produced, the spin trap provided only a spectrum that was consistent with the production of DIPPMPO-OH, as confirmed by simulations. This signal was inhibited by catalase but not by SOD (**Fig. 4B**).

DIPPMPO provided a non-detectable EPR signal when incubated in the presence of unstimulated macrophages. When incubated with PMA-stimulated cells, an EPR spectrum corresponding mainly to the DIPPMPO-OH adduct appeared. This concentration of spin adduct was 3.1 μM after 10 min, was significantly decreased in the presence of PEG-SOD (1.2 μM after 10 min), thus demonstrating that superoxide was the main contributor to the EPR signal observed. PEG-CAT partially inhibited the signal observed with PMA-stimulated macrophages (2.6 μM after 10 min) suggesting that a small part of the signal was caused by the hydroxyl radical. In addition, we found that SOD inhibited the signal to a lower extent than PEG-SOD: the concentrations of spin adduct were 1.87 μM and 1.2 μM after 10 min, respectively. The association of PEG-SOD and PEG-CAT further decreased the signal compared to PEG-SOD alone (**Fig. 4C**).

Cyclic hydroxylamines

Among cyclic hydroxylamines, we selected CMH (a 5-membered ring known to be membrane permeable) [24] and mitoTEMPO-H (a six-membered ring linked to a triphenylphosphonium moiety)[24]. When superoxide was produced in PBS in the presence of one of these two molecules, we observed the appearance of a three line-spectrum typical of a nitroxide [24]. As expected, the increase in nitroxide formation of both molecules was completely inhibited by SOD (**Fig. 5A and S4A**). Interestingly MitoTEMPO-H only produced 6 μM nitroxide compared to CMH that formed 21.3 μM nitroxide in 10 min (**Fig. 4A and S5A**). At 2.5 and 1 mU/mL of XO, CMH formed 11.2 μM and 3.64 μM nitroxide compared to 4 μM and 2 μM nitroxide with mitoTEMPO-H after 10min (**Fig. S2E and S2F**). When the hydroxyl radical was produced in PBS, both cyclic hydroxylamines provided a larger increase in nitroxide concentration compared to the one observed in the presence of superoxide. With CMH, the increase in nitroxide concentration reached a "plateau" after 15 min. This was not observed with mitoTEMPO-H. Surprisingly, the addition of catalase only partially inhibited this increase in signal intensity ($p < 0.001$) (**Fig. 5A and S5A**).

In cell lysates, nitroxides were formed from cyclic hydroxylamines in the presence of superoxide (8 μM for CMH and 1.6 μM for MitoTEMPO-H after 10min), which was completely inhibited by SOD (**Fig. 5B and S5B**). In addition, we observed that the conversion of CMH to its nitroxide analogue was larger compared to mitoTEMPO-H (about five times larger after 10 min) (**Fig. 5B and S5B**). However, it was smaller than in PBS for both molecules. In the presence of the hydroxyl radical, CMH was more slowly converted into nitroxide than what was observed in PBS,

but reached a plateau at the same nitroxide concentration (**Fig 5B**). Similar results than those obtained in PBS were recorded for mitoTEMPO-H (**Fig. S5B**).

An increase in nitroxide was observed when CMH was incubated in the presence of PMA-stimulated macrophages: after 10 min, the concentration was 17.63 μM in PMA-stimulated macrophages compared to 3.58 μM in unstimulated cells. Concentration of nitroxide apparition with PEG-SOD was about the same than observed in unstimulated cells (**Fig. 5C**). Interestingly, SOD was less efficient in inhibiting the nitroxide formation (8.5 μM after 10 min) compared to PEG-SOD (4.9 μM after 10 min) (**Fig. 5C**), suggesting that intracellular and extracellular superoxide were at the origin of the EPR signal recorded. PEG-CAT had a very small inhibitory effect on nitroxide formation (16.57 μM after 10 min) (**Fig.5C**),further suggesting a limited contribution of the hydroxyl radical to oxidize the hydroxylamine. Similar results were observed for mitoTEMPO-H (**Fig.5C and S5C**).

Discussion

While superoxide plays a key role in physiology and pathophysiology [3,8-10], it is still challenging to detect this species with high sensitivity and high specificity. Several promising EPR methods have been described to quantify superoxide production. As these methods have been developed independently of each others, it could be interesting for researchers in the field to have elements of comparison that may help them to choose the most adapted method for a given research purpose. A previous EPR study from Dikalov already compared two spin traps (EMPO and DEPMPO) together with diamagnetic superoxide scavenger CMH [26]. Another study compared the ability of different spin traps (DMPO, BMPO, DEPMPO, DIPPMPO) to detect superoxide [18]. Our present study extends the comparison by including paramagnetic superoxide scavengers (nitroxides TEMPOL and mitoTEMPO, and trityl radical CT-03), spin trap DIPPMPO and diamagnetic scavengers (CMH and mitoTEMPO-H). Our purpose was a practical approach using conditions that have been used and optimized in the literature rather than addressing a mechanistic approach, which has been already described for most probes[20,22,32,33]. Comparison was done in media of increasing complexity: simple PBS buffer, cell lysates (containing redox modulators, such as chemical reductants and enzymes) and cells. It focused on the sensitivity of the methods and the specificity of detection regarding their reactivity towards different radicals (superoxide vshydroxyl) and our ability to specifically inhibit the reactivity of the probes using specific enzymes.

Figure 6 provides a direct comparison in terms of sensitivity and specificity. This figure represents the % of superoxide effectively trapped by the EPR probes (which was inhibited by SOD) in PBS (**Fig. 6A**) and lysates (**Fig. 6B**)after 10 mincompared to the amount of superoxide produced in our system as measured by the cytochrome c assay.

In PBS, the sensitivity of detection of superoxide was significantly better for CMH and for nitroxides (mitoTEMPO and TEMPOL) compared to other probes (**Fig. 6A**). In PBS buffer, the reactivity of nitroxides was fully inhibitable by SOD (**Fig. 2A, S3A**). For the spin trap, the EPR spectrum of the spin adduct was the fingerprint of the radical produced (**Fig. 4A**). Superoxide adduct formation rapidly increased before reaching a plateau after 20 min.For hydroxylamines we found that the formation of the nitroxide after reaction of CMH or mitoTEMPO-H with superoxide was completely abolished in the presence of SOD (**Fig. 5A and S5A**). Of note, those probes are not intrinsically specific for superoxide. When the hydroxyl radical was produced in PBS, we observed a higher EPR signal compared to the situation where superoxide was produced for both compounds. Intriguingly, the signal recorded in the presence of hydroxyl production was only partly inhibitable by catalase.

In cell lysates, the sensitivity of all the probes to detect superoxide anion was lower than in PBS (**Fig. 6B**). TEMPOL significantly provided the best performances in these conditions (**Fig. 2B, 6B**). Of note, the reaction of the hydroxyl radical with nitroxides (TEMPOL and mitoTEMPO) in lysates also led to a decrease of nitroxides initial concentrations (**Fig. 2B, S3B**),suggesting a non-specificity of nitroxides for superoxide. These results are consistent with previous reports [33,34]. Importantly, we noticed that, in cell lysates, using TEMPOL

and mitoTEMPO, the formation of diamagnetic species due to superoxide was only partially inhibitable by SOD (**Fig. 2B, S3B**). The decrease in the initial radical concentration of CT-03 in the presence of superoxide was much lower than for nitroxides (**Fig. 3B**). However, this decay was fully inhibitable by SOD contrarily to nitroxides highlighting the specificity of the reaction of CT-03 towards superoxide. For the spin trap, spectrum arising from a mixture of superoxide and hydroxyl adducts was obtained (**Fig. 4B**) in the presence of superoxide. Importantly, the suppression of that signal by SOD, but not by catalase, indicated that the signal was due to the transformation of the superoxide adduct into the hydroxyl adduct or that superoxide anion is the source of the hydroxyl radical adduct detected. For the cyclic hydroxylamines, a similar observation than in PBS was done in cell lysates where we observed a higher performance of CMH compared to mitoTEMPO-H to detect superoxide (**Fig. S6, 6B**). SOD inhibited nitroxides formation for both compounds (**Fig. 5B and S5B**). Again, the hydroxyl radical produced in cell lysates provided a higher yield in nitroxide formation compared to superoxide, that could not be fully inhibited by catalase.

In RAW 264.7 cells stimulated by PMA, it has previously been described that superoxide is produced both extracellularly and intracellularly, as it is produced by membrane-bound enzyme NOX-2 [18]. Therefore, we used PEG-SOD that could induce the dismutation of superoxide in both compartments. To compare the performances of the probes in RAW 264.7 macrophages, we did not use the cytochrome c assay as because it measures superoxide only in the extracellular compartment. We used the PEG-SOD inhibitable superoxide trapped to allow the comparison (**Fig. 7**). We observed that CMH presented the highest sensitivity to detect superoxide compared to all other probes. Of note, PEG-CAT partially inhibited the decrease in TEMPOL concentration induced by PMA suggesting that a part of the signal decrease of this nitroxide may also be due to the hydroxyl radical (**Fig. 2C**). This is consistent with the fact that trace formation of hydroxyl radical could occur in these stimulated cells [18]. Overall, these results are in agreement with the experiments carried out on lysates demonstrating the non-specificity of the detection of superoxide by TEMPOL. This observation on cells was not found when using mitoTEMPO (**Fig. S3C**), thus showing a better specificity of mitoTEMPO for superoxide in such conditions. PEG-SOD and SOD completely inhibited the trityl decrease, whereas PEG-CAT had no effect. These enzymatic experiments confirmed that CT-03 is able to detect superoxide specifically in cells. In stimulated RAW 264.7 cells, the EPR spectrum obtained with DIPPMPO was the one of the hydroxyl adduct (**Fig. 4C**). It has been previously shown that DIPPMPO-OOH adduct (peroxide) is converted to DIPPMPO-OH (alcohol) by peroxidases (e.g. glutathione peroxidase (GPx)) [17]. This is consistent with the inhibition of DIPPMPO-OH by SOD that we observed. Consistently with the results obtained by Abbas et al. [18], we found that this signal was inhibited by PEG-SOD and partially by PEG-CAT meaning that the larger part of the signal originated from a superoxide production. PEG-SOD was slightly more efficient than SOD in the inhibition of the adduct formation, indicating that DIPPMPO was able to trap superoxide in intracellular and extracellular compartments. The small effect of CAT could potentially be explained because CAT and PEG-CAT can down regulate superoxide production due to redox dependence of superoxide sources such as mitochondria and NADPH oxidase [35]. Several studies described superoxide production using cyclic hydroxylamines in both stimulated and unstimulated cells [24-26,36]. Here, we found that CMH sensitively detected the superoxide anion in PMA-stimulated cells. PEG-SOD and SOD (to a smaller extent) inhibited the apparition of their corresponding nitroxide (**Fig. 5C and S5C**). As PEG-SOD inhibited the signal apparition to the level of unstimulated cells, and as PEG-CAT had almost no influence on the EPR signal, we concluded that the nitroxide obtained was almost exclusively due to the presence of superoxide. Of note, we also observed in this study that mitoTEMPO-H was less sensitive than CMH (**Fig. S6**), results that are consistent with previous results [37]. At this stage, it is difficult to know whether the lower sensitivity of the mito-targeted compounds is due to the triphenylphosphonium moiety (responsible for different

localization and/or reactivity) or to the stability of their structure as the piperidinoxyl radicals are known to be more sensitive to bioreduction than the pyrrolidinoxyl radicals [38].

Based on our systematic comparison in different media, some lessons may be taken to guide the choice of the probes that can be useful for a specific cellular detection of superoxide. While the decay of nitroxides is sensitive to the presence of superoxide, it should be emphasized that the reaction is shared with other ROS (namely the hydroxyl radical), that it was not possible to fully reverse the reaction using specific superoxide inactivators such as SOD or PEG-SOD. Moreover, the concentration of reductants present in cellular media is a factor that also contributes to the decay of nitroxides signal intensity. Therefore, nitroxides should be considered as redox-sensitive probes in general, but are not advised for a specific detection of superoxide. Among paramagnetic scavengers, we found that CT-03 could be a valuable probe as we observed a high specificity of the probe: no cross reactivity with the hydroxyl radical was found, and the use of SOD or PEG-SOD unambiguously demonstrated the involvement of superoxide in cellular measurements. However, the sensitivity of detection was low compared to other probes (**Fig.6**). For this reason a CT-03 based biradical has been recently developed, enabling the measurement of $O_2^{\cdot-}$ by following the increase of the EPR signal corresponding to a CT-03 based monoradical. However, this probe has not been used in cellular systems yet [39]. Spin trap DIPPMPO was sensitive to superoxide production. It should be noticed that the EPR signals obtained in cells and lysates were, not *per se*, a signature of superoxide, as we observed a small to large contribution of the hydroxyl radical adduct. However, as extensively documented by Abbas [18] who used PMA-stimulated RAW 264.7 macrophages, it is possible to assign the origin of the signal by using appropriate controls with PEG-SOD and catalase. Among diamagnetic scavengers, CMH offered the highest sensitivity in all conditions tested, including in cellular systems. In PMA-stimulated RAW 264.7 macrophages, the involvement of superoxide was unambiguously ascribed. However, one should always keep in mind that other ROS, such as the hydroxyl radical, may also contribute to the appearance of the EPR signal, therefore requiring the use of appropriate controls to exclude their potential involvement. Our “practical approach” study suffers from potential limitations that should be emphasized. The concentrations of the probes that were used were based on existing literature. Also, we monitored the production of free radicals in a rather short time window. We cannot exclude that the reactivity of the probes could be different at other concentrations and using a longer time window. It is also important to note that reactivity was measured under a continuous air flow equilibrated at 310 K, and it is likely that our results could not directly be extrapolated to other conditions. Finally, our conclusion is likely limited to the probes that we used in the comparison. Some lessons obtained with a particular probe can not be directly extrapolated to others, even within the same structural family. Still, we believe that our systematic comparison will be very helpful for researchers who will use these probes in their research, and that our procedure for qualifying (or disqualifying) probes could be used when validating other potential EPR candidates for measuring superoxide.

Conclusion

EPR is a highly performant and sensitive tool to detect superoxide production. Our results suggest that, in our conditions and models, the classical nitroxide DIPPMPO as well as the cyclic hydroxylamine CMH are good candidates to sensitively and specifically detect superoxide in complex biological media when used with the appropriate controls. Therefore, these probes could be further used as tools to measure and quantify ROS, provide the nature of the radicals implicated, their localization and could help to better understand the pathological conditions in which ROS are thought to be involved.

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Declaration of interest statement

The authors report no conflict of interest.

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Figure captions

Figure 1

Structures of the EPR-based superoxide sensors used in the present study (top), expected EPR signal (center) and expected evolution of the EPR signal intensity over time (bottom).

Figure 2

Evolution of TEMPOL concentration in the presence of free radicals in different media.

- A)** Evolution of the EPR signal intensity of the low component of TEMPOL (30 μ M) in the presence of superoxide and NADH (1 mM) in PBS buffer, in the presence (blue) or absence (green) of SOD (300 U/mL). Stability of TEMPOL concentration when incubated with the hydroxyl radical in PBS (pH 7.4) in the presence (black) or absence (red) of catalase (300 U/mL). Superoxide was generated using HX (1 mM), DTPA (1mM) and XO (5 mU/mL). Hydroxyl radical was produced with DTPA (0.1 mM), FeSO_4 (0.1 mM) and hydrogen peroxide (1mM).
- B)** Sensitivity for superoxide and specificity towards hydroxyl of TEMPOL in lysates. Evolution of TEMPOL (30 μ M) concentration in the presence of superoxide (green), superoxide and SOD 300 U/mL (blue), hydroxyl (red) and hydroxyl radical with catalase 300 U/mL (black). Superoxide was generated using HX (1 mM), DTPA (1 mM), and XO (5 mU/mL). Hydroxyl radical was produced with DTPA (0.1 mM), FeSO_4 (0.1 mM) and hydrogen peroxide (1 mM).
- C)** Detection of superoxide in PMA-stimulated cells with TEMPOL at 30 μ M (green), in the presence of PEG-CAT 100 U/mL (blue), or PEG-SOD 200 U/mL (red) or SOD 100 U/mL (purple) or PEG-CAT 100 U/mL+ PEG-SOD 200 U/mL (black) or in the presence of unstimulated cells (light blue). Experiments were performed with 20×10^6 cells/mL and 1 mM DTPA in PBS (pH 7.4).

Figure 3

Evolution of CT-03 concentration in the presence of free radicals in different media.

- A)** Sensitivity for superoxide alone (green) or in the presence of SOD 300 U/mL (blue) and specificity towards the hydroxyl radical alone (red) or with catalase 300 U/mL (black) of CT-03 (30 μ M) in PBS (pH7.4). Superoxide was generated using HX (1 mM), DTPA (1 mM), and XO (5 mU/mL). Hydroxyl radical was produced with DTPA (0.1 mM), FeSO_4 (0.1 mM) and hydrogen peroxide (1 mM).
- B)** Evolution of the EPR signal of CT-03 (30 μ M) in lysates when incubated with superoxide in the presence (blue) or absence (green) of SOD (300 U/mL) or incubated with the hydroxyl radical in the presence (black) or absence (red) of catalase (300 U/mL). Superoxide was generated using HX (1 mM), DTPA (1 mM), and XO (5 mU/mL). Hydroxyl radical was produced with DTPA (0.1 mM), FeSO_4 (0.1 mM) and hydrogen peroxide (1mM).
- C)** Detection of superoxide in PMA-stimulated cells with CT-03 30 μ M (green), in the presence of PEG-CAT 100 U/mL (blue), or PEG-SOD 200 U/mL (red) or SOD 100 U/mL (purple) or PEG-CAT 100 U/mL + PEG-SOD 200 U/mL (black) or in the presence of unstimulated cells (light blue). Experiments were performed with 20×10^6 cells/mL and 1 mM DTPA in PBS (pH 7.4).

Figure 4:

- A) Appearance of the specific EPR signal of DIPPMPO (50 mM) in the presence of superoxide (green), no apparition of the spectrum with SOD 300 U/mL (blue). Specific spectrum in the presence of the hydroxyl radical (red). Superoxide was generated using HX (1 mM), DTPA (1 mM), and XO (5 mU/mL). Hydroxyl radical was produced with DTPA (0.1 mM), FeSO_4 (0.1 mM) and hydrogen peroxide (1 mM).
- B) Apparition of a mix of DIPPMPO-OOH and DIPPMPO-OH adducts in the presence of superoxide produced in lysates (green) and no EPR spectrum observed in the presence of SOD 300 U/ml (blue). Specific spectrum in the presence of the hydroxyl radical (red). Superoxide was generated using HX (1 mM), DTPA (1 mM), and XO (5 mU/mL). Hydroxyl radical was produced with DTPA (0.1 mM), FeSO_4 (0.1 mM) and hydrogen peroxide (1mM).
- C) Detection of superoxide in PMA-stimulated RAW264.7 macrophages with DIPPMPO 10 mM (green), in the presence of PEG-CAT 100 U/mL (blue), or PEG-SOD 200U/mL (red) or SOD 100 U/mL (purple) or PEG-CAT 100 U/mL + PEG-SOD 200 U/mL (black) or in the presence of unstimulated cells (light blue). Experiments were performed with 20×10^6 cells/mL and 1 mM DTPA in PBS (pH 7.4).

Figure 5:

Evolution of the apparition of CMH corresponding nitroxide in the presence of free radicals in different media.

- A) Appearance of nitroxide in the presence of superoxide with (blue) or without SOD 300 U/mL (green), or in the presence of the hydroxyl radical with (black) or without catalase 300 U/mL (red) of CMH (0.5 mM) in PBS (pH7.4). Superoxide was generated using HX (1 mM), DTPA (1 mM), and XO (5 mU/mL). Hydroxyl radical was produced with DTPA (0.1 mM), FeSO_4 (0.1 mM) and hydrogen peroxide (1 mM).
- B) Evolution of the EPR signal of CMH (0.5 mM) in lysates when incubated with superoxide in the presence (blue) or absence (green) of SOD (300 U/ml) or incubated with the hydroxyl radical in the presence (black) or absence (red) of catalase (300 U/ml). Superoxide was generated using HX (1 mM), DTPA (1 mM), and XO (5 mU/mL). Hydroxyl radical was produced with DTPA (0.1 mM), FeSO_4 (0.1 mM) and hydrogen peroxide (1 mM).
- C) Detection of superoxide in PMA-stimulated cells with CMH 0.5 mM (green), in the presence of PEG-CAT 100 U/mL (blue), or PEG-SOD 200 U/mL (red) or SOD 100 U/mL (purple) or PEG-CAT 100 U/mL + PEG-SOD 200 U/mL (black) or in the presence of unstimulated cells (light blue). Experiments were performed with 20×10^6 cells/ml and 1 mM DTPA in PBS (pH 7.4).

Figure 6:

% of superoxide detected by our probes after 10 min compared to the quantity of superoxide produced in the media (Superoxide flux: $1.97 \mu\text{M}/\text{min}$). Superoxide was generated using HX (1 mM), DTPA (1 mM), and XO (5 mU/mL).

- A) In PBS: CMH (black), MitoTEMPO-H (red), DIPPMPO (blue) MitoTEMPO (light grey), TEMPOL (grey), CT-03 (green).
- B) In lysates: CMH (black), MitoTEMPO-H (red), DIPPMPO (blue), MitoTEMPO (light grey), TEMPOL (grey), CT-03 (green).

The letters correspond to a significant difference ($p < 0.05$) Anova one-way with CMH (A), MitoTEMPO-H (B), DIPPMPO (C), mitoTEMPO (D), TEMPOL (E), CT-03 (F).

Figure 7:

PEG-SOD inhibitable superoxide trapped after 10 min in PMA-stimulated cells (20×10^6 cells/ml) and 1 mM DTPA in PBS (pH 7.4). CMH (black), MitoTEMPO-H (red), DIPPMPO (blue) MitoTEMPO (light grey), TEMPOL (grey), CT-03 (green).

The letters correspond to a significant difference ($p < 0.05$) Anova one-way with CMH (A), MitoTEMPO-H (B), DIPPMPO (C), mitoTEMPO (D), TEMPOL (E), CT-03 (F).

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