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Comparative EPR study of different macrophage types stimulated for superoxide and nitric oxide production

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Abstract

Despite the major impact of ROS on human health, their quantification remains difficult and requires an analytical approach, such as the EPR spin trap technique. In this study, a comparative EPR analysis of different macrophage types stimulated for superoxide and nitric oxide production was performed. U937 monocytes, J774A.1, RAW 264.7 and primary mouse (PMM) macrophages were included. In contrast to the U937 cells, all macrophages produced significant EPR signals after stimulation. The use of PMA as stimulator and CM-H as spin probe led to the highest response in EPR signals for detection of $O_2^{\bullet -}$ as nitroxide radical. A combination of LPS and IFN-gamma and the spin trap [$Fe(DETC)_2$] turned out to be the best combination for the production and detection of intracellular NO spin adducts. In conclusion, this study established practical experimental conditions for the EPR analysis of $O_2^{\bullet -}$ and NO produced by different types of activated macrophages.

Keywords: *EPR, macrophage, nitric oxide, superoxide, assay optimization*

Introduction

Reactive oxygen species (ROS) such as the free radicals superoxide ($O_2^{\bullet -}$) and nitric oxide (NO) are products of normal cellular metabolism that are generated by tightly regulated enzymes including NO synthase (NOS), xanthine oxidase and NAD(P)H oxidase isoforms [1]. Although free radicals are mostly associated with biological damage, $O_2^{\bullet -}$ and NO also play an important role in cell signal transduction pathways and as defense against infectious agents [1,2]. ROS cause biological damage only when there is an imbalance between pro- and antioxidants, as shown to occur in several cardiovascular, infectious and pulmonary diseases. Atherosclerosis and hypertension are accompanied by increased $O_2^{\bullet -}$ levels [3] and endothelial dysfunction caused by inactivation of NO [4]. Oxidative stress in chronic obstructive pulmonary disease and asthma is caused by accumulation of macrophages and neutrophils in the lower respiratory tract leading to an exaggerated generation of free radicals, such as $O_2^{\bullet -}$ [5,6]. In infectious diseases, free radicals do protect against microorganisms as destruction of the pathogen is accomplished by phagocytosis followed by the generation of $O_2^{\bullet -}$, H_2O_2, NO and $ONOO^-$ [7]. However, certain microbial organisms like *Mycobacterium* spp., *Listeria* spp. and *Leishmania* spp. are capable to survive and multiply in these ROS-producing macrophages. These examples clearly show the pathophysiological importance of $O_2^{\bullet -}$ and NO, but assessing their *in situ* production remains difficult for several reasons. In many tissues, intracellular basal ROS levels remain at the nanomolar level and are hard to measure because of interactions with cellular proteins such as superoxide dismutase (SOD) and reduced glutathione (GSH). Even with an acceptable detection limit, a next pitfall is that the half-life times of ROS are ultra short and largely dependent on

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different factors, e.g. environment conditions. The more common techniques are based on the reaction of the free radical with a substrate leading to the formation of a spectrophotometrically detectable molecule, such as the cytochrome *C* reduction assay and lucigenin chemiluminescence assay for $O_2^{\bullet-}$ and the Griess reaction for NO [1,8–10]. The cytochrome *C* reduction and dihydroethidium assays lack accuracy, while chemiluminescence techniques (e.g. lucigenin) and techniques with fluorescent probes (e.g. dichlorofluorescein and dihydrorhodamine 123) lack specificity [11–14].

A more sensitive and specific method is electron paramagnetic resonance (EPR). With the introduction of relatively cheap and sensitive EPR instruments (e.g. E-Bioscan from Brüker and MS300 from Magnettech), EPR spin trapping can now more easily be applied by research groups for the detection of NO and $O_2^{\bullet-}$ in biological systems [15–17]. A wide range of spin traps, such as DMPO, DEPMPO and EMPO, and spin probes, such as CM-H, CP-H and PP-H, are now available for detection of $O_2^{\bullet-}$ [16–24]. The use of hydroxylamines to form a stable nitroxide radical after reaction with a free radical has already been known for a long time [25,26]. For example, the reaction of CM-H with $O_2^{\bullet-}$ leads to the formation of the nitroxide radical $CM^{\bullet}$, which can easily be measured with EPR. For detection of NO iron, dithiocarbamate complexes, e.g. $[Fe(MGD)_2]$, $[Fe(DTCS)_2]$ or $[Fe(DETC)_2]$, are frequently used spin traps [24,27–32]. Because of the importance of NO in vascular diseases, many articles describe protocols for the detection of NO in vascular tissue, whole blood or endothelial cells [31–34].

Despite this apparent abundance of literature on EPR, comparative studies measuring NO and $O_2^{\bullet-}$ produced by different types of macrophages are still missing. The aim of the present study is to investigate the experimental conditions needed for EPR analysis of $O_2^{\bullet-}$ and NO produced by different types of activated macrophages. NO (as spin adduct) and $O_2^{\bullet-}$ (as nitroxide radical) levels are detected after stimulation of different types of macrophages, i.e. U937 (monocytes), J774A.1, RAW 264.7 and primary mouse macrophages (PMM) with phorbol 12-myristate 13-acetate (PMA), lipopolysaccharide (LPS), interferon-gamma (IFN-gamma) or tumor necrosis factor–alpha (TNF-alpha).

Materials and methods

Culture media, reagents and animals

Diethyldithiocarbamate sodium salt (DETC), (Z)-1-(N-methyl-N-[6-(N-methylammoniohexyl) amino])-diazen-1-ium-1,2-diolate (MAHMA/NO) and N-(dithiocarbamoyl)-N-methyl-D-glucamine (MGD) were purchased from Alexis Inc. The $O_2^{\bullet-}$ spin probes 1-hydroxy-3-carboxyl-2,2,5,5-tetramethylpyrrolidine (CP-H), 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CM-H), 1-hydroxy-4-phosphonooxyl- 2,2,6,6-tetramethylpiperidine (PP-H) and 3-carboxyl-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl (CP) and special EPR Krebs Hepes buffer (KHB) were obtained from Noxygen (Germany). Diethylenetriaminepentaacetic acid (DTPA), superoxide dismutase (SOD from bovine erythrocytes, lyophilized powder, Sigma-Aldrich S5395 without preservatives), polyethylene glycol-linked superoxide dismutase (PEG-SOD from bovine erythrocytes, lyophilized powder, Sigma-Aldrich S9549 without preservatives), trypan blue solution (0.4%) and the stimulators phorbol 12-myristate 13-acetate (PMA), lipopolysaccharide from *Escherichia coli* 0128:B12 (LPS), interferon-gamma from mouse (IFN-gamma) and tumor necrosis factor-alpha from mouse (TNF-alpha) were supplied by Sigma-Aldrich (Belgium). D-MEM, RPMI-1640 medium, L-glutamine and foetal calf serum (FCS) were purchased from Invitrogen (Belgium). Swiss mice were supplied by Janvier (France). Animal experiments were approved by the ethical committee of the University of Antwerp.

Cell cultures

Different cell types were used: monocytes, primary mouse macrophages and two continuous macrophage cell lines. The mouse macrophage cell line RAW 264.7 was cultured routinely in D-MEM supplemented with 10% heat-inactivated FCS (iFCS). The mouse macrophage cell line J774A.1 and monocytic U937 cells were maintained in RPMI-1640 medium supplemented with 5% iFCS. Primary peritoneal macrophages were collected from Swiss mice 2 days after peritoneal stimulation with a 2% starch suspension. Cells were collected and grown in RPMI-1640 medium supplemented with 200 mM L-glutamine and 5% iFCS. For the experiments with the Griess reagent, the same media were used but without the pH indicator phenol red to avoid interference with the dye. Cell viability was evaluated by adding an equal volume of 0.4% trypan blue solution. Control as well as stimulated cells showed $> 90\%$ viability, except after 48 h stimulation with 100 ng/ml LPS and 5 ng/ml IFN-gamma, resulting in $89.2 \pm 4.5\%$ viability for RAW cells.

Calibration curves

To obtain a calibration curve for the nitroxide radical, $CP^{\bullet}$ was diluted in KHB at a dose range of 100, 75, 50, 25, 10, 5 and 1 μM. These concentrations give a linear calibration curve and all the experimental data were within this range. EPR measurements were performed in capillaries at 37°C. A calibration curve for

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the NO spin adduct signal was obtained using MAHMA NONO-ate as NO donor, first dissolved in a 10 mM NaOH solution (donor concentration of 8 mM) and then further ½ diluted in 1 mM phosphate buffer pH 7.4 (final concentrations of the donor: 0.5–16 μM). After incubation with the spin trap [$Fe(MGD)_2$] samples were frozen in liquid nitrogen and measured with EPR.

In vitro production of $O_2^{\bullet-}$ in stimulated cells

Protocol in T25 flasks. Cells were cultivated in T25 flasks (Sarstedt) until confluence and then harvested. The cells were washed twice in KHB followed by centrifugation at 1500 rpm for 5 min and brought into a final volume of 100 μl KHB containing DTPA (100 μM). To activate cellular $O_2^{\bullet-}$ production, 1×10^6 cells were stimulated with PMA, LPS, IFN-gamma or TNF-alpha at different concentrations and incubation times. To identify the $O_2^{\bullet-}$ radical, SOD or PEG-SOD was added. After stimulation, spin probe (1 mM) was added 50 min prior to EPR measurement.

Protocol in a 24-well plate. Cells were grown in 24-well plates (Sarstedt) using the following conditions: RAW 264.7 and J774A.1 at 0.5×10^6 cells/well 1 day prior to the test and primary mouse macrophages at 1×10^6 cells/well 2 days prior to the test. Before adding the stimulator(s), cells were washed with KHB containing DTPA (100 μM). Since media with serum produce high background noise, all stimulation and EPR measurements were performed in buffer. After stimulation for 5 min to 6 h, depending on the experiment, and after 50 min incubation with the spin probe (37°C, 5% CO_2), 50 μl supernatant or 50 μl cell suspension was brought into a capillary tube for measurement at 37°C.

All results were presented as the nitroxide radical concentration reached after 50 min accumulation in stimulated cells minus the concentration reached in non-stimulated (control) cells.

In vitro production of NO in stimulated cells

Cells were stimulated in T25 culture flasks for 1–6 h with the following stimulators: LPS (10 ng/ml, 100 ng/ml and 1 μg/ml), IFN-gamma (5 ng/ml), TNF-alpha (2 ng/ml), PMA (10 μM) or a combination of LPS and IFN-gamma. The cells were then further incubated with [$Fe(DETC)_2$] or [$Fe(MGD)_2$] for 1 h before harvesting. After removal of the supernatant, re-suspended cells were brought into a 1 ml syringe and frozen in liquid nitrogen. [$Fe(DETC)_2$] was prepared according to Kleschyov and Münzel [33]. Briefly, sodium DETC (7.2 mg) and $FeSO_4.7H_2O$ (4.5 mg) were dissolved in two separate volumes (10 ml) of deoxygenated Krebs solution. Equal volumes of these parent solutions were mixed. The [$Fe(DETC)_2$] colloid solution was used immediately after preparation. [$Fe(MGD)_2$] was made in a similar way: 4.5 mg $FeSO_4.7H_2O$ and 15 mg MGD were dissolved in separate volumes (10 ml) of deoxygenated Krebs solution.

All results were given as the NO spin trap adduct concentration reached after 1 h accumulation of the [NO-$Fe(DETC)_2$] or [NO-$Fe(MGD)_2$] complex in stimulated cells minus the concentration reached in non-stimulated (control) cells.

EPR spectroscopy

EPR measurements were performed on the bench top EPR spectrometer MiniScope MS300 with the temperature controller TC-H02 (Magnettech, Germany). For NO detection, samples were brought in a liquid nitrogen Dewar (Magnettech, Germany) and recordings were made at 77°K. Instrument settings were 10 mW of microwave power, 5 G of amplitude modulation, 100 kHz of modulation frequency and 80 G sweep width. For $O_2^{\bullet-}$ detection, recordings were performed in a capillary tube (Blaubrand, colour-code green) at 37°C. Instrument settings were 10 mW of microwave power, 1 G of amplitude modulation, 100 kHz of modulation frequency, 20 G or 100 G sweep width. The signal intensity can be expressed in terms of area under the curve (AUC) or by peak height. In this study, peak height was used for detection of signal intensity. AUC is the double integration of the EPR signal. The peak height Delta Y is the arbitrary distance between lowest and highest point of the curve on the *y*-axes (Figures 1 and 8) (Analysis EPR spectrum data progressing, part of Windows® EPR spectrometer software, Magnettech, Germany).

Griess reaction

A Griess reagent kit (Invitrogen G-7921) was used for the determination of extracellular nitrite in stimulated macrophages. After incubation with a stimulator, the supernatant was transferred to a 96-well plate (150 μl/well). After addition of 20 μl Griess reagent and 130 μl demineralized water, samples were incubated for 30 min and absorbance was measured at 550 nm (Labsystems Multiskan MCC/340). Standard calibration curve was set up by diluting the nitrite standard of the kit.

Statistical analysis

All results were expressed as the mean ± SEM of at least three independent experiments. Statistical analyses were carried out with GraphPad Prism 4 software. The statistical tests used in the present study are noted in the figure legends. $p < 0.05$ was considered statistically significant.

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Results

In vitro detection of $O_2^{\bullet-}$

To optimize the EPR analysis of $O_2^{\bullet-}$ formed by macrophages, two different culture protocols were evaluated. First, the cells were grown in T25 culture flasks to maximize the cell number and thus the EPR response. This protocol requires a lot of cells and reagents and may therefore be of more limited practical use. In a second protocol, the cells were grown in 24-well plates and EPR measurements were performed on both supernatant and re-suspended cells. A minor practical disadvantage of the latter method is the more difficult harvesting protocol compared to T25 culture flasks.

Calibration of the nitroxide radical signal

To set up a calibration curve for the nitroxide radical, CP• was selected because it is a stable radical and structurally related to the applied hydroxylamine spin probes. The calibration curve (Figure 1) with delta Y as arbitrary value for EPR intensity was used in all further experiments to quantify nitroxide radical concentrations. Delta Y is the peak height of the EPR signal, which is the distance between the highest and the lowest point of the signal.

Comparative study of hydrophilic and lipophilic spin probes

According to the literature, the hydroxylamine spin probes PP-H, CM-H and CP-H are most frequently used for detection of $O_2^{\bullet-}$ in a cellular environment (Figure 2) [17,21–23]. Although their structures are quite similar, the compounds differ greatly in water solubility. CM-H contains a methoxy-carbonyl function and is the most lipophilic, thereby able to react with intra- and extracellular $O_2^{\bullet-}$. CP-H is more hydrophilic, while the phosphate containing PP-H is the most hydrophilic, only reacting with extracellular $O_2^{\bullet-}$ [21–23].

In the comparative study of these spin probes, RAW 264.7 macrophages were stimulated for $O_2^{\bullet-}$ production with 10 μM PMA for 5 min. After 50 min incubation with 1 mM of spin probe, nitroxide radical concentrations in the supernatant (Figure 3A) and re-suspended cells (Figure 3B) were measured. A two-way ANOVA Bonferroni post-test demonstrated that both supernatant and suspended cells contained the same nitroxide radical levels for a certain spin probe ($p > 0.05$). The highest nitroxide radical levels were found for CM-H, followed by CP-H and PP-H. For practical reasons, measurements were done in the supernatant with CM-H in all further 24-well plate experiments. A similar activity profile of the spin probes (CM-H > CP-H > PP-H) was found with the T25 culture flask protocol (data not shown) in which re-suspended cells were used.

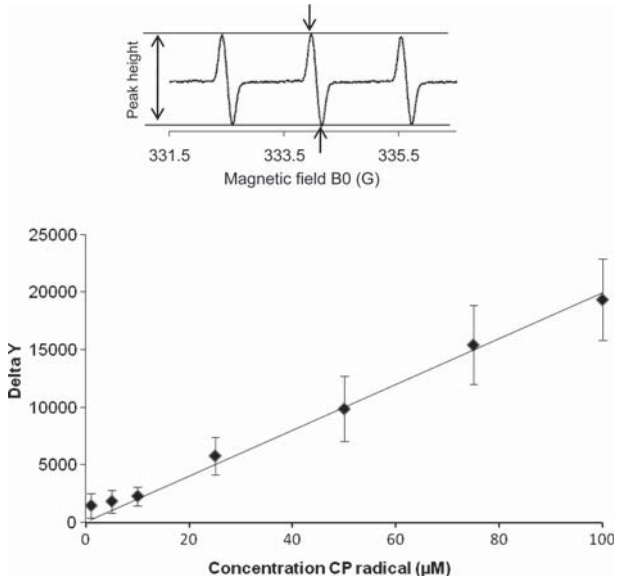

Figure 1. Calibration curve for nitroxide radicals obtained by testing different CP radical concentrations (1–100 μM) and measuring the delta Y value (average of four replicates, $R^2 = 0.99$). The insertion explains the peak height or Delta Y (the three line spectra of the nitroxide radical is shown).

Evaluation of $O_2^{\bullet-}$ stimulators for RAW 264.7 cells

The stimulators PMA (10 μM), IFN-gamma (50 ng/ml), TNF-alpha (20 ng/ml), LPS (100 ng/ml) and a combination of LPS and IFN-gamma were evaluated for $O_2^{\bullet-}$ production. RAW 264.7 cells were incubated with stimulators for 1–6 h, while also 5 min incubation time was included for PMA. The spin probe was added and EPR spectra were recorded after 50 min. The optimal spin probe incubation time was selected based on time experiments (data not shown).

In the 24-well protocol (Figure 4), PMA led to a significant higher response compared to all other stimulators after 1 h incubation ($p < 0.001$).

However, after 6 h, the PMA signal was lower with only a significant difference between PMA and TNF-alpha ($p < 0.01$; data not shown). When the nitroxide

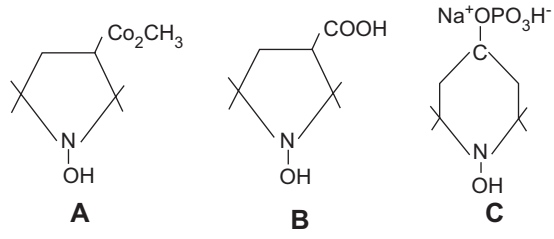

Figure 2. Chemical structures of hydroxylamine spin probes for $O_2^{\bullet-}$: (A) CM-H, (B) CP-H, (C) PP-H.

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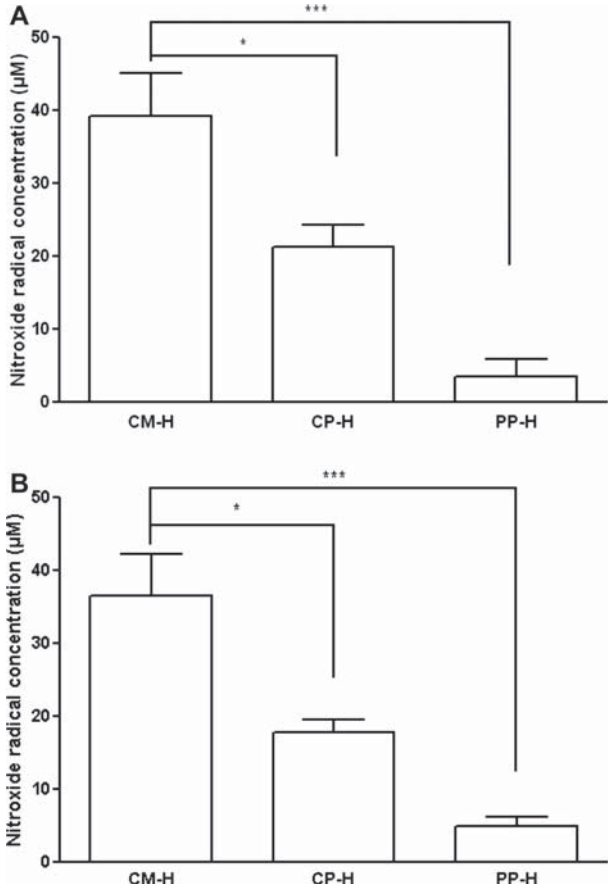

Figure 3. Change in nitroxide radical levels in the supernatants (A) and in re-suspended RAW 264.7 cells (B) after stimulation with 10 μM PMA for 5 min, followed by incubation with 1 mM CM-H, CP-H or PP-H for 50 min. Cells were cultivated in 24-well plates. There was a significant difference between CM-H and the other spin probes ($^{***}p < 0.001$ compared with PP-H and $^{*}p < 0.05$ compared with CP-H, one-way ANOVA followed by Bonferroni's multiple comparison test). For each spin probe, there was no significant difference between cells and supernatants ($p > 0.05$, two-way ANOVA followed by Bonferroni's multiple comparison test).

radical levels after stimulation with PMA for 5 min, 1 h and 6 h were compared, 6 h incubation led to a significant lower signal ($p < 0.001$; data not shown). In the T25 protocol (Figure 5), nitroxide radical levels in re-suspended RAW 264.7 cells were highest after 5 min PMA, while stimulation for 1 h with other compounds remained significantly lower. PMA stimulation showed a lower but non-significant EPR signal after 1 h incubation compared to 5 min, while in the above 24-well protocol, stimulation for 5 min or 1 h led to similar nitroxide radical levels. In all further experiments, PMA was used at 10 μM for 5 min prior to addition of the spin probe.

Stimulation of different monocyte/macrophage cultures

The $O_2^{\bullet -}$ production of the following cell cultures was compared using the T25 protocol: the macrophage cell lines RAW 264.7 and J774A.1, one monocyte cell line U937 and PMM. All three macrophage cell types gave high nitroxide radical responses after stimulation with 10 μM PMA. There was only a significant difference in EPR signal between J774A.1 macrophages and U937 monocytes ($p < 0.05$). Because of the low $O_2^{\bullet -}$ response of the monocytes, this cell line was excluded from the 24-well plate experiments. In the supernatants (24-well protocol), RAW 264.7 cells showed significantly higher EPR signals compared to J774A.1 and PMM (Figure 6).

Specificity of CM-H

Although CM-H gave the highest EPR response of all spin probes, it was pivotal to determine its specificity, since it can react with $O_2^{\bullet -}$ as well as with some other ROS. Therefore, SOD was added to calculate

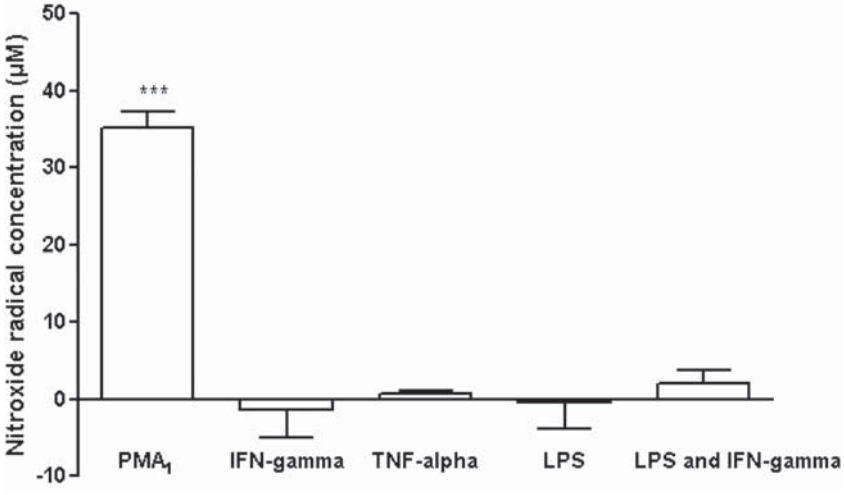

Figure 4. Change in nitroxide radical concentration in supernatant of stimulated RAW 264.7 cells (24-well protocol – 1 h stimulation) with PMA_1 10 μM, IFN-gamma 50 ng/ml, TNF-alpha 20 ng/ml, LPS 100 ng/ml or the combination of IFN-gamma and LPS. CM-H was used as a spin probe and RAW 264.7 as reference macrophage cell line. PMA seemed to be the best stimulator ($^{***}p < 0.001$, one-way ANOVA followed by Bonferroni's multiple comparison test).

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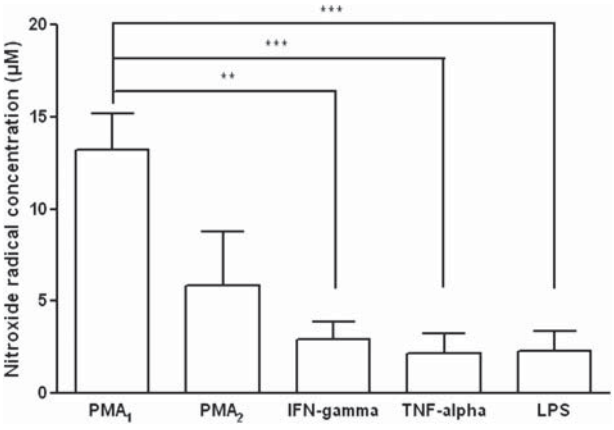

Figure 5. Change in nitroxide radical concentration in resuspended, stimulated RAW 264.7 cells (T25 protocol) with PMA_1 = 10 µM and 5 min incubation; PMA_2 = 10 µM and 1 h incubation; IFN-gamma = 50 ng/ml and 1 h incubation; TNF-alpha = 20 ng/ml and 1 h incubation; LPS = 100 ng/ml and 1 h incubation. CM-H was used as a spin probe ($^{**}p$ <0.01 $^{***}p < 0.001$, one-way ANOVA followed by Bonferroni's multiple comparison test).

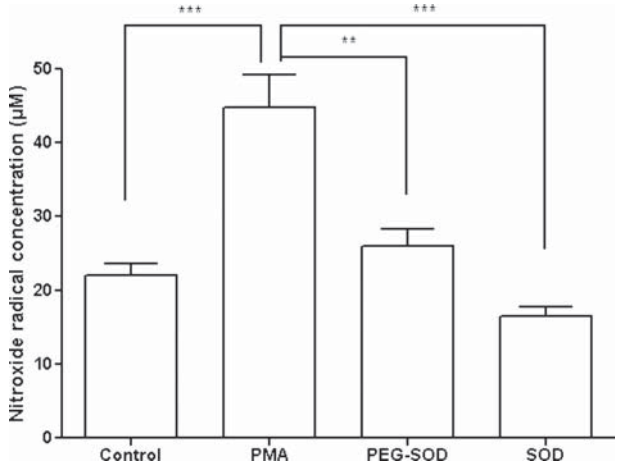

Figure 7. Specificity of the spin probe CM-H for $O_2^{\bullet-}$. Inhibition of the $O_2^{\bullet-}$ levels with SOD or PEG-SOD led to a significant decrease in nitroxide radical levels ($^{***}p < 0.001$, $^{**}p < 0.01$). After inhibition, the EPR signal was similar to the control level ($p > 0.05$, one-way ANOVA followed by Bonferroni's multiple comparison test).

the amount of non-$O_2^{\bullet-}$ trapped radicals while PEG-SOD was applied for intracellular $O_2^{\bullet-}$. EPR measurements were performed on re-suspended cells for which no significant differences between the control group and the groups treated with PMA and SOD or PMA and PEG-SOD were found ($p > 0.05$). After stimulation, nitroxide radical concentrations significantly increased (PMA vs PMA – SOD or PEG-SOD: $p < 0.001$ and $p < 0.01$, respectively, Figure 7).

In vitro detection of NO

For detection of NO, a totally different protocol was applied. After treatment, samples were frozen in liquid nitrogen allowing EPR measurements independent of cell harvesting.

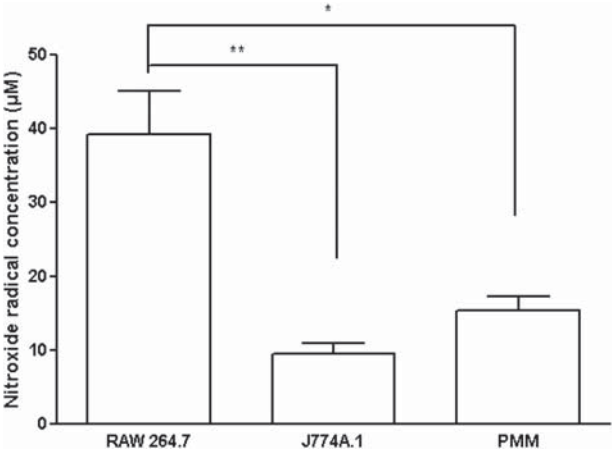

Figure 6. Change in nitroxide radical concentration in supernatants of RAW 264.7, J774A.1 and primary mouse macrophages (PMM) after stimulation with PMA (5 min, 10 µM) and with CM-H as spin probe in the 24 well plate protocol ($^{*}p < 0.05$, $^{**}p < 0.01$, one-way ANOVA followed by Bonferroni's multiple comparison test).

Calibration of the NO spin adduct signal

To set up a calibration curve for the NO spin adduct, the NO donor MAHAMA NONO-ate, giving two moles NO per mol donor, was applied. The calibration curve was made under the same conditions as used in the following NO experiments (Figure 8). The delta Y value is the peak height of the EPR signal. This arbitrary unit is the distance between the highest and the lowest point in the EPR signal.

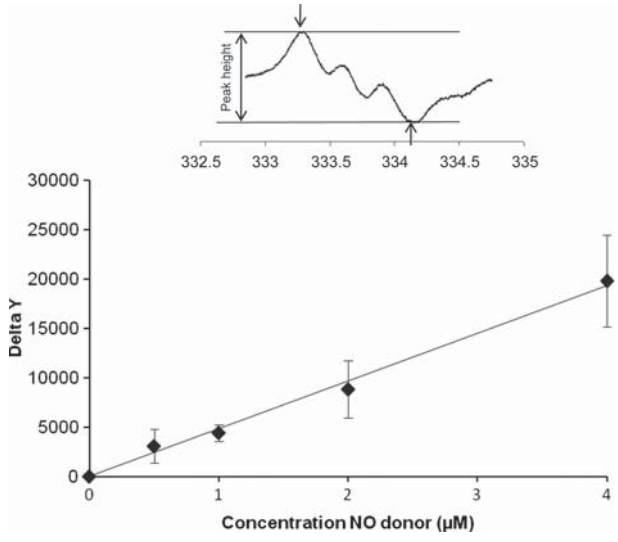

Figure 8. Calibration curve of the [NO-Fe(MGD)$_2$] EPR signal by testing different concentrations of the MAHAM NONO-ate donor and measuring the Delta Y value (average of four replicates, R^2 = 0.99). The insertion explains the peak height or Delta Y (three line spectrum of [NO-Fe(MGD)$_2$] adduct in liquid nitrogen is given).

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NO spin trapping by [Fe(DETC)$_2$] vs [Fe(MGD)$_2$]

Frequently used spin traps for NO detection are dithiocarbamate-Fe^{2+} complexes that bind NO by interaction between radical and Fe^{2+} [24,31–33,35]. [Fe(DETC)$_2$] and [Fe(MGD)$_2$] are structurally similar molecules trapping NO, but because of the more lipophilic structure of DETC, [Fe(DETC)$_2$] complex can react with intracellular NO in contrast to [Fe(MGD)$_2$].

These spin traps were selected because the difference in lipophilic characteristics made it possible to compare intra- and extracellular levels of the NO spin adducts. After addition of the spin trap, NO can react with the iron and the accumulation of the NO spin adduct was measured.

LPS and IFN-gamma stimulated RAW 264.7 macrophages were incubated with the spin traps for 1 h. The signal of [NO-Fe(DETC)$_2$] (134 ± 3 μM NO spin adduct) was significantly higher compared to that of [NO-Fe(MGD)$_2$] (2 ± 1 μM NO spin adduct), endorsing the importance of intracellular NO spin adduct detection by the [Fe(DETC)$_2$] spin trap.

Evaluation of NO stimulators for RAW 264.7 cells

RAW 264.7 cells were incubated with LPS, IFN-gamma, TNF-alpha and PMA. TNF-alpha at 2 ng/ml and PMA at 10 μM resulted only in minimal [NO-Fe(DETC)$_2$] signals (data not shown). Seven combinations of LPS and IFN-gamma were evaluated: IFN-gamma 5 ng/ml, LPS 10 ng/ml, LPS 100 ng/ml and LPS 1 μg/ml, IFN-gamma 5 ng/ml + LPS 10 ng/ml, IFN-gamma 5 ng/ml + LPS 100 ng/ml and IFN-gamma 5 ng/ml + LPS 1 μg/ml. The low EPR response of LPS at a concentration of 1 μg/ml was due to cytotoxicity, especially in combination with IFN-gamma, while the highest NO spin adduct levels were obtained at 100 ng/ml LPS with or without 5 ng/ml IFN-gamma (Figure 9).

The combination of 100 ng/ml LPS and 5 ng/ml IFN-gamma was withheld for further experiments because of the higher reproducibility. To determine the optimal period of stimulation, cells were incubated with LPS 100 ng/ml and IFN-gamma 5 ng/ml for 6 h, 24 h or 48 h. Differences between 6 h and 24 h stimulation were minimal (14 ± 2 μM NO spin adduct for 6 h, 13 ± 6 μM NO spin adduct for 24 h) while incubation for more than 48 h led to a lower EPR response and cytotoxicity. For practical reasons, the 6 h protocol was used in all further experiments.

Stimulation of different cell lines

The generation of NO was measured in RAW 264.7, J774A.1, U937 and PMM cells after incubation with LPS 100 ng/ml and IFN-gamma 5 ng/ml for 6 h

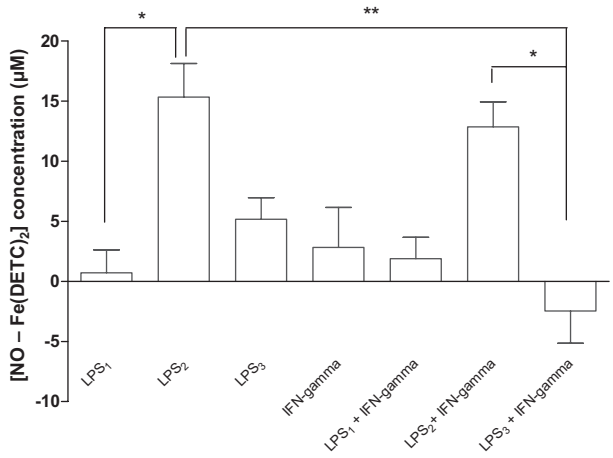

Figure 9. Change in [NO-Fe(DETC)$_2$] signal after 6 h stimulation with LPS 10 ng/ml (LPS$_1$), 100 ng/ml (LPS$_2$) or 1 μg/ml (LPS$_3$), IFN-gamma (5 ng/ml) or combinations. The significant differences between LPS$_3$ and IFN-gamma compared to LPS$_2$ ($^{**}p < 0.01$) and LPS$_2$ + IFN-gamma ($^{*}p < 0.05$) can be explained by cytotoxicity of high LPS concentrations in combination with IFN-gamma (one-way ANOVA followed by Bonferroni's multiple comparison test).

(Figure 10). Similar to the $O_2^{\bullet -}$ experiments, monocytes did not produce a significant EPR signal. RAW 264.7 cells gave the highest EPR response and were therefore selected for further testing.

Specificity of [Fe(DETC)$_2$]

To determine the specificity of [Fe(DETC)$_2$], the NOS inhibitor L-NAME was added together with the stimulators. There was a significant difference between stimulated cells that were treated with L-NAME and those who were stimulated but not treated with L-NAME ($p < 0.001$). There was no significant difference between the control group and the cells treated with L-NAME ($p > 0.05$, Figure 11).

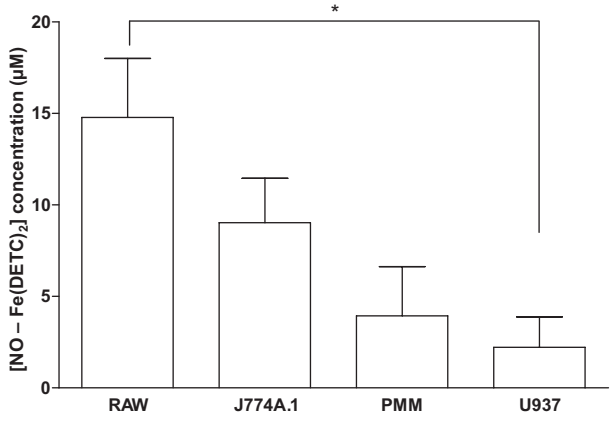

Figure 10. Change in [NO-Fe(DETC)$_2$] signal of RAW 264.7 and J774A.1 mouse macrophages, PMM and U937 monocytes. RAW 264.7 gave the highest EPR signal after 6 h incubation with 100 ng/ml LPS and 5 ng/ml IFN-gamma ($^{*}p < 0.05$, Kruskal Wallis test followed by Donn's multiple comparison test).

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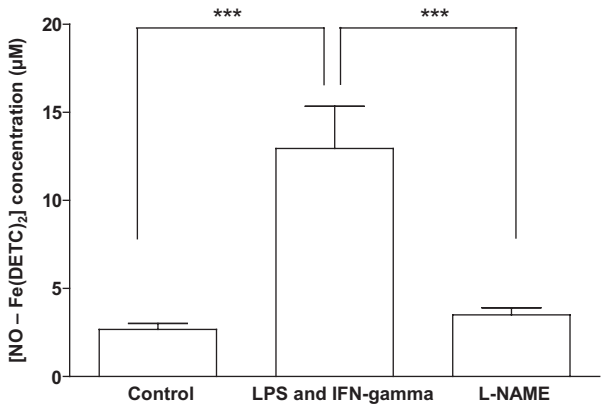

Figure 11. Specificity of the spin trap [$Fe(DETC)_2$] for NO. After inhibition of NOS with L-NAME, the EPR signal was similar to the control level and significant different from stimulated cells ($^{***}p < 0.001$, one-way ANOVA followed by Bonferroni's multiple comparison test).

Nitrite detection with the Griess reaction assay

The Griess reaction assay is often used for the measurement of nitrite in the incubation medium. Mostly, authors correlate these nitrite levels to the amount of NO that is formed by the cells [14,36–39]. These nitrite levels reflect the accumulation of nitrite in the supernatant up to the time point at which the measurements take place. After 6 h incubation with the stimulators, nitrite levels were rather low: 4 ± 2 µM. After 24 h and 48 h incubation the nitrite accumulated in the supernatant and higher levels were obtained: 95 ± 20 µM and 267 ± 33 µM nitrite, respectively.

The EPR method, which detects [NO-$Fe(DETC)_2$] generated within 1 h (duration in which the spin trap can react with NO), gave comparable results for the three time points: 12 ± 3 µM (6 h stimulation), 9 ± 5 µM (24 h stimulation) and 9 ± 3 µM (48 h stimulation).

Conclusion and discussion

Macrophages are one of the main mediators of the immune response exhibiting functions like phagocytosis and production of $O_2^{\bullet-}$ and NO. Quantification of both free radicals remains difficult since most techniques measure them indirectly [8,9]. An analytical approach that permits direct detection of free radicals is the EPR spin trap technique, which allows direct measurement of reaction products of the free radical with the spin trap or probe. Therefore, it is evident that this method does not deliver exact quantitative data of the measured radicals itself but of the spin adducts (e.g. [NO – $Fe(DETC)_2$]) or oxidation products (e.g. nitroxide radicals). The specific aim of this study was to compare different spin traps or probes, different stimulators and incubation times to establish a practical protocol for the detection of $O_2^{\bullet-}$ (as nitroxide radical) and NO (as spin adduct) in different types of macrophages, i.e. U937, J774A.1, RAW 264.7 and primary mouse macrophages. The cyclic hydroxylamines CM-H, CP-H and PP-H were selected as spin probes for $O_2^{\bullet-}$, since they are more sensitive than the commonly used nitrone spin traps DMPO and DEPMPO [22,23]. Radical-trapped cyclic hydroxylamines are more resistant to reduction by ascorbate and low molecular thiols compared to the nitroxides TEMPONE-H and DMPO [8,21,22].

For the detection of $O_2^{\bullet-}$, the 24-well plate protocol proved to be more practical than the T25 protocol since it allows measurements of resuspended cells and supernatant in capillary tubes at 37°C. The phorbol ester PMA seems to be the best stimulator as it only takes 5 min of incubation to adequately stimulate macrophages for $O_2^{\bullet-}$ production, measured as nitroxide radical. For measurement of extracellular $O_2^{\bullet-}$, cells can be cultivated in 24-well plates followed by EPR quantification of the supernatant with CP-H or PP-H spin probe. The optimal protocol for the measurement of $O_2^{\bullet-}$ production by macrophages should consist of a macrophage cell line, CM-H as spin probe and PMA as stimulator (Table I). Even with a limited amount of cells, supernatant from the 24-well plate protocol can still be used because no significant differences in nitroxide radical levels between supernatant and cell suspension were present.

It should be emphasized that the measured nitroxide radical levels can differ according to the applied protocol and cell line. For example, nitroxide radical

Table I. Overview of the obtained protocols for detection of NO spin adduct and nitroxide radical levels produced by stimulated macrophages.

	NO		$O_2^{\cdot-}$	
	Cells		Cells	Supernatant
Cell compartment	Intracellular	Extracellular	Intracellular	Extracellular
Spin trap/probe	[$Fe(DETC)_2$]	[$Fe(MGD)_2$]	CM-H	CM-H, CP-H or PP-H
Reaction product measured	NO- spin adduct		Nitroxide radical	
Stimulator	100 ng/ml LPS + 5 ng/ml IFN-gamma		10 µM PMA	
Incubation time	6 h		5 min	
Measurement temperature	77K		37°C	

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levels obtained from the T25 flask protocol were lower compared to the 24-well protocol on supernatant. The many interventions in the T25 flask protocol, like harvesting, centrifugation and washing with KHB, may influence the cellular levels of radicals. In re-suspended cells, J774A.1 cells exhibited the highest EPR response, while in the supernatant RAW 264.7 had the highest response. In both protocols, the monocytic U937 cell line was hard to stimulate by PMA. These data suggest that the right choice of a spin probe depends on the experimental conditions of the test (e.g. intra- or extracellular levels of the free radical, *in vitro* or *in vivo* experiments).

For measurement of NO, cells must be cultivated in T25 flasks. In some studies, L-arginine was added as an external NO substrate to measure NO levels in stimulated macrophages [39]. In this study, the L-arginine concentrations in the cell media are high enough (0.398 mM in DMEM and 1.15 mM in RPMI) to minimize $O_2^{\bullet -}$ production by NOS. After incubation with a stimulator and a spin trap, cells were harvested and frozen in liquid nitrogen, allowing NO measurements even weeks after collection of the cells. The optimal protocol involves stimulation for 6 h with LPS 100 ng/ml and IFN-gamma 5 ng/ml (Table I). To detect NO spin adducts in the supernatant, $[Fe(MGD)_2]$ can be used as a spin trap, while for intracellular NO spin adducts, $[Fe(DETC)_2]$ is the best spin trap. The lipophilic characteristics of the spin trap $[Fe(DETC)_2]$ makes it possible to insert in the lipophilic parts of the cellular membrane and to form the spin NO adduct with intracellular NO. More hydrophilic Fe^{2+} complexes such as $[Fe(DTCS)_2]$ and $[Fe(MGD)_2]$ are more useful for *in vitro* extracellular or *in vivo* measurements [28,40,41].

The Griess reaction was used to measure the nitrite levels in the supernatant. After 6 h, 4 ± 2 μM nitrite was formed, after 24 h, 95 ± 20 μM and after 48 h, 267 ± 33 μM. Our experiments show that accumulation of nitrite in the supernatant is almost linear after a certain lag period. With the EPR method, NO is assessed in a direct way by measuring the spin adduct and the data reflect $[NO\text{-}Fe(DETC)_2]$ formation for 1 h (the contact period of NO and spin trap) at a certain time point. To compare both methods, it is necessary to convert the EPR measurements to cumulative data by using the average of each consecutive pair of measurements: after 6 h, 18 μM NO was formed (assuming a lag period of 3 h for induction of iNOS), after 24 h, 202 μM NO (18 μM + 10.25 μM/h during 18 h) and after 48 h, 412 μM (202 + 8.75 μM/h during 24 h). After the delay observed with the Griess assay, both methods follow almost the same linear curve. These data indicate that both methods can be used for detection of NO in a cellular environment. However, the Griess reaction has the disadvantage of a rather high detection limit and the low sensitivity, and it should be emphasized that nitrite is an intermediate product, which is partly oxidized to the more stable nitrate [42–44]. Therefore, it is less certain whether the nitrite concentration in the medium is directly proportional to the NO generated by the cells. In our opinion, EPR detection with a lipophilic spin trap is the best choice for intracellular NO detection.

In conclusion, comparison of different spin probes/traps, stimulators and incubation times resulted in sensitive and specific protocols for the analysis of $O_2^{\bullet -}$ or NO production in different types of stimulated macrophages. The proposed protocol can be used as a first basic approach to start-up EPR measurements in a cellular environment without the need to screen all spin probes/traps, cell lines or potential stimulators.

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