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PAPER IN FOREFRONT

Microchip electrophoresis with laser-induced fluorescence detection for the determination of the ratio of nitric oxide to superoxide production in macrophages during inflammation

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Abstract It is well known that excessive production of reactive oxygen and nitrogen species is linked to the development of oxidative stress-driven disorders. In particular, nitric oxide (NO) and superoxide ($O_2^{\bullet-}$) play critical roles in many physiological and pathological processes. This article reports the use of 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate and MitoSOX Red in conjunction with microchip electrophoresis and laser-induced fluorescence detection for the simultaneous detection of NO and $O_2^{\bullet-}$ in RAW 264.7 macrophage cell lysates following different stimulation procedures. Cell stimulations were performed in the presence and absence of cytosolic (diethyldithiocarbamate) and mitochondrial (2-methoxyestradiol) superoxide dismutase (SOD) inhibitors. The NO/$O_2^{\bullet-}$ ratios in macrophage cell lysates under physiological and proinflammatory conditions were determined. The NO/$O_2^{\bullet-}$ ratios were 0.60 ± 0.07 for unstimulated cells pretreated with SOD inhibitors, 1.08 ± 0.06 for unstimulated cells in the absence of SOD inhibitors, and 3.14 ± 0.13 for stimulated cells. The effect of carnosine (antioxidant) or Ca^{2+} (intracellular messenger) on the NO/$O_2^{\bullet-}$ ratio was also investigated.

Keywords Bioanalytical methods · Inflammation · Macrophages · Microchip electrophoresis · Nitric oxide · Superoxide

Introduction

Reactive nitrogen and oxygen species (RNOS) play a variety of roles in biological systems [1, 2]. In mammals, specialized enzymes, such as NADPH oxidase and nitric oxide synthase (NOS), are involved in the production of RNOS as part of the immune system. Excessive production of these reactive species can lead to oxidative and nitrosative stress, resulting in damage to important biomolecules [3]. Moreover, this damage has been linked to neurodegenerative diseases, cardiovascular disorders, and cancer [4–6].

Nitric oxide (NO) is a water-soluble, free-radical gas that acts as an intracellular and intercellular messenger in all vertebrates [7]. NO is able to modulate cytokine and chemokine release [8] during the immune response [9], and plays important roles in both the cardiovascular system [10] and the nervous system [11]. NO has a half-life of 3–6 s in vivo, and is synthesized by a complex family of NOS enzymes through the conversion of L-arginine to L-citrulline [7].

The superoxide anion ($O_2^{\bullet-}$) is a reactive oxygen species naturally produced in the human body when oxygen (O_2) gains an excess electron during various enzymatic reactions in mitochondria; it is involved in many physiological and pathological signaling processes [12]. Overproduction of $O_2^{\bullet-}$ can lead to cell death due to oxidative damage to DNA, lipids, carbohydrates, and proteins [13]. In living organisms, the intracellular enzyme superoxide dismutase (SOD) protects the cell from the deleterious effects of $O_2^{\bullet-}$ by catalyzing the conversion of $O_2^{\bullet-}$ to O_2 and hydrogen peroxide [14].

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Simultaneous production of intracellular NO and $O_2^{\bullet-}$ can lead to the formation of peroxynitrite [15]. This dangerous molecule has the ability to nitrate, nitrosylate, and oxidize proteins, DNA, and lipids, inhibiting their functions and causing cytotoxicity within the cell [16]. Additionally, peroxynitrite has been linked to neurodegenerative disorders, cardiovascular disease, and cancer [7]. Therefore, the simultaneous detection of NO and $O_2^{\bullet-}$ is necessary to obtain a thorough understanding of intracellular nitrosative and oxidative stress.

Macrophages are cells involved in the primary immune defense mechanism that, when activated in vivo under proinflammatory conditions, can lead to a higher expression of inducible NOS (iNOS) coupled with the production of a large amount of NO and, thus, RNOS [17, 18]. It is well known that a combination of lipopolysaccharides (LPS) and interferon gamma (IFN-γ) results in the stimulation of macrophages to produce a large amount of NO via iNOS [19]. In addition, high amounts of intracellular $O_2^{\bullet-}$ can be generated by incubation of macrophages with phorbol 12-myristate 13-acetate (PMA) [20].

A method for the simultaneous detection of $O_2^{\bullet-}$ and hydrogen peroxide in stimulated macrophages using microchip electrophoresis (ME) with laser-induced fluorescence (LIF) detection was reported by Li et al. [21]. Our group has also used ME–LIF for the determination of the intracellular production of NO in lymphocytes [22], as well as detection of $O_2^{\bullet-}$ in macrophages [23]. ME has several advantages over conventional methods for the analysis of cultured cells, especially for the detection of RNOS. These advantages include short separation times, isolation of the intended product from interfering substances, high throughput, and the ability to easily integrate multiple detection platforms. Additionally, ME systems are ideal for single-cell analysis because they can be automated and permit on-chip cell manipulation and lysis [24–27].

In the present work, the use of ME–LIF for the simultaneous detection of intracellular NO and $O_2^{\bullet-}$ in RAW 264.7 macrophage cells is reported. These RNOS are detected by ME–LIF in the cell lysates of macrophages following incubation of the cells with both 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM DA) and MitoSOX Red for NO and $O_2^{\bullet-}$, respectively. Changes in the NO/$O_2^{\bullet-}$ ratio were then investigated under physiological and proinflammatory conditions. The resulting method provides an additional tool for understanding the physiopathological role of RNOS in oxidative stress-driven disorders.

Materials and methods

Materials and reagents

Murine RAW 264.7 cells (ATCC® TIB-71™), Dulbecco's modified Eagle's medium (DMEM), DMEM free of phenol red, fetal bovine serum, and penicillin–streptomycin antibiotic solution were purchased from American Type Culture Collection (Manassas, VA, USA). L-Carnosine, diethyldithiocarbamate (DDC), 2-methoxyestradiol (2-ME), PMA, anhydrous dimethyl sulfoxide (DMSO), phosphate-buffered saline (PBS), trypan blue solution, LPS, sodium dodecyl sulfate (SDS), calcium chloride, and bovine serum albumin (BSA) were all supplied by Sigma-Aldrich (St. Louis, MO, USA). Sodium hydroxide, hydrochloric acid, 25-mL polystyrene culture flasks, boric acid, ethanol (95%), and C-Chip disposable hemocytometers were obtained from Fisher Scientific (Pittsburgh, PA, USA). IFN-γ was supplied by Calbiochem (Gibbstown, NJ, USA). DAF-FM DA and MitoSOX Red were purchased from Life Technologies (Carlsbad, CA, USA). Poly(ether sulfone) membrane (3 kDa) centrifuge filters were purchased from VWR International (West Chester, PA, USA). Polydimethylsiloxane (PDMS) microdevices were prepared from a Sylgard 184 elastomer kit (Ellsworth Adhesives, Germantown, WI, USA). All water used was ultrapure (18.3 MΩ cm) (Milli-Q Synthesis A10, Millipore, Burlington, MA, USA).

Cell culture and preparation

RAW 264.7 macrophages were cultured in DMEM containing 10% (v/v) fetal bovine serum, L-glutamine (2 mM), penicillin (50 IU/mL), and streptomycin (0.3 mg/mL). The cells were cultured in 25-cm^2 polystyrene culture flasks at a density of 5×10^6 cells per flask, maintained in a humidified environment at 37 °C, 5% CO_2, and 95% air, and passaged every 2 to 3 days depending on the cell confluence to avoid overgrowth.

Stimulation protocol for the simultaneous detection of NO and $O_2^{\bullet-}$

The protocol used for cell sample preparation is shown in Fig. 1. On the day of the experiment, cells were harvested with use of a cell scraper, counted with a C-Chip disposable hemocytometer, and plated at a density of 1.2×10^7 cells per flask. Stock solutions of LPS (1 mg/mL) and IFN-γ (200,000 U/mL) were prepared in 10 mM PBS and in 10 mM PBS with 0.1% BSA, respectively. Once the cells had adhered to the flask surface, they were stimulated to increase the production of NO by dilution of the LPS stock solution to 100 ng/mL and the INF-γ stock solution to 600 U/mL in 5 mL of cell culture medium. Immediately after the stimulation, the macrophages were incubated for 20 h in a humidified environment at 37 °C, 5% CO_2, and 95% air. A stock solution of 5 mM DAF-FM DA was prepared in 99% sterile DMSO. After the 20-h incubation with LPS plus INF-γ, the medium was replaced with 5 mL of DMEM free of phenol red containing 10 μL DAF-FM DA for 60 min (10 μM DAF-FM DA final

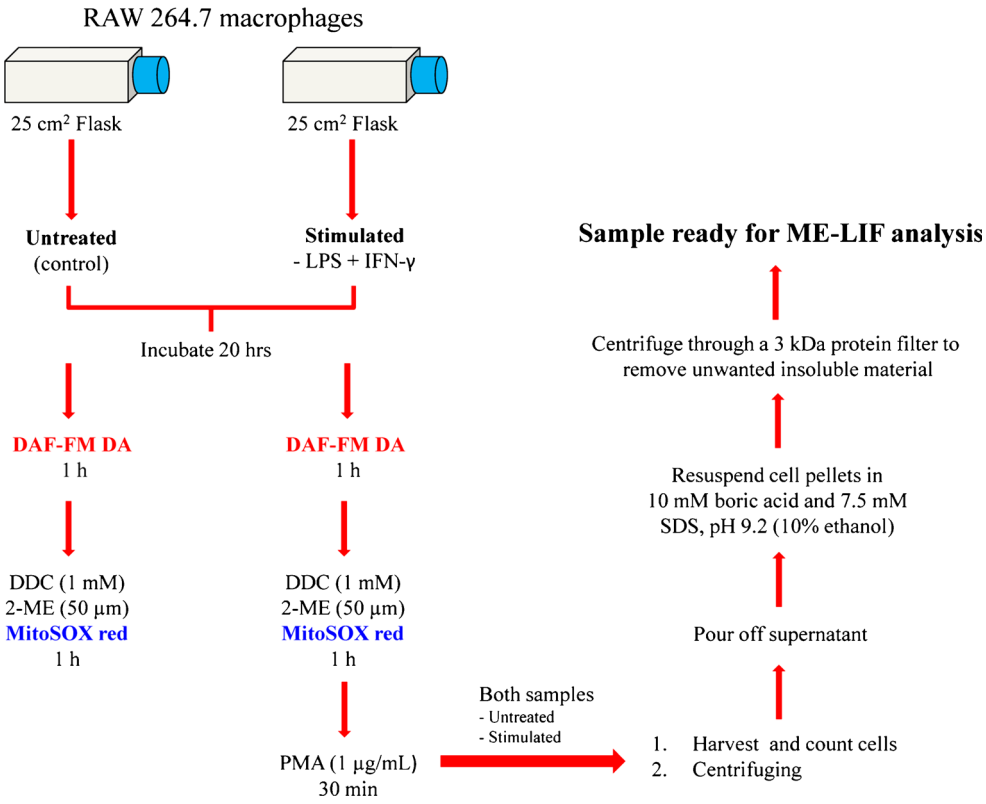


Fig. 1 Flowchart of the protocol used for preparation of RAW 264.7 cell lysates. *DAF-FM DA* 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate, *DDC* diethyldithiocarbamate, *IFN-γ* interferon gamma, *LPS* lipopolysaccharide, *2-ME* 2-methoxyestradiol, *ME-LIF* microchip electrophoresis and laser-induced fluorescence, *PMA* phorbol 12-myristate 13-acetate, *SDS* sodium dodecyl sulfate

concentration) [22]. A 5 mM MitoSOX Red stock solution was prepared in 99% sterile DMSO [23]. Additionally, a stock solution of 100 mM DDC was prepared in 10 mM PBS, and stock solutions of 16.5 mM 2-ME and PMA (1 mg/mL) were prepared in DMSO. Each flask of cells containing 5 mL of culture medium was then incubated for 1 h with a combination of the cytosolic SOD inhibitor DDC (1 mM final concentration), mitochondrial SOD inhibitor 2-ME (50 μM final concentration), and MitoSOX Red (10 μM MitoSOX Red final concentration). Finally, the cells were stimulated with PMA (1 μg/mL final concentration) for 30 min. During the incubation of the macrophages with DAF-FM DA and MitoSOX Red, the flasks were covered with aluminum foil to minimize any photobleaching of the dyes.

Native untreated cells from the same population were used as a control. These were incubated under the same conditions as the cells described earlier, except that no stimulants was added. Another set of untreated cells were incubated in the presence of both SOD inhibitors (to increase the detectable concentration of $O_2^{\bullet-}$).

At the end of the stimulation process, the cells were harvested, and 100 μL of the cell suspension was removed for cell counting. The suspension was then centrifuged at 1137 *g* for 4 min. The supernatant was removed, and the cell pellet was washed twice with 1 mL of cold 10 mM PBS at pH 7.4. Cells were lysed with 50 μL of pure ethanol. The lysate solution was filtered with a 3 kDa molecular weight cutoff filter with centrifugation at 18,690 *g* for 10 min. Then 10 μL of the filtered cell lysates was added to a 90 μL solution of 10 mM boric acid and 7.5 mM SDS at pH 9.2 (10% ethanol final concentration) and immediately analyzed with the microfluidic device. Peak identification of NO or $O_2^{\bullet-}$ was accomplished with the same stimulation protocol, except that the cells were incubated in the presence of DAF-FM DA or MitoSOX Red only.

Alternative stimulation protocol for measuring changes in the NO/$O_2^{\bullet-}$ ratio

The effect of specific NO or $O_2^{\bullet-}$ stimulation procedures was evaluated through the analysis of changes in the NO/$O_2^{\bullet-}$ ratio in the macrophages. The protocol used for this analysis is the same as that described for the simultaneous detection of both analytes, except that the cells were stimulated with LPS plus INF-γ (NO stimulation) or PMA ($O_2^{\bullet-}$ stimulation).

Effect of pretreatment with carnosine or calcium on NO/$O_2^{\bullet-}$ ratio changes

To investigate changes in the NO/$O_2^{\bullet-}$ ratio due to the presence of carnosine or calcium ions, cells were incubated with either carnosine (10 μL of 500 mM stock solution in 10 mM PBS; 1 mM final concentration) 1 h before the stimulation

with LPS plus INF-γ or calcium chloride (25 μL of 200 mM stock solution in deionized water; 1 mM final concentration) 1 h before the stimulation with PMA. Native untreated (nonstimulated) cells from the same population were incubated under identical conditions, in the presence of carnosine or calcium chloride, and used as controls.

Cell density and viability

Cell density and viability were measured with a C-Chip disposable hemocytometer and Trypan blue exclusion assay, respectively. The cell suspension was diluted either 1:3 or 1:5 (stimulated and untreated, respectively) with 0.4% Trypan blue solution.

Microchip fabrication and instrumental setup

The fabrication of hybrid PDMS–glass microfluidic devices has been described previously [22]. Briefly, a silicon master containing the design of the microchip was fabricated with SU-8 photoresist and soft lithography. A 10:1 (w/w) PDMS prepolymer to curing agent mixture was degassed in a vacuum desiccator and poured onto the master. The PDMS was cured overnight in an oven at 70 °C. Then the cured PDMS was peeled off the master, and 3-mm reservoirs were punched in the substrate with a biopsy punch (Harris Uni-core, Ted Pella, Redding, CA, USA). To make the final microfluidic device, the PDMS substrate was reversibly bonded to a borosilicate glass substrate (Precision Glass and Optics, Santa Ana, CA, USA). For these experiments, a microchip with a simple-T design was used with a 5 cm separation channel, 0.75 cm side arms, and 40 μm by 15 μm channels throughout.

Before operation, the microchip was conditioned with 0.1 M sodium hydroxide and run buffer. The run buffer consisted of 10 mM boric acid and 7.5 mM SDS at pH 9.2. A separation field was generated with a high-voltage power supply (Ultravolt, Ronkonkoma, NY, USA). A 1 s gated injection was used for sample introduction. A gate was established by application of +2400 V and +2200 V to the buffer and sample reservoirs, respectively.

For LIF detection, the microchip was placed on the stage of an Eclipse Ti-U inverted microscope (Nikon Instruments, Melville, NY, USA). A 488-nm diode laser (Spectra-Physics, Irvine, CA, USA) was aligned 3.75 cm below the sample–buffer intersection as the excitation source. Fluorescence signals were collected with a photomultiplier tube (Hamamatsu, Bridgewater, NJ, USA) and amplified with a SR570 low-noise preamplifier at 1 μA/V (Stanford Research Systems, Sunnyvale, CA, USA). Data were acquired with a digital-to-analog converter (National Instruments, Austin, TX, USA) and a homemade LabView (National Instruments, Austin, TX, USA) program. Data analysis was accomplished with Origin 8.6 (OriginLab, Northampton, MA, USA).

Comparison of the sensitivity of DAF-FM DA and MitoSOX Red probes

The fluorescence quantum yield of deacetylated form of DAF-FM DA (DAF-FM) is approximately 0.005, but increases about 160-fold to approximately 0.81 after reaction with NO [28]. In the case of MitoSOX Red, the literature does not present a uniform view of the fluorescence quantum yield before and after reaction with $O_2^{\bullet-}$. To ensure that the ratio accurately depicts the relative concentration of NO to $O_2^{\bullet-}$ in the cell, the response factors for the products of the two probes were determined as described previously [22, 23]. The ratio of the slope of the response curve for NO-specific product (DAF-FM T) to that for $O_2^{\bullet-}$-specific product (2-OH-MitoE$^+$) was determined to be 1.2. All NO/$O_2^{\bullet-}$ ratios were corrected for the difference in sensitivity (quantum yield) between the two products. This provided a more accurate assessment of the relative amounts that are produced (more details can be found in Fig. S1).

Results and discussion

Optimization of stimulation protocol and electrophoretic separation

Before the NO/$O_2^{\bullet-}$ ratio was monitored, it was crucial to ensure that the method would be able to generate separable, measurable, and reproducible signals for both NO and $O_2^{\bullet-}$ in complex matrices such as RAW 264.7 macrophage cell lysates. Several factors needed to be considered because of their possible influence on the ability to detect both analytes by ME–LIF. Initially, the cell stimulation protocol was optimized for generation of NO and $O_2^{\bullet-}$. In the first studies, cells were stimulated for 20 h with LPS plus IFN-γ, followed by the addition of PMA (500 ng/mL final concentration) and incubation for an additional 60 min. However, this protocol was not optimal in terms of cell viability and NO and $O_2^{\bullet-}$ production. The PMA-stimulation time was then decreased from 60 to 30 min, and the concentration was doubled to 1 μg/mL. The MitoSOX Red probe was incubated with cells for 1 h before stimulation with PMA. This new protocol was found to be the best for cell viability and NO and $O_2^{\bullet-}$ production.

Once the optimal cell stimulation protocol had been established, attention was focused on the separation and detection conditions. Our group previously reported the detection of NO using DAF-FM DA and ME–LIF using a run buffer consisting of 10 mM boric acid and 7.5 mM SDS at pH 9.2 [22]. A separate method was developed for $O_2^{\bullet-}$ that used a similar run buffer but with a lower SDS concentration (3.5 mM) [23]. To determine the optimal concentration of SDS needed for the simultaneous detection of NO and $O_2^{\bullet-}$, several SDS concentrations (3.5, 5.5, and 7.5 mM) in combination with 10 mM boric acid at pH 9.2 were investigated as background electrolytes. It was found that 7.5 mM SDS

provided the best resolution for DAF-FM-T, 2-OH-MitoE$^+$, and potential interferences.

The previous ME–LIF methods mentioned earlier used a detection distance of 4.5 cm from the T intersection of the simple-T microchip. Therefore, initial experiments in these studies used the same detection distance. However, it was found in these experiments that a distance of 3.5 cm provided a faster separation and better resolution, so it was used for all further studies. The migration times for DAF-FM T and 2-OH-MitoE$^+$ under the different experimental conditions are reported in Table 1. The relative standard deviation for migration times was below 5% for both DAF-FM T and 2-OH-MitoE$^+$ within the sample type. However, there was a drift to longer migration times with the stimulated samples, which could be due to changes in the sample matrix effects and fouling of the PDMS substrate. The final optimized method including sample preparation is described in detail in "Materials and methods."

Simultaneous detection of NO and $O_2^{\bullet-}$ in macrophage cell lysates: determination of changes in the NO/$O_2^{\bullet-}$ ratio as a function of the stimulation protocol

Before the investigation of the effect of inflammation on the NO/$O_2^{\bullet-}$ ratio in macrophage cell lysates, the identity of the NO- and $O_2^{\bullet-}$-product-specific fluorescence peaks had to be verified. Figure 2a shows representative electropherograms of the simultaneous detection of NO and $O_2^{\bullet-}$ in RAW 264.7 macrophage cell lysates. The identification of NO and $O_2^{\bullet-}$ peaks was performed by incubation of stimulated cells in the presence of one probe (DAF-FM DA or MitoSOX Red for NO and $O_2^{\bullet-}$, respectively). DAF-FM T and 2-OH-MitoE$^+$ represent the fluorescence reaction products of DAF-FM DA with NO and MitoSOX Red with $O_2^{\bullet-}$ [22, 23], respectively. Although DAF-FM is very selective for NO, it has been shown that it can react with dehydroascorbate (DHA), giving DAF-FM DHA [29–31]. In earlier studies, the DAF-FM DHA peak was effectively separated from the NO-specific peak in cell lysates [22]. In these studies, a peak for DAF-FM DHA was observed only in the electropherogram of a stimulated cell sample (Fig. 2c).

Figure 2a shows a representative electropherogram for untreated macrophages. As can be seen by this electropherogram, the amount of NO and $O_2^{\bullet-}$ produced by native macrophages is very small because of the natural occurrence of endogenous intracellular scavenging molecules, such as SOD. Cytosolic and mitochondrial SOD regulates the intracellular concentration of $O_2^{\bullet-}$, which can make $O_2^{\bullet-}$ difficult to detect in the cell lysate samples [32]. Therefore, to reduce the degradation of intracellular $O_2^{\bullet-}$ by SOD, two different SOD inhibitors, 2-ME and DDC, were introduced into the cells, along with MitoSOX Red, and the cells were incubated for 1 h before analysis (Fig. 1). Figure 2b shows a representative electropherogram obtained for unstimulated macrophages pretreated with 2-ME and DDC, with a corresponding increase in the 2-OH-MitoE$^+$ peak in relation to the DAF-FM T peak. An electropherogram of the cell lysate obtained for cells stimulated with LPS, IFN-γ, and then PMA in the presence of SOD inhibitors is shown in Fig. 2c. In this case, the products for the NO and $O_2^{\bullet-}$ signals were increased, indicating an expected enhancement in intracellular production of both species, with a greater amount of NO being produced. This yielded a higher NO/$O_2^{\bullet-}$ ratio than for the SOD inhibitors alone.

A bar graph showing the comparison of the NO/$O_2^{\bullet-}$ ratios obtained under the different experimental conditions is provided in Fig. 2d. The ratios obtained for unstimulated cells pretreated with SOD inhibitors (0.60 ± 0.07), unstimulated cells without SOD inhibitors (1.08 ± 0.06), and stimulated macrophages (3.14 ± 0.13) show that, along with peak areas, the NO/$O_2^{\bullet-}$ ratio changes as a function of the stimulation conditions. In these experiments, the number of viable cells was determined before analysis because the stimulation process can reduce the amount of cell division [33], increase cell differentiation [34], and also cause cell death [35]. Figure S2 shows the variation in cell numbers as a function of the different stimulation protocols used in the present study.

Table 1 Migration times for *DAF-FM T* (NO product) and *2-OH-MitoE*$^+$ ($O_2^{\bullet-}$ product) for untreated and stimulated cells

Cell treatment	Migration time (s)	
	DAF-FM T	2-OH-MitoE$^+$
Untreated	21.82 ± 0.11	27.87 ± 0.15
Unstimulated + SOD inhibitors	22.81 ± 0.59	29.41 ± 0.45
LPS + IFN-γ + PMA + SOD inhibitors	23.64 ± 1.13	30.90 ± 1.42

All the migration times are given as number ± standard deviation

IFN-γ interferon gamma, *LPS* lipopolysaccharide, *PMA* phorbol 12-myristate 13-acetate, *SOD* superoxide dismutase

Changes in the NO/$O_2^{\bullet-}$ ratio for macrophages stimulated with LPS plus IFN-γ versus macrophages stimulated with PMA

NO [36] and $O_2^{\bullet-}$ [37] have been implicated in the development of several neurodegenerative disorders and cardiovascular disease. Therefore, a series of experiments were performed to investigate the effect of two different stimulation protocols on the intracellular NO/$O_2^{\bullet-}$ ratio. Figure 3a shows the comparison between NO and $O_2^{\bullet-}$ peak areas obtained for cells stimulated with LPS plus INF-γ (NO stimulation) or PMA

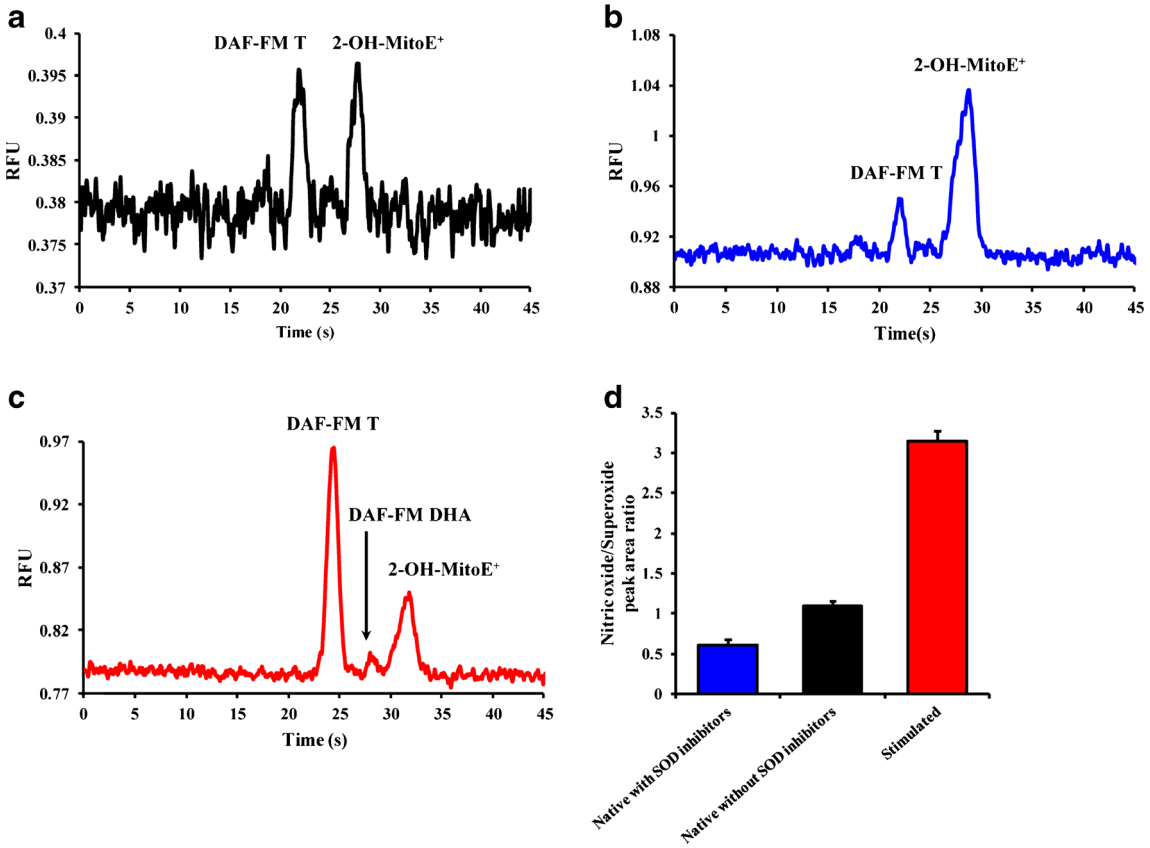


Fig. 2 Representative electropherograms of (**a**) native macrophage cell lysate, (**b**) macrophage cell lysate treated with DDC and 2-ME, and (**c**) cell lysate stimulated with LPS plus IFN-γ plus PMA in the presence of superoxide dismutase (SOD) inhibitors. (**d**) A histogram comparing the $NO/O_2^{\bullet-}$ peak area ratios between unstimulated cells in the presence or absence of SOD inhibitors, and cells stimulated with LPS plus IFN-γ plus PMA in the presence of SOD inhibitors. Standard deviations are represented by *vertical bars*. *DAF-FM T*, *2-OH-MitoE*$^+$, *RFU* relative fluorescence units

($O_2^{\bullet-}$ stimulation). As expected, the NO peak area was higher than that of $O_2^{\bullet-}$ for the samples stimulated with LPS plus INF-γ, whereas the opposite situation was observed for samples treated with PMA. The average $O_2^{\bullet-}$ peak area was more than three times higher in the case of iNOS-activated cells (LPS + INF-γ) than in the case of cells stimulated by PMA. These data imply that increased NO production or iNOS activation may catalyze $O_2^{\bullet-}$ production, which agrees with previous reports in the literature [38–41].

A bar graph showing the effect of the stimulation protocol on the $NO/O_2^{\bullet-}$ ratio is shown in Fig. 3b. The $NO/O_2^{\bullet-}$ ratio for cells stimulated with LPS plus INF-γ was 2.31 ± 0.41 and the ratio for cells treated with PMA was 0.19 ± 0.06.

Effect of carnosine or calcium on the $NO/O_2^{\bullet-}$ ratio in native and stimulated macrophages

Carnosine is an endogenous dipeptide that exhibits antioxidant properties and protects cells against free radicals. It has been clearly demonstrated that carnosine is able to scavenge RNOS [42]. Caruso et al. [43] recently reported that carnosine can catalyze the conversion of NO to nitrite, thereby causing a decrease in the apparent intracellular NO concentration. We have also shown that significant amounts of carnosine are taken up by macrophages when it is incorporated in the cell culture medium [44]. In these studies, the effect of Ca^{2+} on the $NO/O_2^{\bullet-}$ ratio was also investigated. Ca^{2+} is an intracellular second messenger involved in signal transduction and many pathological processes [45]. Ca^{2+}, along with RNOS, participates in the regulation and integration of many cellular functions [46]. Increases in cytoplasmic Ca^{2+} concentration have been correlated with increased amounts of $O_2^{\bullet-}$ [46–48].

The effect of pretreatment of the cells with either carnosine or Ca^{2+} on the $NO/O_2^{\bullet-}$ ratio in macrophage cell lysates was investigated under native and proinflammatory conditions. Figure 4 depicts the change in $NO/O_2^{\bullet-}$ ratio due to pretreatment of the cells with carnosine or Ca^{2+} in unstimulated (Fig. 4a) and stimulated (Fig. 4b) cells. When compared with the control (unstimulated cells), the samples pretreated with carnosine showed a decrease in the $NO/O_2^{\bullet-}$ ratio (from 0.60 ± 0.07 to 0.35 ± 0.04). This ratio decrease was even more prominent for cells pretreated with Ca^{2+} (from 0.60 ± 0.07 to 0.29 ± 0.05). A comparable trend was observed for stimulated

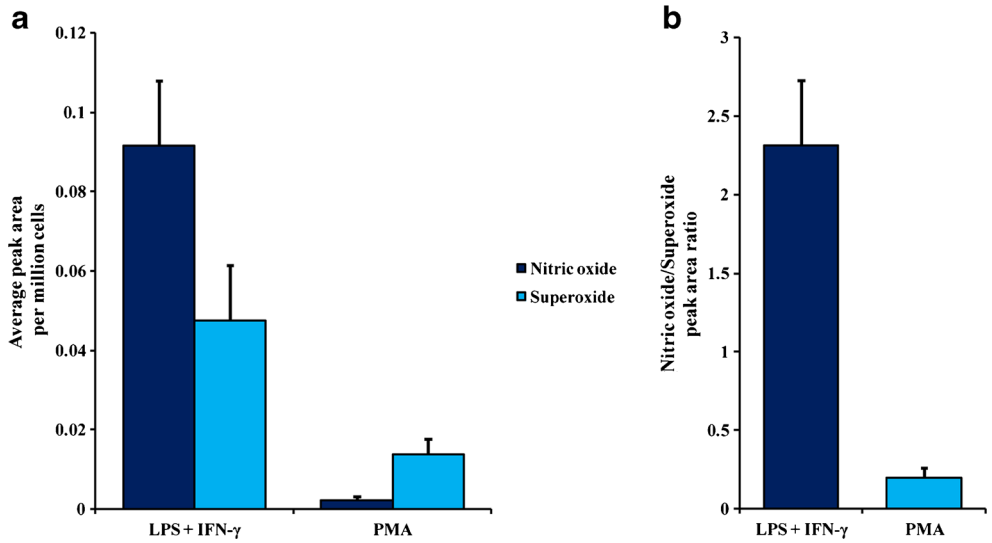


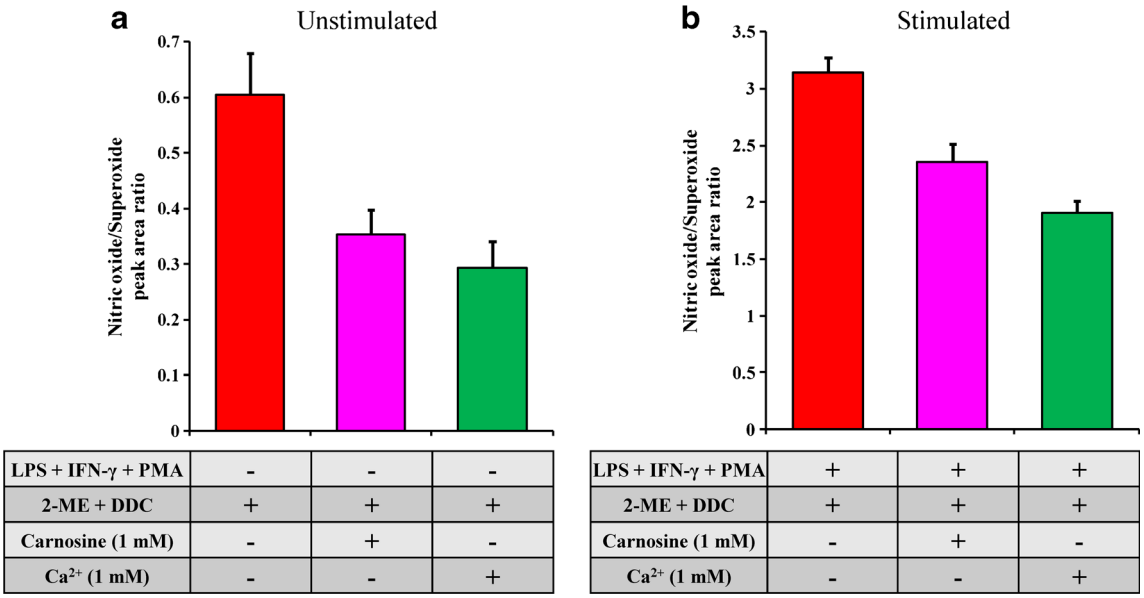
Fig. 3 (**a**) The average peak area per million cells of NO and $O_2^{\bullet-}$ in cell lysate samples stimulated with LPS plus INF-γ (NO stimulation) or PMA ($O_2^{\bullet-}$ stimulation). (**b**) Comparison of the NO/$O_2^{\bullet-}$ peak area ratios between the two different stimulation protocols. Standard deviations are represented by *vertical bars*

samples, where the difference in the NO/$O_2^{\bullet-}$ ratio between each sample was slightly lower (from 3.14 ± 0.13 for cells stimulated with LPS plus INF-γ plus PMA to 2.35 ± 0.16 and 1.91 ± 0.10 for cells challenged with the same stimuli and pretreated with carnosine or Ca^{2+}, respectively).

We believe that the decrease in the NO/$O_2^{\bullet-}$ ratio caused by pretreatment with carnosine is due to a decrease in NO production (with little or no change in $O_2^{\bullet-}$ production). Previous studies by our group and others have shown that carnosine reduces iNOS-facilitated NO production in cells [43, 49, 50]. In contrast, it is proposed that Ca^{2+} pretreatment caused a decrease in the NO/$O_2^{\bullet-}$ ratio because of an increase in intracellular $O_2^{\bullet-}$ production (and not a decrease in NO production). High intracellular Ca^{2+} concentrations have been shown to enhance the production of $O_2^{\bullet-}$ in both the cytoplasm and mitochondria [46–48].

Conclusions

In this investigation, ME–LIF was used for the simultaneous detection of NO and $O_2^{\bullet-}$ using the fluorescent probes DAF-FM DA and MitoSOX Red. This method was also used to study the variations of the NO/$O_2^{\bullet-}$ ratio in RAW 264.7 macrophage cell lysates under physiological and proinflammatory conditions. Additionally, the effect of the natural antioxidant



LPS + IFN-γ + PMA	-	-	-
2-ME + DDC	+	+	+
Carnosine (1 mM)	-	+	-
Ca^{2+} (1 mM)	-	-	+

LPS + IFN-γ + PMA	+	+	+
2-ME + DDC	+	+	+
Carnosine (1 mM)	-	+	-
Ca^{2+} (1 mM)	-	-	+

Fig. 4 Changes in the NO/$O_2^{\bullet-}$ ratio due to pretreatment with carnosine or Ca^{2+} in (**a**) unstimulated cells and (**b**) cells stimulated with LPS plus IFN-γ plus PMA. Standard deviations are represented by *vertical bars*

carnosine and the second messenger Ca^{2+} in modulating this ratio was investigated. These results highlight the roles played by different stimulation protocols in influencing the release and bioavailability of NO with respect to $O_2^{\bullet-}$. It is well known that NO and $O_2^{\bullet-}$ production is related to many nitrosative and oxidative stress-driven disorders; thus, the development of new cell stimulation protocols along with the application of this method in single-cell analysis formats will provide new perspectives that can be used for a better understanding of the role of RNOS in neurodegenerative and cardiovascular disease.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animals This article does not contain any studies with human participants performed by any of the authors.

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