Oxygen levels modulation of the macrophage oxidative response: nitric oxide and peroxynitrite production

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INTRODUCTION

The macrophage cytotoxic response is dependent on oxygen (O_2) as a substrate for oxidant production. Indeed, O_2 is utilized by (the inducible) nitric oxide synthase (iNOS) and NADPH oxidase-2 (NOX-2), to produce nitric oxide ($^{\bullet}NO$) and superoxide ($O_2^{\bullet-}$), respectively (Figure 1). Furthermore, the combination reaction between these species leads to peroxynitrite ($ONOO^-$), a strong oxidant that can cause biological damage through oxidation and nitration. Since oxygen concentration varies between tissue and culture conditions, it has been of great interest to study these processes *in cellula,* considering the physiological environment. Previous reports from our group showed that oxidant production depends on oxygen partial pressure (pO_2) when evaluated during short-time exposures (Figure 2). Herein, we show the effects of long-term exposure to a range of physiological pO_2 on the activity and expression of iNOS.

Peroxynitrite formation, assessed through the boronate-based probe, coumarin-boronic ester (CBE), also shows a similar tendency with pO_2 in short-term exposures (scheme 1). Nevertheless, CBE is limited to only certain methodologies because of its spectroscopic properties. Moreover, other probes such as fluorescein-boronate (Fl-B) are sensitive to drastic variations in intracellular pH and pO_2. Therefore, we are developing a novel fluorescent boronate-based probe (Red-B) derived from xanthene, to achieve an accurate cellular detection of peroxynitrite in different cellular conditions.

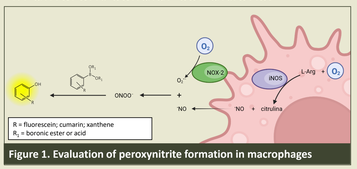


Figure 1. Evaluation of peroxynitrite formation in macrophages

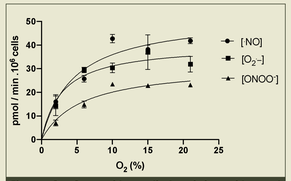
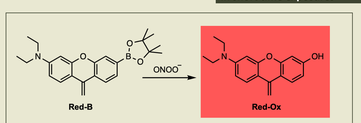


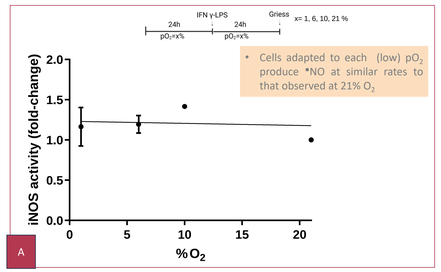
Figure 2. Superoxide radical, nitric oxide and peroxynitrite formation rates at different pO_2 evaluated in short time exposures

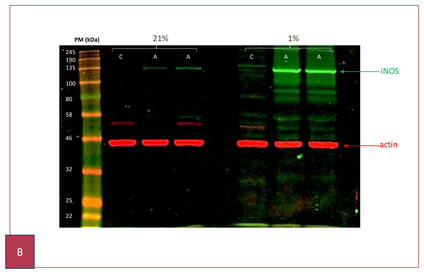


Scheme 1. Reaction between peroxynitrite and Red-B.

RESULTS

1. OXYGEN AS A MODULATOR OF •NO PRODUCTION





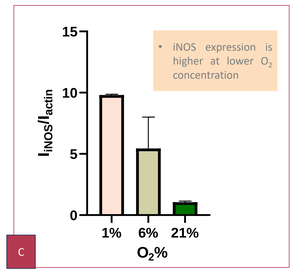
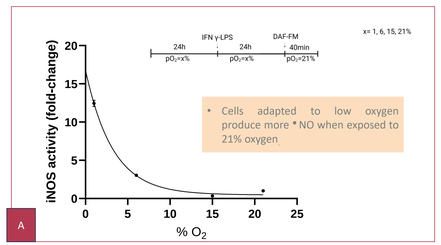


Figure 3. Oxygen modulation in the production of nitric oxide. A) J7741-A1 macrophages were incubated at different pO_2(1,6,10,21%) for 24h and then activated with LPS and IFN-γ for another 24h, • NO production was determined by Griess assay. • NO fluxes were normalized by cell number and expressed as a percentage to that observed at 21% O_2 . **B)** Macrophages incubated as in A) were lysed to evaluate iNOS expression through immunoblotting. **C)** Ratio iNOS/actin intensity of the western blot.



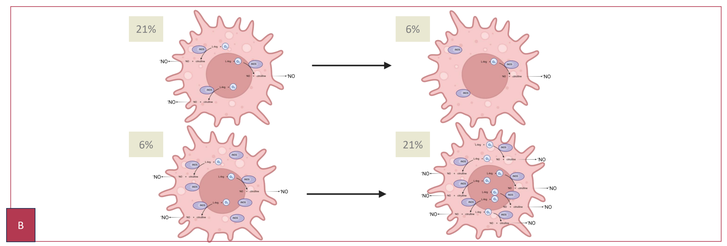
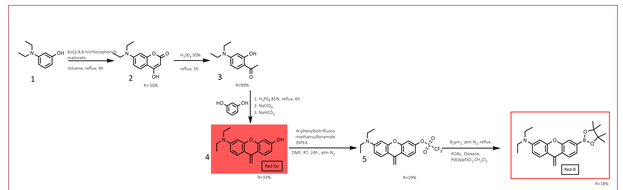
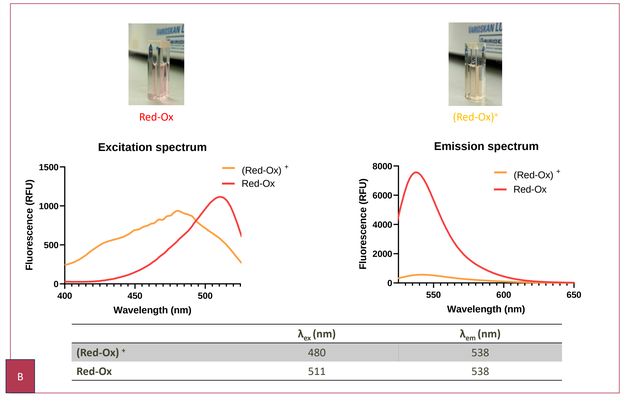


Figure 4. • NO flux in cells cultured at different oxygen concentrations, during their return to normoxia (pO_2=21 %). A) J774-A1 macrophages were incubated in different oxygen concentrations (pO_2=1,6,10,15,21%) for 24h and then activated with LPS and IFN-γ for another 24h. After that, • NO flux was determined with fluorescent probe DAF-FM . • NO fluxes were normalized by cell number and expressed as a percentage to that observed at 21% O_2 **B)** Hypothesis to explain the results showed in 3 y 4.

2. Red-B: FLUORESCENT BORONATE-BASED PROBE FOR PEROXYNITRITE DETECTION





	λ_{ex} (nm)	λ_{em} (nm)
(Red-Ox) +	480	538
Red-Ox	511	538

B

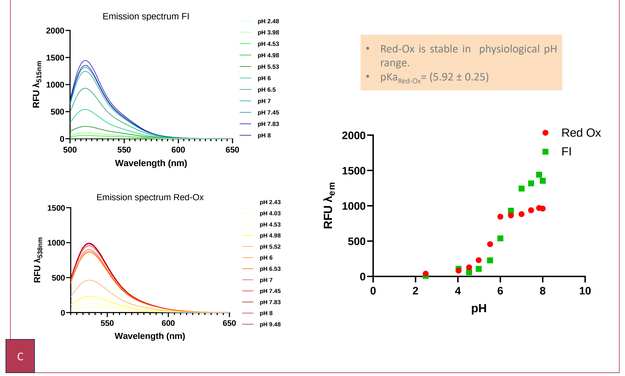


Figure 5. Synthesis and characterization of Red-B and Red-Ox. A) Red-B synthetic route involves 5 steps. Red-Ox was obtained in 3 steps. **B)** Spectroscopic characterization of Red-Ox, showing its emission and excitation spectra and their differences with its protonated form (Red-Ox)+. **C)** Fluorescence dependence on pH for Fluorescein (Fl) and Red-Ox.

CONCLUSIONS

- Cells exposed 48 h to each pO_2 (1, 6, 10 and 15%) produce $^{\bullet}NO$ at similar rates to that observed at 21% O_2 despite the limited availability of substrate.
- An increase in iNOS expression can be observed as oxygen concentration decrease. This could be a compensation mechanism for the lack of substrate.
- After returning cells to 21% O_2, cells adapted to lower pO_2 showed an increase in $^{\bullet}NO$ production which can be explained by the increase its expression.
- Red-B was synthesized in 5 reaction steps. The corresponding fluorophore Red-Ox, was obtained in a 3-step synthetic route and exhibits optimal spectroscopic properties (λ_{exc}=511nm; λ_{em}=538nm) and shows low pH dependence in physiological range (pKa = (5.92 ± 0.25)).
- Prolonged incubation at physiological O_2 concentrations results in macrophage adaptation, enabling similar $^{\bullet}NO$ production rates as those observed at 21% oxygen. Future experiments with Red-B will allow as to determine if this trend also applies to $ONOO^-$ formation.

REFERENCES

- Casella , AC. Prolo, C., *et al.* (2023) Superoxide, Nitric Oxide and Peroxynitrate production under different physiological oxygen tensions. *FRBM, submitted.*
- Jeffrey Man, H. S., Tsui, A. K. Y., & Marsden, P. A. (2014). Nitric oxide and hypoxia signaling. In *Vitamins and Hormones* (Vol. 96, pp. 161–192). Academic Press Inc.
- Carreau, A. *et al.* (2011). Why is the partial oxygen pressure of human tissues a crucial parameter? Small molecules and hypoxia. *Journal of Cellular and Molecular Medicine*, *15*(6), 1239.
- Prolo, C., Rios, N., *et al.* (2018). Fluorescence and chemiluminescence approaches for peroxynitrite detection. *FRBM*, *128*, 59–68.
- Rios, N., *et al.* Sensitive detection and estimation of cell-derived peroxynitrite fluxes using fluorescein-boronate., *101*, 284–295.

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