Differential modulation of S-nitrosoproteome of *Paracoccidioides brasiliensis* by Nitric Oxide: change in S-nitrosylation levels and fungal redox status

Marina V. Navarro¹, Daniele G. Castilho¹, Alison F.A. Chaves¹, Juliana C. Calado¹, Palloma M. Conceição², Leo K. Iwai³, Beatriz F. de Castro², Isis Casula², Wagner L. Batista^{1,2}.

¹ Department of Microbiology, Immunology and Parasitology, Universidade Federal de São Paulo, São Paulo, Brazil.

² Department of Pharmaceutical Sciences, Universidade Federal de São Paulo, Diadema, São Paulo, Brazil,

³ Laboratory of Applied Toxinology, Center of Toxins, Immune-response and Cell Signaling, Instituto Butantan, São Paulo, SP, Brazil

INTRODUCTION

Paracoccidioides brasiliensis and *P. lutzii* are the causative agents of paracoccidioidomycosis (PCM), a systemic mycosis endemic in Latin America. These fungi are considered facultative intracellular pathogens, able to survive and replicate inside macrophages. After infection, the survival depends on its adaptability to host conditions, such as nitrosative stress caused by nitric oxide (NO) produced by the immune cells. Currently, NO is viewed as a remarkably important signaling molecule, involved in regulating stress responses. Protein S-nitrosylation, characterized by covalent attachment of nitroso group to cysteine thiol, has emerged as a central mechanism of NO-dependent cellular regulation. We have previously reported that low NO concentrations leads to Ras S-nitrosylation and induces cell proliferation in *P. brasiliensis*. However, others targets of NO in this fungus are unknown. In present study, using a combination of the biotin switch assay and label-free LC-MS/MS analysis we revealed the S-nitroso-proteome profile of *P. brasiliensis* isolate 18 (Pb18) under different NO concentration. Immunoblotting and enzymatic assays were performed to assess protein S-nitrosylated profiles and redox profile respectively. In order to determine S-nitrosylated proteins, first it was performed biotinylation assay followed by protein reduction, alkylation, digestion and peptide desalting. Next, samples were injected in Orbitrap Velos mass spectrometry connected to an Easy-nLCII. The MS raw files were processed and searched using MaxQuant software. All validated proteins were submitted to Blast2GO algorithm. Immunoblotting results showed distinct band patterns among samples treated with different NO concentrations were observed. S-nitrosylation levels increased when cells were treated with 0.25 μ M NO, suggesting the participation of this post-translational modifications in the fungus growth induced by low concentrations of RNS. Changes in the fungus redox state according with NO concentrations were also observed by enzymatic assays and evaluation of the GSH (reduced glutathione) and GSSG (oxidized glutathione) levels. Using a mass spectrometry-based approach, we mapped 335 S-nitrosylated proteins, where 48 and 6 were exclusively found on the groups treated with 0.25 and 10 μ M of NO₂ respectively. With this approach, we observed that proteins treated with NO low concentrations presented a proliferative response pattern, with several proteins involved with cellular cycle regulation and growth and would represent, as last analysis, molecules with an important role to fungi virulence. On the other hand, proteins stimulated with NO high concentrations exhibited a survival response pattern. It has been demonstrated that the distinct redox-dependent events (cell proliferation or survival) were promoted by different NO concentrations, which may help us understand RNS antifungal properties and identify potential molecular targets to future development of new drugs.

MATERIALS AND METHODS

RESULTS

Figure 1: Cell viability of *P. brasiliensis* isolate under varying concentrations of reactive nitrogen species. 1.8×10^5 viable cells were treated with different concentrations of nitric oxide with pH: 5.6 for 5 h at 37°C. The treated cells were subjected to serial dilution and 30 μ L of each dilution was plated on YPD agar. The Petri dish were incubated for 7 days at 37°C.

Figure 2: Nitration and S-nitrosylation profile of *P. brasiliensis* proteins before and after treatment with NO. Protein extracts were analyzed by Dot blotting (A) and Western blotting (B) with anti-nitro-Tyr antibody and Dot blotting (C) and Western blotting (D) with anti-nitroso-Cys antibody. The graphs were obtained by densitometric analysis of the bands.

Figure 3: Enzymatic activity of (A) superoxide dismutase (SOD) and (B) catalase of *P. brasiliensis* samples treated with different concentrations of nitrite. The results are representative of three independent experiments with $n = 5$ and $*p=0,0001$.

Figure 4: Enzymatic activity of (A) glutathione peroxidase (GPx) and (B) glutathione reductase (GR) after nitrosative stress. The results are representative of three independent experiments with $n = 5$ and $*p=0,0004$.

Figure 5: Estimative of (A) total glutathione, (B) glutathione, oxidized glutathione, (C) reduced glutathione and (D) GSH/GSSG ratio after nitrosative stress. The ratio GSH/GSSG is used to estimate biological system redox state. Under oxidative stress GSH is converted to GSSG leading to a decrease of this ratio. The results are representative of three independent experiments with $n = 5$ and $*p=0,0038$.

Figure 6: Venn diagram. The diagram shows the number of proteins detected on *P. brasiliensis* extracts before and after treatment with 0.25 and 10 μ M oxide nitrite. The diagram was generated from JVENN bioinformatics software (bioinfo.genotoul.fr/jvenn/index.html). The graph shows the list size of identified proteins and the protein list was generated from MaxQuant software.

Figure 7. Functional categorization for molecular function of Blast2GO.

CONCLUSIONS

- ✓ Stimulus with diferente concentrations of RNS → alterations in cellular metabolism of Pb yeast cells;
 - ✓ ↓ RNS → ↑ cellular growth;
 - ✓ ↑ RNS → ↓ cellular viability;
- ✓ NO modulate redox post-translational modifications in *P. brasiliensis*;
- ✓ Such modifications (S-nitrosylation and / or nitration) can activate or inhibit signaling pathways, modulating different phenomena (proliferation or cell survival) observed in our model.