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New insights in the study of s-nitrosylation & s-nitration: strategies and problems

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Introduction

The study of the physiological role of reactive nitrogen species (RNS) is attracting increasing interest in the biomedical community. The most common protein modifications by RNS are S-nitrosylation or covalent incorporation of a nitrosile radical to a thiol group, and nitration, or incorporation of a NO_2 group, generally to tyrosine residues. It is currently thought that nitrosative stress is one of the most relevant pathogenic factors in cardiovascular pathology, including atherosclerosis, hypertension, diabetes and cardiac insufficiency, and NO has become recognized as a key signaling molecule in plants. Moreover, there are evidences that exposing endothelial cells to the immunosupressor cyclosporin A (CsA) increases production of RNS, and particularly peroxinitrite, that stimulates nitration of proteins, being the protein MnSOD one of the candidates to CsA-induced nitration (Horcajo-Redondo *et al.*, 2005). Here we have developed and used proteomics techniques to identify specific sites of S-nitrosylation and S-nitration in protein extracts from different species. The growing interest in the study of these modifications have led to the creation of a Spanish working group, the "*Nitrosoteam*",

with the objective of increasing the knowledge of the modifications in different kinds of samples by using a common strategy.

Material and methods

We used the "*biotin-switch*" method (Martínez-Ruiz *et al.*, 2005) to selectively enrich nitrosylated peptides in human and plant protein extracts. The Selected MS/MS Ion Monitoring (SMIM) technique using a linear ion trap mass spectrometer (*LTQ, Thermo-Fischer Scientific, San Jose, USA*) (Jorge *et al.*, 2007) was used to identify a specific site of nitration in a MnSOD peptide.

Results

Here we present the first results of the "*Nitrosoteam*". The first goal was to apply the "*biotin-switch*" method to the purification of peptides instead of proteins, what is an innovation of the technique. We first used synthetic peptides as probes and after optimization of the mass spectrometer and the database searching conditions we proceeded to the study of *in vitro* cell culture extracts without blocking free cysteine thiols with MMTS. Elution of peptides from the avidin column was made with either acid or reducing conditions. The acidic elution had the advantage that biotin remained attached to the peptide, whereas in the reducing elution more peptides could be identified. Some problems in the different steps of the experiment have emerged. We observed that the biotin moiety attached to the peptides produced a lot of fragments that could not be assigned (Figure 1). Besides, the manipulation of the samples produced different atypical adducts that make the identification more difficult. For studying the nitration of a specific site in MnSOD, we had to optimize the mass spectrometer conditions in order to identify beyond any doubt the modification site using multiple fragmentation stages (MS^3).

Conclusions

Several technical problems were resolved during the course of this study to analyze S-nitrosylation and S-nitration sites by MS approaches. Our preliminary results give us good indications about the viability and specificity of the technique and encouraged the "*Nitrosoteam*" to apply these developments to the analysis of physiological samples.

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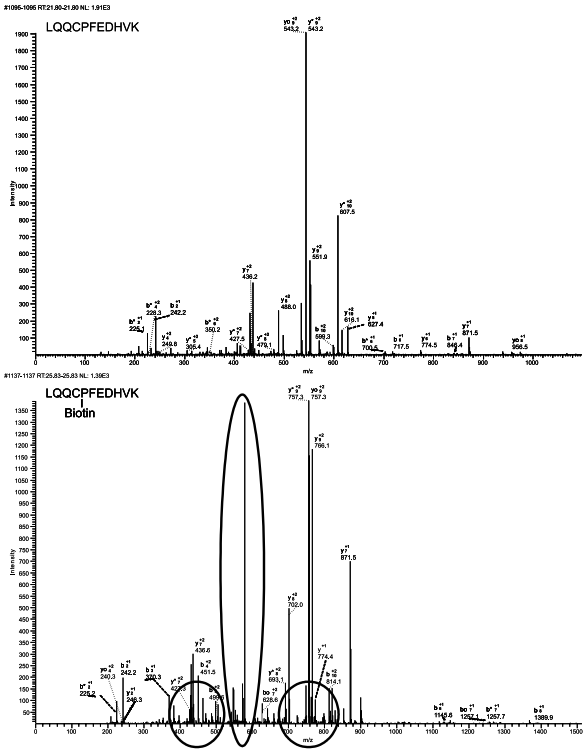

Figure 1: *Fragmentation spectra of a non biotinilated (upper panel) and a biotinilated peptide (lower panel) showing the presence of multiple fragments produced by biotin that could not be assigned (circles).*