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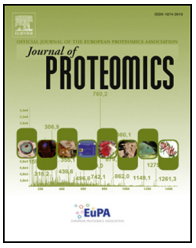

Proteomic characterization of nitrated cell targets after hypobaric hypoxia and reoxygenation in rat brain

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ABSTRACT

This study analyzes the nitrated protein profile of the rat-brain cortex in a model of hypoxia/reoxygenation, identifying the nitrated proteins and assessing spot changes. The proteins identified were grouped into categories, according to their function: 1) metabolism: pyruvate kinase (PK), α-enolase, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), phosphoglycerate mutase 1 (PGAM1), and glutamine synthetase (GS); 2) cytoskeletal proteins: α-tubulin, β-tubulin, γ-actin, and glial fibrillary acidic protein (GFAP); 3) chaperones: heat-shock protein 71 kDa (HSP71); and 4) carrier proteins: voltage-dependent anion-selective channel protein 1 (VDAC-1) and Atp6v1a. PK, α-enolase, and GS nitration rates were upregulated, increasing progressively during reoxygenation and peaking at 24 h. GAPDH and PGAM1 nitration levels fell after hypoxia/reoxygenation. α-Tubulin, β-tubulin, γ-actin, and GFAP nitration rates augmented at 24 h, but diminished at 5 d. HSP71 suffered from nitration immediately after hypoxia, but not during reoxygenation. VDAC-1 tyrosine nitration was identified only in the control group, whereas detectable Atp6v1a nitration levels were observed only immediately after hypoxia. The data have been deposited to the ProteomeXchange with identifier PXD001049.

Our findings suggest a hypothetically crucial linkage between nitration-related protein modifications and metabolic and cell-structure alterations. These changes are probably needed for the remodeling and plasticity processes activated by the hypoxic brain response.

Abbreviations: 2-DE, two-dimensional gel electrophoresis; CNS, central nervous system; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; GS, glutamine synthetase; HIF-1, hypoxia-inducible factor 1; HSP71, heat-shock protein 71 kDa; iNOS, inducible nitric oxide synthase; NO, nitric oxide; NOS, nitric oxide synthase; n-Tyr, 3-nitro-L-tyrosine; $^{\cdot}OH$, hydroxyl radical; $ONOO^{-}$, peroxynitrite; PGAM1, phosphoglycerate mutase 1; PK, pyruvate kinase; PMF, mass-fingerprinting analysis; RNS, reactive nitrogen species; ROS, reactive oxygen species; VDAC-1, voltage-dependent anion-selective channel protein 1.

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Biological significance

For the first time the spectrum of nitrated proteins in the hypoxic brain as well as its changes during reoxygenation are described. Our findings suggest a hypothetically crucial linkage between nitration-related protein modifications and metabolic and cell-structure alterations. These changes are probably needed for the remodeling and plasticity processes activated by the hypoxic brain response.

The biological relevance of these findings is linked to the important role developed by the signaling molecule NO in the hypoxic brain, and could be interpreted in two different but complementary ways: first, as a mechanism of damage due to nitration impacts over some key proteins affecting its structure and function; and second, as a regulation mechanism involved in the hypoxic response. Hence, based on the modified proteins identified and their functions, it would be possible to design new tools and therapies to prevent brain damage in low-oxygen-pressure atmospheres.

1. Introduction

Hypobaric hypoxia, a type of hypoxia brought on by a reduction in total atmospheric pressure, occurs spontaneously as altitude increases and can be experimentally induced in hypobaric chambers [1–3]. During both hypoxia and the following reoxygenation (recovery of the normal oxygen levels in blood and tissues) some major changes take place in the oxidative cell status, such as the generation of high concentrations of oxygen-reactive and nitrogen-reactive species (ROS and RNS, respectively), as well as the specific expression of a number of genes which participate in the adaptive response to hypoxia [4]. Although the neurological and physiopathological alterations associated with hypobaric hypoxia are well known [5–8], the molecular mechanisms underlying these alterations have not been fully explored.

The nitric oxide (NO) system plays a crucial role in the central nervous system (CNS) response to hypobaric hypoxia, since, depending on the NO concentration, and particularly on its capability to nitrate proteins, it can trigger opposite neuroprotector/neurotoxic actions [6,9–11]. Thus, when NO is produced in high concentrations or during long periods, it not only acts as an intra- and intercellular messenger, but also exerts deleterious effects [12]. NO is synthesized by three protein species of the nitric oxide synthase (NOS), among these being the inducible nitric oxide synthase (iNOS) protein species, the activity of which is not regulated, this being the main factor responsible for the toxic effects of NO. Thus, after iNOS induction, higher NO levels are detected; NO can then easily react with the superoxide anion, producing the strongly oxidant peroxynitrite ($ONOO^-$) [13–15]. Peroxynitrite is highly soluble in lipids, and has a wide range of targets, triggering the oxidation of –SH, lipid peroxidation, and DNA as well as RNA breaks [16–18]. $ONOO^-$ may be broken down into nitrogen dioxide (NO_2) and hydroxyl radical ($^{\cdot}OH$). $^{\cdot}OH$ is a powerful oxidant as well, and contributes strongly to oxidative cell damage [19]. Peroxynitrite can also act on itself by triggering the intrinsic apoptotic pathway [20] or inducing protein nitration.

Protein nitration is a post-translational modification strongly increased under oxidative-stress conditions [21] that transforms protein tyrosine residues into 3-nitro-L-tyrosine (n-Tyr) [22]. In this sense, tyrosine nitration has been considered a marker of protein degradation [23]; particularly, the proteolytic cleavage rate is reportedly higher for nitrated proteins [24–26]. Protein nitration is not an unspecific but a selective process that affects specific proteins, altering their activities [27]. In this sense, peroxynitrite-dependent protein modification might modulate signaling events by altering the phosphorylation/dephosphorylation equilibrium [28]. It has been proposed that n-Tyr resembles phosphotyrosine and its formation may irreversibly block tyrosine phosphorylation-dependent signaling. Moreover, given that an *in vivo* denitration process might also occur [29], the nitration impacts, and even the posterior denitration may trigger major pathways, modulating enzymatic activity [30], regulating the specific enzyme protein levels, and promoting biological effects with key roles in the pathogenesis of many diseases [31]. However, protein nitration can be a specific or even reversible process that, depending on its target, can result in either activation or inhibition [32]. In this sense, it has been demonstrated that tyrosine nitration is a highly selective modification that can develop physiological roles under certain conditions [32].

Ischemic or hypoxic situations induce high NO production, and consequently peroxynitrite formation [33]. A NO-mediated mechanism of programmed cell death, based on protein nitration, underlies hypoxic brain damage. In turn, this molecule induces both the formation of mitochondrial permeability transition pores [34] and a rapid modification of cytochrome c structure, probably through tyrosine nitration, allowing its release to cytosol [35].

In short, both *in vitro* and *in vivo* studies, including proteomic approaches, show that protein tyrosine nitration is a selective process that appears to play an important role in hypoxia, where a small amount of proteins are found to be nitrated and only one or a few tyrosine residues are modified in each case [36].

The present work is part of a more comprehensive study that has analyzed the changes in the whole-brain proteomic profile after hypobaric hypoxia [2]. This previous work described for the first time the protein profile affected by hypobaric hypoxia in the brain cortex of rat, and pointed to energy metabolism and cytoskeletal proteins as the main targets of the hypoxic-derived damage. Hence, delving into this field, the present work seeks: 1) to reveal by means of proteomic approaches the target proteins of hypobaric hypoxia-related nitration, as well as the corresponding changes in their