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**REGULATION OF CELLULAR FUNCTIONS
BY NITRIC OXIDE PATHWAYS**

**REGOLAZIONE DELLE FUNZIONI CELLULARI
INDOTTE DA OSSIDO NITRICO**

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TABLE OF CONTENTS

ABSTRACT

1. INTRODUCTION	1
1.1 Function and regulation of Nitric Oxide.....	1
2. Oxidative/nitrosative modifications of proteins	4
2.1 Oxidative/nitrosative modifications of protein thiols.....	5
2.2 Glutathionylation.....	9
2.3 Metalloproteins.....	11
<i>Metallothioneins</i>	12
<i>Specificity protein 1</i>	14
2.4 Aim of the work.....	15
2.5 Results and discussion.....	16
<i>Identification of MT cysteine residues hypothetically susceptible to S-glutathionylation</i>	16
<i>S-glutathionylation of MT induced by oxidant agents</i>	18
<i>S-glutathionylation of MT in peripheral blood mononuclear cell extracts</i>	22
<i>Levels of S-glutathionylated endogenous MTs in different age groups</i>	23
<i>S-glutathionylation of endogenous Sp-1 in PBMC</i>	25
3. NO signalling through NOS interaction with target proteins	27
3.1 Mitochondrial NOS interaction with CcOX.....	28
3.2 Aim of the work.....	31
3.3 Results and discussions.....	32
<i>hnNOS alpha cloning and mutagenesis</i>	32
<i>Over-expression of wild type and mutant nNOS in PC12 cells</i>	32
<i>Mitochondrial localization of nNOS and co-localization with CcOX</i>	35
<i>Molecular modeling</i>	38
<i>Analysis of nNOS/CcOX interaction in transfected cells after NGF treatment</i>	40

4. Conclusions	46
ACKNOWLEDGMENTS.....	49
REFERENCES.....	50
APPENDIX.....	64

ABSTRACT

Nitric oxide (NO) is a highly diffusible gas that is synthesized by three distinct isoforms of nitric oxide synthases (NOS). Two isoforms are constitutively expressed and generate NO for cell-signalling purposes: neuronal NOS (nNOS) and endothelial NOS (eNOS), and the third member of the family is an inducible isoform (iNOS) which releases NO in larger quantities during inflammatory or immunological defence reactions and is involved in host tissue damage. NO has been shown to act as a multifunctional gaseous modulator in many cellular events. It is an important messenger molecule synthesized in a variety of tissues and involved in various physiological and pathological functions.

The regulatory actions of NO are explained both by the physiological intracellular concentrations of NO and by some well known NO-dependent cell signalling and regulatory pathways. During this research project we focused on two of these regulatory pathways: the NO-dependent protein modifications of cysteine residues and the reversible inhibition of mitochondrial cytochrome c oxidase (CcOX), the terminal complex of the mitochondrial respiratory chain.

Modulation of thiol-disulfide status of critical cysteines on enzymes, receptors, transport proteins, and transcription factors is recognized as an important mechanism of signal transduction and an important consequence of oxidative/nitrosative stress associated with aging, cardiovascular and neurodegenerative diseases. Within these contexts, a prevalent form of cysteine modification is reversible formation of protein mixed disulfides with glutathione (GSH), the major non-protein thiol compound in cells. Protein *S*-glutathionylation increases globally during overt oxidative stress, but selective/local generation of reactive oxygen and nitrogen species mediates physiological redox signaling. Moreover, reversible modifications, as *S*-glutathionylation, have been suggested to have a dual role: protection from cysteine irreversible oxidation and modulation of protein function.

Among the wide list of proteins demonstrated to be susceptible to oxidative cysteine modifications, as *S*-nitrosylation, we focused on Metallothionein (MT) and the transcription factor Sp1, two zinc-binding metalloproteins that play fundamental roles in cellular functions, and whose activity has been shown to be affected upon ageing. Thus we investigated the susceptibility to *S*-glutathionylation of these proteins. Analysis of the three-dimensional structure of both MT and Sp1 showed the presence of some Cys residues likely targets for *S*-glutathionylation, both for their solvent accessibility and

electrostatics induced reactivity glutathionylable cysteine residues. Western blot and dot blot assays performed after *in vitro* exposure to GSNO, diamide and H₂O₂ (oxidant agents acting through different mechanisms) revealed that both MT and Sp1 can be susceptible to S-glutathionylation upon oxidative/nitrosative stress conditions. Moreover, this effect was completely reversed by treatment with the reducing agent DTT, indicating the involvement of protein-mixed disulphides. Together our findings support a potential functional role for S-glutathionylation in protecting these proteins from irreversible oxidation that could occur during oxidative/nitrosative stress into the cell, and may represent an important antioxidant mechanism in the ageing process.

NO has been observed to act as an effective signal molecule that regulates mitochondrial events, including oxygen consumption and reactive oxygen species production. Binding of NO with the terminal electron acceptor of the mitochondrial electron transport chain, CcOX, plays crucial roles in mediating the physiological effects of NO. Interaction between NO and CcOX is bidirectional resulting not only in the modulation of the mitochondrial enzyme activity by NO, but also in the regulation of NO concentrations by CcOX.

Recently, we reported that mtNOS is physically associated with CcOX, and that this binding is mediated by the PDZ motif of mtNOS. In order to further analyze the role played by NO in CcOX activity modulation, in the present work we explored whether and how the interaction between the mtNOS PDZ motif and the subunit Va of CcOX can be regulated. Through molecular modeling simulations we individuated a potentially critical specific tyrosine residue (Tyr77) in the PDZ of nNOS (whose alpha isoform is identical to mtNOS). Considering that the importance of phosphorylation/dephosphorylation in regulating NOS activity and, more recently, mitochondrial processes has been recognized and several protein kinases and phosphatases have been identified in mitochondria, we investigated whether nNOS PDZ Tyr77 phosphorylation could be implicated in the mtNOS/CcOX interaction modulation. To this purpose we utilized different experimental approaches such as nNOS alpha cloning and mutagenesis, expression of wild type and mutant nNOS in mammalian cells, and, finally, analysis of nNOS/CcOX interaction in wild type and transfected cells by means of confocal microscopy and co-immunoprecipitation assays. Together our results suggest phosphorylation as a likely mechanism of mtNOS/CcOX interaction regulation. Moreover it seems that this modulation is mediated by the action of some src tyrosine kinases.

1. INTRODUCTION

Nitric oxide (NO) has been firmly established as an ubiquitous signalling molecule in the regulation of key functions in the nervous, immune and cardiovascular systems. It is generated by a family of enzymes termed NO synthases (NOS) through sequential oxidation that converts the amino acid L-arginine to L-citrulline, in the presence of O₂, NADPH and others appropriate cofactors (Moncada and Higgs, 1993). Three different forms of NOS were originally identified: two of these, the so-called 'neuronal' (nNOS) and 'endothelial' (eNOS) isoforms are expressed constitutively (cNOS) and generate NO for cell-signalling purposes. The third one is an inducible isoform (iNOS) which releases NO in larger quantities during inflammatory or immunological defence reactions and is involved in host tissue damage.

Both the regulation and functions of the cNOS isoforms are largely determined by their subcellular localization, which in turn is cell type specific. nNOS is present not only in neurons but also in skeletal muscle, neutrophils, pancreatic islet cells, endometrium, respiratory gastrointestinal epithelia, astrocytes (Nathan and Xie, 1994) and cardiomyocytes (Miethke *et al.*, 2003). While eNOS has been localized in endothelial cells, cardiomyocytes (Shultz *et al.*, 1992), platelets (Muruganandam and Mutus, 1994) and also in a small population of neurons in the central nervous system (CNS) (Dinerman *et al.*, 1994). Both these isoforms are regulated by the levels of various necessary cofactors and substrates within the cell, as well as through modifications of the enzymes by processes such as phosphorylation. Unlike cNOS, the inducible isoform can be transcriptionally triggered in response to a wide range of stimuli, most prominently endotoxin and endogenous proinflammatory mediators, in many cell types including astrocytes, dendritic cells, endothelial cells, fibroblast, macrophages, cardiomyocytes, neurons and vascular smooth muscle cells (Nathan and Xie, 1994).

1.1 Function and regulation of Nitric Oxide

Nitric oxide, first identified as endothelium-derived relaxing factor in blood vessels (Furchgott and Zawadzki, 1980; Palmer *et al.*, 1987), has been recognized, during the following years, to play a pivotal role in intercellular communication, as well as in intracellular signalling, in many tissues (Moncada *et al.*, 1989; Murad, 1994a; Kerwin *et al.*, 1995).

Two principal mechanisms have been firmly established as primary cellular targets able to explain the profound effects mediated by NO.

Activation of soluble guanylate cyclase (sGC) by NO was the first identified cellular target for transduction of NO-mediated signals (Ignarro, 1991; Murad, 1994b). The reversible coordination bonding of NO with the heme center of sGC changes its conformation and activates it to produce cyclic GMP (cGMP), another small molecule that gives way to the activation of specific kinases. This transduction pathway is involved in the response of many different cell types to NO signal and can affect the function of a wide array of proteins, as well as modulate the function of other cellular messengers, such as cyclic AMP and calcium (Hanafi *et al.*, 2001; Contestabile *et al.*, 2003; Guix *et al.*, 2005). Cellular activities regulated by cGMP are multifarious (Fiscus, 2002; Pilz and Casteel, 2003), as is the number of putative protein targets for reaction with NO. Thus, the modalities of cellular transduction of NO signal may well account for the broad spectrum of effects regulated by NO in many cells.

Other roles of NO rely on a similar kind of reversible coordination bonding that can change or modulate protein activities. Protein nitration is the result of reaction of peroxynitrite anion, formed by reaction of NO with superoxide radical, with residues such as tryptophan and particularly tyrosine (Kelm *et al.*, 1997; Hanafi *et al.*, 2001). Nitric oxide also reacts with thiol groups of several amino acidic residues, especially with cysteine, forming *S*-nitrosylated derivatives (Stamler, 1994; Hanafi *et al.*, 2001). Both nitration and *S*-nitrosylation affect the function of the target proteins, ensuring that a multiplicity of cell-specific effects stem from the same initial signalling molecule (Hanafi *et al.*, 2001; Jaffrey *et al.*, 2001; Contestabile *et al.*, 2003; Guix *et al.*, 2005). The two main modalities able to elicit cellular responses to NO, i.e., through cGMP messenger or through conformational modification of protein function by direct chemical reaction, are not mutually exclusive even if they may preferentially occur at different concentrations of NO (Hanafi *et al.*, 2001). NO is considered to be a ubiquitous endogenous system which is involved in opposite actions: the maintenance of homeostasis on one hand and the mediation of pathological processes on the other one. NO is a free-radical gas with an unshared electron that is freely membrane diffusible. Depending on the environment in which NO is generated, it can mediate regulatory physiological functions such as vasodilatation and neurotransmission (Moncada and Higgins, 1993) or react with superoxide radicals to generate destructive reactive nitrogen species (RNSs) such as dinitrogen trioxide (N₂O₃) and peroxynitrite (ONOO⁻) as in neurodegenerative conditions (Bolaños *et al.*, 1997).

cNOSs are responsible for a low basal level of NO synthesis (in the range of pM-nM) that can be slightly and rapidly increased for a short period of time in response to certain signals. Low basal level of NO continuously produced by cNOS in absence of stimulation, participates in the regulation of daily activities of cells and exerts a large variety of activities essential for cells normal functioning in vascular endothelium (Kubes *et al.*, 1991; Magazine *et al.*, 1996; Schini-Kerth, 1999), in the heart (Ritchie *et al.*, 1998; Simko and Simko, 2000; Massion and Balligand, 2003), in the central nervous system (Colasanti *et al.*, 1995; Peunova and Enikopolov, 1995; Hudetz *et al.*, 1998). Recent evidence indicates that NO can also affect mitochondrial function. In mitochondria of several cell types, low concentration of NO reversibly binds Cytochrome c oxidase (CcOX), the terminal electron acceptor of respiratory chain, in competition with oxygen (Cleeter *et al.*, 1994) thus modulating O₂ uptake and availability, ATP synthesis and preventing overproduction of reactive oxygen species (ROS). CcOX inhibition by NO also results in the maintenance of the mitochondrial membrane potential and prevention of apoptosis at the early stages of the process (Beltran *et al.*, 2000). There are findings indicating that NO through its interaction with components of the electron-transport chain might function not only as a physiological regulator of cell respiration, but also to augment the generation of reactive oxygen species by mitochondria, and thereby trigger mechanisms of cell survival or death (Moncada and Erusalimsky, 2002).

The wide range of activities exerted by both basal and transiently increased levels of constitutive NO clearly show the essential role played by this molecule in the physiology of cells and systems. Loss of constitutive NO availability is always associated with cell dysfunction and diseases.

Differently from cNOS, iNOS produces large amounts of NO (in the range of μ M), after an induction/latency period of about 3-4 hours from stimulation and over a long period of time, around 24-48 hours (Stefano *et al.*, 1998; Radomski *et al.*, 1990). Inducible NOS expression is generally induced as a physiological answer to pathological conditions (MacMicking *et al.*, 1997; Bodgan *et al.*, 2000; Serbina *et al.*, 2003). Indeed, high concentration of NO as produced by iNOS, is a cellular tool used to solve dangerous situations. Should iNOS expression become deregulated and prolonged, this tool would result cytotoxic itself. Chronic iNOS expression has been demonstrated in several diseases such as cardiovascular, neurodegenerative diseases and cancer. Beside the pathological involvement, iNOS-derived NO has been demonstrated to participate also to

physiological events linked to cell differentiation, function that is strictly connected to cell developmental stage and subcellular localization.

nNOS and/or eNOS can crosstalk with the machinery that regulates iNOS expression using NO as a modulator (Colasanti and Suzuki, 2000). NO was reported to inhibit iNOS activity through a mechanism that involves the heme iron prosthetic group of the enzyme (Griscavage *et al.*, 1993). More recently, NO has been shown to suppress the transcription of the gene encoding iNOS in a variety of cell types by inactivating the transcriptional nuclear factor κ B (NF κ B) (Mariotto *et al.*, 1995; Colasanti *et al.*, 1995; Park *et al.*, 1997; Katsuyama *et al.*, 1998). So far, two mechanisms have been identified by which NO can inhibit the actions of NF κ B. First, NO can suppress NF κ B activation by inducing and stabilizing the NF κ B inhibitor I κ B (Peng *et al.*, 1995), as well as interfering with I κ B phosphorylation (Katsuyama *et al.*, 1998). Second, NO has been reported to interfere directly with the binding of NF κ B to its response element present in the promoter region of the gene encoding iNOS both *in vitro* (Matthews *et al.*, 1996) and *in vivo*.

Taken together, these data clearly show the essential role of NO and the sophisticated modulation that underlies its biological functions. Concentration, time, place of NO production and isoforms crosstalk are responsible for the beneficial to deleterious nature of this molecule. All conditions under which one or more of these features are deregulated lead to loss of constitutive NO bioavailability and chronic iNOS expression with pathological consequences. This is particularly evident in oxidative and nitrosative stress normally occurring during the aging process and in disease related to aging.

2. Oxidative/nitrosative modifications of proteins

Nitric oxide is a free radical, which makes it very unstable compared to many other chemical species. This implies not only that it is rapidly decomposed but also that it is able to react with other species, producing a variety of radical and non radical compounds, collectively named “reactive nitrogen species” (RNS).

The process by which milder physiological levels of reactive oxygen species (ROS) and RNS induce modifications to proteins that are discrete,

site-specific and reversible is term “redox signalling”. Redox signals may abrogate or enhance activity of the target protein, and have been implicated in physiological signalling processes that include kinase signalling, channel function, apoptotic proteolysis, and regulation of transcription (Ueda *et al.*, 2002; Hool and Corry, 2007; Janssen-Heininger *et al.*, 2008). When RNS and ROS levels exceed the cellular antioxidant capacity, a deleterious condition known as oxidative/nitrosative stress occurs. It describes a status in which cellular antioxidant defences are insufficient to keep the levels of ROS/RNS below a toxic threshold. This may be either due to excessive production of ROS/RNS, impairment of cellular antioxidant defences or both. Unchecked, excessive ROS/RNS generation can lead to the disruption of redox signalling and/or to the molecular damage of cellular components including proteins, leading ultimately to cell death via apoptosis or necrosis (Jones, 2006).

One of the major sources of ROS in cells are mitochondria. In particular, recent findings show that the two major sites of superoxide production are at complex I (Herrero and Barja, 1997; Genova *et al.*, 2001; Liu *et al.*, 2002) and complex III (Nohl and Stolze, 1992) of the electron transport chain. Excessive ROS production is often deleterious to mitochondrial function.

ROS/RNS can cause specific, oxidative modifications on sensitive proteins that may lead to a change in the activity or function of the oxidized protein (Finkel and Holbrook, 2000). Most protein oxidation products are commonly considered as biomarkers of oxidative/nitrosative stress/damage (Dalle-Donne *et al.*, 2005; Dalle-Donne *et al.*, 2006). And recent scientific studies have advanced the notion of chronic inflammation as a major risk factor underlying aging and age-related diseases (Yu and Chung, 2006).

2.1 Oxidative/Nitrosative modifications of protein thiols

Under conditions of oxidative/nitrosative stress, the thiols of cysteine residues within proteins are among the most susceptible oxidant-sensitive targets and can undergo a diverse array of redox alterations in response to ROS and/or RNS increase or exposure (Fig. 1). All the modifications to protein thiols can potentially affect protein activity, with the degree depending on the importance of the cysteine residue in carrying out protein function. Actually, the oxidative modifications of the cysteine sulfhydryl group have recently attracted renewed interest, because Cys is present in the active site of many proteins and in protein motifs that function in protein regulation and trafficking, cellular signalling, and control of gene

expression (Biswas *et al.*, 2006; Jacob *et al.*, 2006). Some proteins may not contain cysteine residues important in protein function; however, modification of thiols may cause a conformational change that alters protein activity. Thus, because many redox alterations to protein thiols are readily reversible, thiol redox alteration, like phosphorylation, has been suggested to be an important mechanism of turning on and off proteins, i.e., protein redox regulation, particularly in response to oxidative and nitrosative stress. The exposure of cysteines on the protein surface is a functional necessity to prevent redox changes from spreading through the entire protein molecule. The surface-oriented Cys residues are normally kept reduced and may therefore serve as “redox sensors” of the cells.

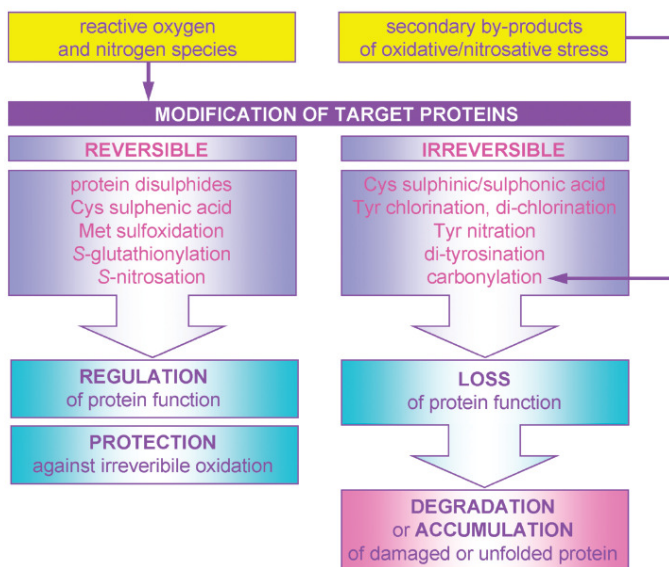


Fig. 1 Consequences of ROS/RNS and oxidative/nitrosative stress on protein function and fate. ROS/RNS may cause oxidative/nitrosative modifications on sensitive target proteins. Reversible modifications, usually at Cys and Met residues, may have a dual role of modulation of protein function and protection from irreversible modification. Irreversible modifications are usually associated with permanent loss of protein function and may lead to the degradation of the damaged proteins by proteasome and other proteases or to their progressive accumulation. (from Giustarini *et al.*, 2004)

Although the thiol moiety on the side chain of cysteine is particularly sensitive to redox reactions, not all cysteinyl thiols are important as redox sensors, as most protein thiols do not react with oxidants under the conditions and at the concentrations they are found in cells. Nevertheless, some cysteine residues are susceptible to oxidation. The two major determinants of the susceptibility of thiols to redox regulation are the accessibility of the thiol within the three-dimensional structure of the protein and the reactivity of the cysteine, which is influenced by the surrounding amino acids. The vast majority of cytoplasmic proteins contain cysteine sulfhydryls with a pKa value greater than 8.0 and, in the reducing environment of the cytoplasm, remain almost completely protonated at physiological pH. As a result, they are unlikely to be reactive with ROS/RNS. However, redox-sensitive proteins have specific Cys residues that exist as thiolate anions at neutral pH, due to a lowering of their pKa values as a result of charge interactions with neighbouring positively charged (i.e., basic) amino acid residues, becoming “active cysteines,” which are therefore more vulnerable to oxidation (Rhee *et al.*, 2000).

These “active cysteines” can be susceptible to various reversible and/or irreversible redox reactions (Fig. 1), which are largely dependent on the species and concentration of the oxidants they contact. Most thiol modifications are unstable and can easily be reversed or replaced by other, more stable modifications. In the presence of increasing ROS concentrations and an oxidative cellular environment, PSH can be oxidized into sulphenic (PSOH), sulphinic (PSO₂H) or sulphonic (PSO₃H) acids (Fig. 2). Whereas the formation of PSOH can be reversed, for example by reduced glutathione (GSH), the two latter species are usually irreversible. Oxidation of PSH can also promote the formation of inter- or intramolecular disulphides between protein thiols or between protein thiols and low-molecular-mass thiols (usually referred to as *S*-thiolation). In the presence of RNS, PSH can be modified by either *S*-nitrosylation (forming *S*-nitrosylated proteins, PSNOs) or *S*-glutathionylation (Schafer *et al.*, 2001; Giustarini *et al.*, 2006). These modifications can alter or regulate the function of numerous proteins containing cysteines of structural importance, within their catalytic centre or as part of protein–protein interaction interfaces.

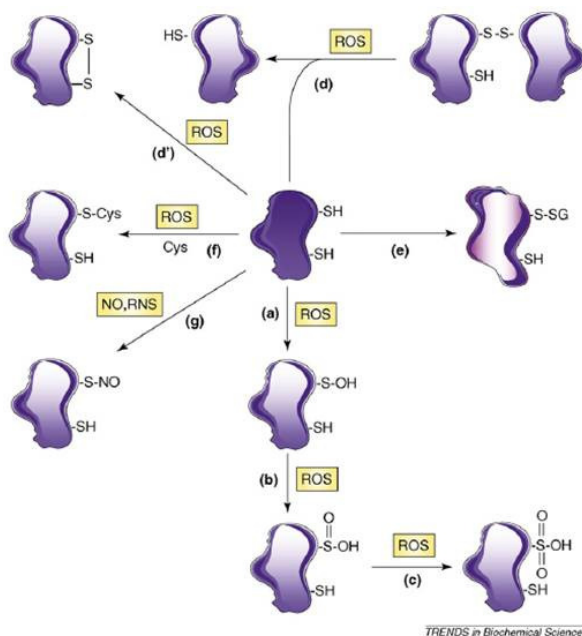


Figure 2. Oxidative modifications of protein thiols. The thiol group of cysteinyl side chains is susceptible to several oxidative modifications. The oxidation of a protein cysteine residue can result in the formation of a cysteinyl radical (P-S \cdot , not shown) or a sulphenic (PSOH) (a), sulphinic (PSO $_2$ H) (b) or sulphonic (PSO $_3$ H) (c) acid (the latter of which is always irreversible). Alternatively, oxidation can result in a disulphide bridge (cystine) (d, d0). Disulphides can form under oxidative conditions between two adjacent proteins (intermolecular cystine or interprotein disulphide) (d) or between two adjacent sulphhydryl groups within a protein (intramolecular cysteine or intraprotein disulphide) (d0), causing changes in protein aggregation and conformation. Reaction between PSHs and low-molecular-mass thiols such as GSH and free cysteine can yield protein-glutathione (e) or protein-cysteine (f) mixed disulphides, respectively (i.e. S-glutathionylated or S-cysteinylylated proteins). Protein thiols found in consensus motifs wherein the cysteine is adjacent to basic and acid residues or aromatic residues can serve as sites of S-nitrosylation, which can be mediated by NO, S-nitrosothiols or several higher N-oxides (g), or catalysed by transition metals. (from Dalle-Donne *et al.*, 2009)

In order to protect itself against hostile oxidative environments, the organism has developed various antioxidant defenses that include the classical antioxidant enzymes, SOD, GSH peroxidase, and catalase, as well as non-enzymatic ROS scavengers, vitamin E, vitamin C, β -carotene, and

uric acid (Lykkesfeldt *et al.*, 1998). Among others, GSH is the most abundant and effective biological anti-oxidative reductant (Cross *et al.*, 1977). Both GSH and GSH reductase levels have been found to decrease with age (Cho *et al.*, 2003).

The tripeptide GSH (γ -Glu-Cys-Gly) in mammalian is the predominant non-protein thiol and has essential roles as an antioxidant and intracellular redox buffer, in ROS scavenging and in detoxification of electrophilic xenobiotics and heavy metals. In mammalian cells, almost 90% of glutathione is in the cytosol (1–10 mM), 10% in the mitochondria (5–10 mM) and a small percentage in the endoplasmic reticulum and the nucleus, but all glutathione is synthesized within the cytoplasm. It is mainly (>98%) in the thiol-reduced form (GSH), and the disulphide (GSSG) content is usually <1% of total glutathione. GSSG forms upon oxidation and is reduced by NADPH-dependent glutathione reductase. And the ratio of GSH to GSSG is critical to cellular redox balance. Cellular GSH to GSSG redox state varies in association with proliferation, differentiation and apoptosis or necrosis (Hansen *et al.*, 2006). GSH participates as an antioxidant both by acting as a thiol buffer, directly reacting with protein thiols and ROS/RNS, and also by serving as a substrate for the glutathione peroxidases (Hurd *et al.*, 2005). Changes in the cellular redox status (mainly due to a decrease in the GSH/GSSG ratio and/or depletion of GSH by the metabolism of drugs or other xenobiotic substances) as well as an increase in ROS and/or RNS generation (e.g., during inflammation), i.e., oxidative or nitrosative stress, may induce reversible formation of mixed disulfides between protein sulfhydryl groups (PSH) and glutathione (a mechanism called *S*-glutathionylation) on multiple proteins, which makes of cellular glutathione a crucial modulating factor for an ever increasing number of proteins.

2.2 Glutathionylation

GSH can be reversibly bound to protein cysteinyl residues (PSHs) generating *S*-glutathionylated proteins (PSSG). Briefly, the addition of GS- to low-pKa Cys residues in proteins is a post-translational modification of key importance in redox regulation. *S*-Glutathionylation results in protein-specific functional changes (activation or deactivation), which are important in the regulation of signalling mediators involved in cellular processes. This protects sensitive protein thiols from irreversible oxidation during oxidative stress (Adachi *et al.*, 2004a; Adachi *et al.*, 2004b; Clavreul *et al.*, 2006a; Clavreul *et al.*, 2006b) and might also serve as a storage form of GSH to

prevent loss of GSH under oxidative conditions, because GSSG would otherwise be exported from the cell. Albeit *S*-glutathionylation is usually considered a modification occurring in response to oxidative or nitrosative stress, it also occurs under basal physiological conditions (Wang *et al.*, 2001; Silva *et al.*, 2008) indicating its possible involvement in the modulation of protein activity (redox regulation) and redox signalling. This has rapidly increased the interest in *S*-glutathionylated proteins in the past few years and the list of proteins demonstrated to be *S*-glutathionylated increases continually. The fact that PSSG are involved in numerous physiological processes such as growth, differentiation, cell cycle progression, transcriptional activity, cytoskeletal functions, and metabolism, suggests that *S*-glutathionylation is a general mechanism of redox regulation. In fact, whereas post-translational modifications such as phosphorylation, acetylation, and ubiquitinylation have been well established and understood for many years, the concept of protein *S*-glutathionylation as a post-translational regulative modification, as opposed to a biomarker of oxidative damage, has gained acceptance only more recently (Giustarini *et al.*, 2004; Fratelli *et al.*, 2005; Ghezzi, 2005).

Interestingly, protein *S*-glutathionylation has several characteristics required for a signalling mechanism. However, some basic criteria should be met: (i) reversibility, (ii) specificity (i.e. *S*-glutathionylation should occur on specific cysteine residues of particular proteins), (iii) physiological stimulus (i.e. *S*-glutathionylation should occur in intact cells in response to a physiological stimulus and elicit a physiological response) and (iv) target protein activity and related cell function should be modified (Shelton *et al.*, 2005). Partial fulfilment of these criteria has been convincingly demonstrated for several proteins in different cell types (Clavreul *et al.*, 2006a; Fiaschi *et al.*, 2006; Pan and Berk, 2007).

S-glutathionylation can be reversed, and therefore regulated, by deglutathionylation, by means of reactions catalysed by the thiol-disulphide oxidoreductases (Grx, also known as thioltransferases) (Shelton *et al.*, 2005). *S*-glutathionylation can also be reversed via direct thiol-disulphide exchange reactions with GSH after restoration of a reducing GSH:GSSG ratio, but this mechanism, in general, is not efficient relative to Grx-catalysed exchange. Grx also catalyses the GSSG-dependent PSSG formation and, in model studies with isolated proteins, can also enhance the rate of *S*-glutathionylation in the presence of glutathione thiyl radical (GS \cdot), despite high GSH content (Starke *et al.*, 2003).

A wide range of proteins have been identified as potentially regulated by reversible *S*-glutathionylation (either inhibited (Cross and Templeton, 2004;

Chen and Ogut, 2006; Pan and Berk, 2007) or activated (Adachi *et al.*, 2004a; Clavreul *et al.*, 2006b; Chen *et al.*, 2007). Various studies enabled the identification of different metabolic enzymes susceptible to redox regulation by *S*-glutathionylation, as the glycolytic enzyme GAPDH which was shown to be inactivated in an “in vitro” system using the purified human enzyme and GSSG (Cotgreave *et al.*, 2002). Other glycolytic enzymes can undergo *S*-glutathionylation in primary rat hepatocytes and in human HepG2 hepatoma cells exposed to artificial oxidant conditions (H₂O₂, diamide or menadione) (Fratelli *et al.*, 2003), thus indicating that *S*-glutathionylation could coordinate cellular metabolism in response to oxidative stress by modulating glycolysis. In the post-ischemic myocardium, it has been found that *S*-glutathionylation activates mitochondrial complex II in vivo (Chen *et al.*, 2007). Recent work has illustrated that mitochondrial complex I within intact, oxidatively stressed (with the thiol oxidant diamide) mammalian heart mitochondria is *S*-glutathionylated only on Cys531 and Cys704 of the 75 kDa subunit, which are on the surface of mammalian complex I and exposed to the mitochondrial GSH pool (Hurd *et al.*, 2008). Complex I *S*-glutathionylation also occurs in response to mild oxidative stress caused by increased superoxide production from the respiratory chain (Hurd *et al.*, 2008). Furthermore, many signalling molecules and transcription factors fundamental for cell growth, differentiation and apoptosis are regulated by *S*-glutathionylation. For instance, *S*-glutathionylation plays a key part in the regulation of the kinase activity of PTP1B (Rinna *et al.*, 2006) and MEKK1 in response to oxidative stresses (Cross *et al.*, 2004). *S*-glutathionylation is apparently also implicated in the regulation of the nuclear factor κB (NF-κB) signalling pathway at multiple levels (Reynaert *et al.*, 2006; Qanungo *et al.*, 2007).

Cytoskeletal arrangements and intracellular trafficking can also be regulated by *S*-glutathionylation. Indeed, growth-factor-mediated actin polymerization into filaments, translocation to the cell periphery and membrane ruffling are physiologically regulated by *S*-glutathionylation and de-glutathionylation, the effect of which on actin assembly has been demonstrated *in vitro* (Wang *et al.*, 2001; Dalle-Donne *et al.*, 2003).

2.3 Metalloproteins

Metalloproteins comprise about a third of all structurally characterized proteins, and a significant portion of metalloproteins contain a Zn²⁺ ion that is coordinated in a tetrahedral configuration by protein-specific

combinations of Cys thiols and/or His imidazole nitrogens. This coordination forms zinc-finger domains within transcription factors that are essential in protein–nucleic-acid interactions. In addition, Cys thiols participate in coordinating active-site Zn^{2+} within metalloproteins and in metal-thiolate prosthetic complexes in a range of other proteins.

Metallothioneins

Metallothioneins (MTs) are a family of small (~7 kDa) metal-binding proteins (Kagi and Schaffer, 1988; Vasak and Hasler, 2000) with the highest known metal content after ferritins. Four distinct mammalian MT isoforms, designated MT-1 through MT-4, have been detected and investigated so far (Vasak and Trace, 2005). MT-1 and MT-2 are found in all organs, whereas MT-3 is expressed mainly in the central nervous system, and MT-4 is most abundant in stratified tissues. All four isoforms are composed of a single polypeptide chain of 61–68 amino acids, 20 of which are highly conserved cysteine residues, while histidine and aromatic amino acids are completely absent in all of them. The high amount of cysteine residues in MTs allow these proteins to coordinate a wide range of mono (i.e. Cu^+ , Ag^+) or divalent metal ions (i.e. Zn^{2+} , Cd^{2+} , Hg^{2+}), although they are mainly found in association with zinc (Duncan *et al.*, 2006). X-ray crystallography and NMR spectroscopy show that the protein is arranged in two domains designated α (C-terminal domain) and β (N-terminal domain), whom cysteines are organized in “cluster” formations that envelope the metal atoms (Fig. 3) (Braun *et al.*, 1992; Romero-Isart and Vasak, 2002). Mammalian MTs usually bind seven divalent metal ions and up to twelve monovalent copper ions, partitioned into the two metal-thiolate clusters (Bogumil *et al.*, 1998; Faller *et al.*, 1999).

Although the physiological role of MTs remains a matter of intense study and debate (Palmiter, 1998), they have been proposed to play an important role in zinc homeostasis (Li *et al.*, 1980; Kagi and Schaffer, 1988) and heavy metal detoxification (Cherian *et al.*, 1994). In addition, it has been reported that MTs act as radical scavengers under oxidative stress (Thornalley and Vasak, 1985; Sato and Bremmer, 1993). Accumulating evidence indicates that cells with low levels of intracellular MT are more susceptible to DNA damage and apoptotic death after exposure to stress stimuli including oxidative stress, whereas prior induction of MT appears to confer protection.

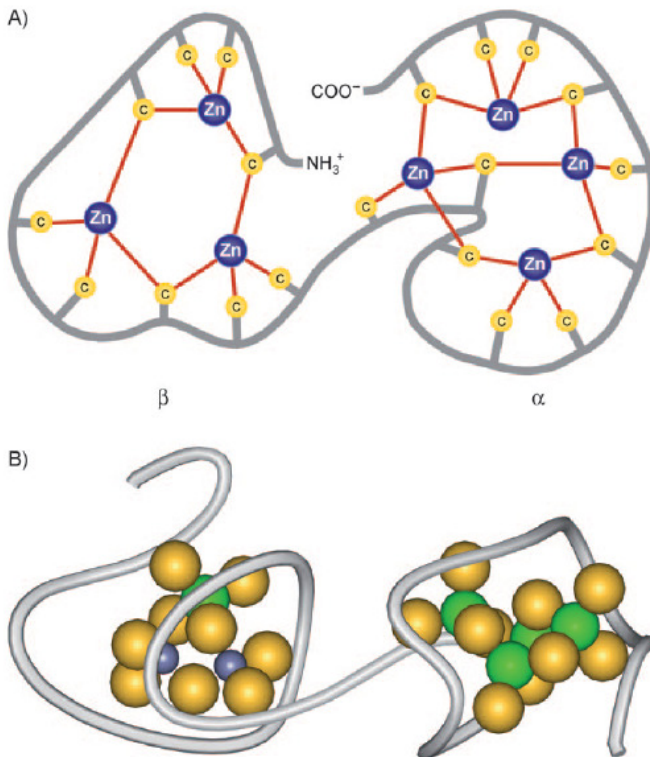


Figure 3. A) The α - and β -zinc-binding domains of MT: three metals are bound in an M_3Cys_9 N-terminal domain, while an $\text{M}_4\text{Cys}_{11}$ four metal cluster is formed in the C-terminal domain. B) The structure of rat $\text{Cd}_5\text{Zn}_2\text{MT-2}$ (PDB ID: 4MT2). Four cadmium atoms are bound in the α domain with one cadmium and two zinc atoms bound in the β -domain: Cd (green) with Zn (blue) and S (gold). (from Bell and Vallee, 2009).

MTs play pivotal roles in metal-related cell homeostasis due to their affinity for metals, in particular zinc and copper for cell growth and development, and cadmium and mercury to avoid the toxicity of the latter two metals (Vallee and Falchuk, 1993). MTs bind zinc with high binding affinity and also distribute cellular zinc, because zinc undergoes rapid inter- and intracluster exchange (Otvos *et al.*, 1993). In general, all isoforms of zinc-bound MTs are antioxidant agents because the zinc-sulphur cluster is

sensitive to changes of the cellular redox state. Oxidizing sites induce transfer of zinc from its binding sites in MTs to those of lower affinity in other proteins (Maret and Vallee, 1998), as for superoxide dismutase activation (Suzuki and Kuroda, 1995). Therefore, the redox properties of Zn-MTs are crucial for their protective role against cytotoxic effect of ROS, ionizing radiation, and heavy metals toxicity (Palmiter, 1998).

Furthermore, the biological role of MTs is crucial in antioxidative and immune responses during ageing and age-related diseases (Nath *et al.*, 2000). Among the different functions, MTs constantly retrieve Zn²⁺ from plasma and tissues. This, induce low Zn ion bioavailability and, consequently, impair immune responses (Mocchegiani *et al.*, 1998). Thus, MTs may not be the donors of Zn²⁺ in ageing but rather sequesters of Zn²⁺, resulting in down-regulation of many biological functions related to Zn, such as metabolism, gene expression and signal transduction. Furthermore, it is suggested that low Zn ion bioavailability is crucial for entire immune efficiency in ageing (Mocchegiani *et al.*, 1998). Moreover, because of the preferential binding of MTs with Zn rather than copper in ageing (Hamer, 1986; Mocchegiani, 2002), different roles of MTs (from protective to dangerous) may be further supported because of Zn deficiency in ageing, and Zn ion bioavailability is essential for immunity in ageing and age-related disease (Wellingtonhausen *et al.*, 1997).

Another possible key player in the role of MTs in signal transduction might be NO, which was shown, both *in vitro* (Kroncke *et al.*, 1994; Misra *et al.*, 1996; Aravindakumar *et al.*, 1999) and *in vivo* (Schwarz *et al.*, 1995; Pearce *et al.*, 2000), to interact with MTs and thereby releases bound zinc and cadmium.

Specificity protein 1

Specificity protein 1 (Sp-1) was the first mammalian transcription factor to be identified and cloned (Kadonaga *et al.*, 1987). It is member of a family of zinc finger transcription factors, which includes at least four Sp isoforms (Philipsen and Suske, 1999; Black *et al.*, 2001). These transcription factors play important roles in a wide variety of physiological processes including cell growth, differentiation, apoptosis, angiogenesis, and immune response, to name just a few.

Sp-1 is a 785-amino-acid, 100- to 110-kDa, protein which structure includes a transcription activation domain at the N-terminal and three C₂H₂-type zinc fingers located at the C-terminal domain through which interacts with DNA (Kadonaga *et al.*, 1988), and that are highly conserved in all Sp family. It is

a nuclear transcription factor that regulates gene expression via multiple mechanisms. It binds GC-rich motifs with high affinity (Briggs *et al.*, 1986) and can regulate the expression of TATA-containing and TATA-less genes *via* protein-protein interactions or interplay with other transcription factors (Ammanamanchi *et al.*, 1998). Sp-1 was once thought to serve mainly as a constitutive activator of housekeeping genes. However, growing evidence indicates that phosphorylation, acetylation, sumoylation, ubiquitylation, and glycosylation are among the posttranslational modifications that can influence the transcriptional activity and stability of Sp-1.

Also of note for this study is the finding that zinc finger transcription factors are involved in redox signalling pathways, possibly as redox sensors (Adler *et al.*, 1999; Knapp and Klann, 2000). Moreover, there is evidence demonstrating that Sp-1 transcriptional regulatory effects can be modified by high levels of oxidative stress (Shafer *et al.*, 2003). It has been observed that the DNA-binding activity of Sp-1 is affected directly by the redox status of the cell, possibly due to the critical functional role of its free-SH groups (Ammendola *et al.*, 1994; Wu *et al.*, 1996; Larabee *et al.*, 2005). The DNA-binding activity of Sp-1 shows a dramatic age-dependent decline. Indeed, it has been observed that Sp-1 binding activity decreases in nuclear extracts from 30-month-old rat tissues compared to those from young ones, although its gene appears to be normally expressed (Ammendola *et al.*, 1992).

2.4 Aim of the work

Nitric oxide is a signalling molecule involved in a wide variety of physiological processes. Increased NO levels can promote cellular components damage and it's well established that accumulation of oxidatively modified proteins is an important hallmark of biological ageing and pathological situations associated with oxidative/nitrosative stress. Thiols on sensitive proteins are commonly assumed to be a major target for NO, and the biological role for NO in the S-nitrosylation of many proteins is emerging as an important regulatory system.

Modifications induced by chronic oxidative/nitrosative stress conditions, if irreversible, are usually associated with permanent loss of function and may lead to the elimination or to the accumulation of the damaged proteins. Thus, reversible modifications, as S-glutathionylation, have been suggested to have a dual role: protection from cysteine irreversible oxidation and modulation of protein function.

Among the wide list of proteins demonstrated to be susceptible to oxidative cysteine modifications, as *S*-nitrosylation, we focused on some metalloproteins that play fundamental roles in cellular functions, and whose activity has been shown to be affected during ageing and age-related disorders.

In this context, aim of the first part of this research project was to study the effects of NO signalling at the level of protein modifications upon oxidative/nitrosative stress. In particular, we assume that a role can be devised for *S*-glutathionylation, an oxidative reversible protein modification. Thus, we wondered whether the *S*-nitrosylable Cys residues of MTs and Sp1 might be highly reactive to *S*-glutathionylation as well.

2.5 RESULTS AND DISCUSSION

Identification of MT cysteine residues hypothetically susceptible to *S*-glutathionylation

It is accepted that the structural environment of the NO-reactive cysteine is critical for other well-known modifications of cysteine residues, i.e., *S*-nitrosylation (Ascenzi *et al.*, 2000). Indeed, the solvent accessibility of the thiol group largely determines cysteine reactivity. In addition, the presence of basic residues in the vicinity of cysteines facilitates deprotonation of the thiol group, thus increasing its reactivity. It is tempting to speculate that the same “activated” cysteine residues may be highly reactive to *S*-glutathionylation as well. Thus, we first examined whether MT protein could contain cysteine residues hypothetically susceptible to the *S*-glutathionylation reaction.

Rat liver MT three-dimensional structure has been analyzed (PDB code 4MT2) (Braun *et al.*, 1992) to reveal structural characteristics that allow hypothesizing the likely *S*-glutathionylation sites. First, the accessible surface area of Cys residues Sc atom has been measured. As can be seen from Table 1 and Fig. 1, only for three Cys residues the measured values are significantly different from zero: Cys7, Cys13, and Cys59. Two out of these latter residues (i.e., Cys7 and Cys13) are located in the N-terminal domain of MT which has been previously shown to be the selective target of *S*-nitrosothiol formation (Zangger *et al.*, 2001). Then, MT structure has been inspected to analyze the structural microenvironment of Cys residues as far as the presence of basic and acidic residues is concerned. From Fig. 1, it can

Table 1
Solvent accessible surface area of rat MT Cys residues S_{γ} atom^a

Residue	Surface area
Cys5	0.000
Cys7	4.388
Cys13	15.503
Cys15	0.000
Cys19	0.000
Cys21	0.337
Cys24	0.000
Cys26	0.000
Cys29	0.000
Cys33	0.052
Cys34	0.000
Cys36	0.490
Cys37	0.000
Cys41	0.000
Cys44	0.000
Cys48	0.000
Cys50	0.000
Cys57	0.000
Cys59	6.342
Cys60	0.000

^a Surface area values are expressed in \AA^2 units.

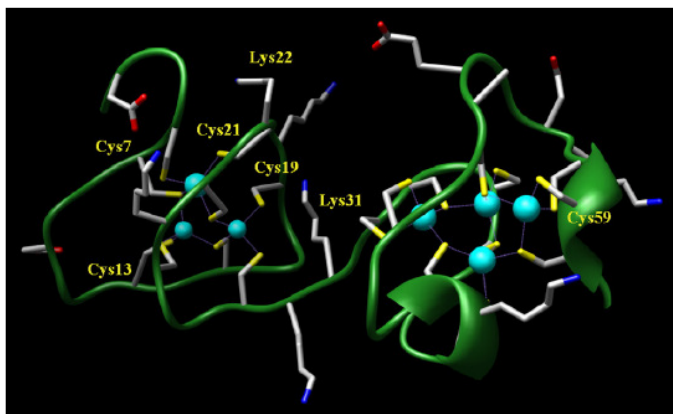


Figure 1. Schematic representation of the three-dimensional structure of rat MT. For clarity only the Cys residues and the charged residues are shown in stick representation. Metal ions are shown as spheres. The figure was made using Chimera (Pettersen *et al.*, 2004).

be seen that two Cys residues located in the N-terminal domain of MT, Cys19, and Cys21, are less than 5 Å away from two Lys residues, Lys31 and Lys22. No other cysteine residue has charged residues in its immediate structural neighbourhood.

Based on the above considerations, it seems likely that Cys residues of the N-terminal domain, given their solvent accessibility, are the likely targets of *S*-glutathionylation, taking also into account the higher bulkiness of glutathione as compared to NO. Further, the observation that two Cys residues of MT N-terminal domain have basic residues in their immediate neighbourhood indicates that these residues can be more reactive, especially in the case that partial apo forms of MT are present.

S-glutathionylation of MT induced by oxidant agents

In order to verify whether MT could be susceptible to *S*-glutathionylation under oxidative/nitrosative stress conditions, we exposed commercially available MT purified from rabbit liver to the oxidant agent *S*-nitrosoglutathione (GSNO). GSNO is the *S*-nitrosothiol of GSH and it could be the most relevant biological molecule to carry out nitrosation reactions under physiological conditions (Meyer *et al.*, 1994; Arnelle and Stamler, 1995; Padgett and Whorton, 1995). Moreover it has been shown to cause rapid, extensive, and reversible *S*-glutathionylation of a number of cell proteins (Kosower and Kosower, 1995; Fratelli *et al.*, 2002; Dalle-Donne *et al.*, 2005; Rossi *et al.*, 2006).

To this purpose purified MT was incubated *in vitro* with increasing concentrations of GSNO (0, 0.25, 0.5, 1, 2, and 5 mM) for 30 min at 37 °C (Fig. 2). Western blot analysis was performed under non reducing conditions, and probing the membrane with a monoclonal antibody raised against MT and a monoclonal anti-GSPro antibody, which specifically reacts with glutathione bound to proteins. Fig. 2A shows a dose-dependent increase of *S*-glutathionylated MT that correlates with the raising GSNO concentration. Densitometric analysis revealed that the signal gradually increased and reached a peak in correspondence of 2 mM GSNO treatment (Fig. 2C). In addition, glutathionylated MT was found to run mainly as a 14 kDa band (Fig. 2A), with a minor portion running at 28 kDa in correspondence of the highest GSNO concentration (data not shown). Probing the membrane with the anti-MT antibody, a 6–7-kDa immunoreactive band was mainly detected in untreated MT (Fig. 2B, lane 1) as well as in GSNO-treated MT (Fig. 2B, lanes 2–6), consistent with the reported molecular weight of the MT's monomeric form. In addition, it is

interesting to note that the treatment with GSNO induced the progressive appearance of lower mobility immunoreactive bands, with molecular masses (multiple of 6–7 kDa) apparently corresponding to different oligomeric forms of MT. As reported by the densitometric analysis in Fig. 2D, the MT dimer running at 14 kDa was the main product. These results are in line with previous data concerning the propensity of MT protein to form stable complexes and aggregates under oxidative stress conditions (Hou *et al.*, 2000; Oppermann *et al.*, 2001; Suzuki *et al.*, 1998; Vallee *et al.*, 1991; Whitacre, 1996; Wilhelmsen *et al.*, 2002), and suggest that *S*-glutathionylation mainly induces dimerization of MT.

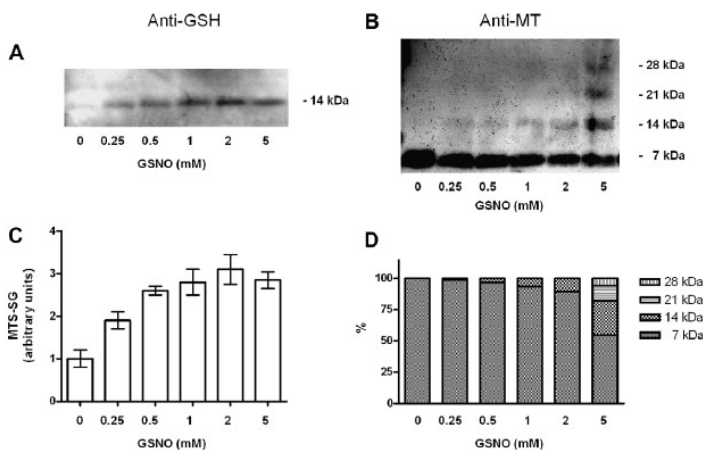


Figure 2. Effect of GSNO on MT protein thiols. Western blot analyses of *S*-glutathionylation of MT induced by GSNO treatment. Treatment of MT with GSNO induces a dose-dependent *S*-glutathionylation of MT (A) and the progressive appearance of immunoreactive bands with molecular masses (multiple of 6–7 kDa) apparently corresponding to different oligomeric forms of MT (B). For quantification, glutathionylated MT (C) and MT (D) bands have been analyzed with the Multi-Analyst PC version 1.1 program. After setting to 1 the ratio MTs-SG/MT from untreated MT (lane 1), values for the other samples have been calculated relative to it. Results are representative of three independent experiments. (MTs-SG: glutathionylated MT)

To confirm the results obtained by SDS-PAGE (Fig. 2) and actually to show at once the total amount of immunoreactive MT, dot blot analysis of MT glutathionylation has been performed. Again, MT was exposed to varying concentrations of GSNO; after 30 min of incubation, samples were treated with the alkylating agent NEM, to block residual free thiols. Samples were then analyzed by dot-immunobinding assay using monoclonal antibodies raised against GSPro or MT. As expected, results (shown in Fig. 3A) were similar to those observed through Western blot analysis: indeed treatment with GSNO induced progressively higher levels of MT *S*-glutathionylation, as validated by densitometric analysis (Fig. 3B). To verify whether the increased glutathionylation of MT detected in samples might be correlated with the formation of protein-mixed disulphides, a parallel set of samples were incubated with the reducing agent DTT after exposure to GSNO, to reduce any *S*-thiolated cysteine. In Fig. 3A is shown that treatment with 10 mM DTT completely reverses *S*-glutathionylation of MT, as demonstrated by the disappearance of the immunostained spots, suggesting that mixed disulfides are formed by the presence of GSNO.

Thus, both Western blot and dot blot analyses have unequivocally shown that MT can be *S*-glutathionylated *in vitro*. Furthermore, it appears that glutathionylation is accompanied by polymerization of MT, suggesting that modification of Cys residues could be one of the structural determinants at the basis of the well-known tendency of MT to aggregate.

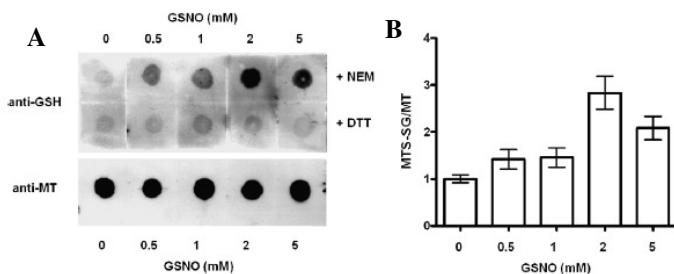


Figure 3. Dot blot analyses of MT *S*-glutathionylation MT induced by GSNO treatment. Treatment of MT with GSNO induces a dose-dependent *S*-glutathionylation of MT (A). Dot blot analysis confirms the GSNO-induced *S*-glutathionylation of MT in the presence of 5 mM NEM, an effect being reversed by treatment with DTT. (B) Estimates of the amounts of glutathionylated MT (MTs-SG) have been obtained by dividing the optical density of the area of the relative spot with the same area of the MT protein spot (A). Results are representative of three independent experiments.

Next, the effect of the highly specific thiol oxidant diamide (Kosower and Kosower, 1995) and of the physiologically occurring oxidant H_2O_2 were tested under identical conditions. Such agents act by different oxidizing mechanisms and, as GSNO, have been reported to promote *S*-glutathionylation on a number of cysteine-containing proteins (Kosower and Kosower, 1995; Fratelli et al., 2002; Dalle-Donne et al., 2005; Rossi et al., 2006). Diamide and H_2O_2 promote formation of glutathione-protein mixed disulfides in different ways: H_2O_2 forms GSSP, via GSSG, according to the reaction, $GSSG + PSH \rightarrow GSSP + GSH$; whereas diamide reacts first with protein -SH groups, giving PS-diamide adducts and then, after reaction with GSH, GSSP.

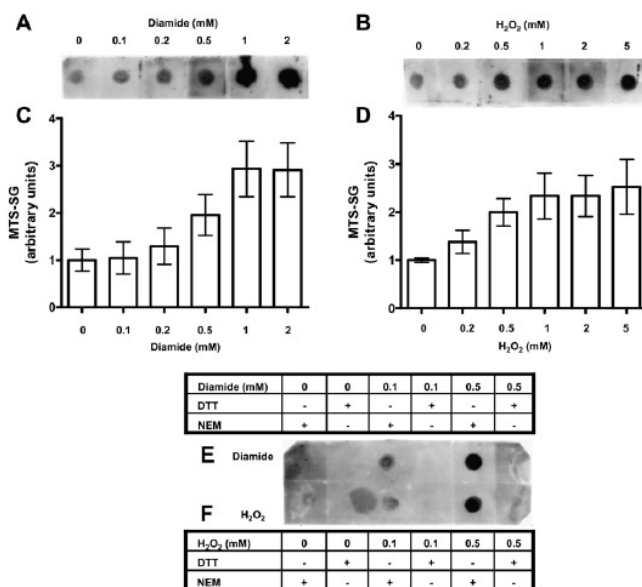


Figure 4. Effect of diamide and H_2O_2 on MT protein thiols. Dot blot analysis of *S*-glutathionylation of MT induced by treatment with diamide and H_2O_2 . Treatment of MT with diamide (A) and H_2O_2 (B), both in the presence of 1 mM GSH, is able to induce a dose-dependent increase of *S*-glutathionylation. DTT (10 mM) completely reverses *S*-glutathionylation of MT induced by diamide (E) and H_2O_2 (F). Estimates of the relative amounts of glutathionylated MT induced by diamide (C) and H_2O_2 (D) have been obtained by measuring the optical density of the area of MTS-SG spot. After setting to 1 the ratio MTS-SG/MT from untreated MT (lane 1), values for the other samples have been calculated relative to it. Results are representative of three independent experiments. (MTs-SG: glutathionylated MT)

To this end, MT from rabbit liver was exposed to different concentrations of diamide (0, 0.1, 0.2, 0.5, 1, and 2 mM) and of H₂O₂ (0, 0.2, 0.5, 1, 2, and 5 mM), both in the presence of 1 mM GSH for 30 min at 37°C. Samples were then processed for dot-immunobinding assay as described above. Consistent with data obtained with GSNO treatment, both diamide (Fig. 4A and B) and H₂O₂ (Fig. 4C and D) induced a dose-dependent increase of S-glutathionylated MT in presence of GSH. Again, samples treated with 10 mM DTT showed a complete reversion of diamide and H₂O₂-induced S-glutathionylation, as demonstrated by the loss of the immunostained spots (Fig. 4E and F, respectively), thereby providing strong evidence, also in this case, that S-glutathionylation of MT protein is related to the formation of protein-mixed disulphides induced by oxidants exposure. As a result, our analyses showed that MTs can be S-glutathionylated *in vitro* by different oxidizing agents acting through diverse mechanisms.

S-glutathionylation of MT in peripheral blood mononuclear cell extracts

Data presented so far demonstrate that S-glutathionylation of MT can be induced *in vitro* by a number of oxidizing agents. The question now is whether MT can be found glutathionylated *in vivo*. To address this point, peripheral blood mononuclear cell (PBMC) lysates were immunoprecipitated with a polyclonal antibody against MT, and immunoprecipitates were processed by Western blot analysis, under non reducing conditions. An MT-immunoreactive band was present in all extracts of PBMC (Fig. 5A). Noteworthy, MT was found to be highly aggregated under our experimental conditions, and it was also found to be reactive toward the anti-GSPro antibody (Fig. 5B). The glutathionylation level was completely reversed by the subsequent addition of 10 mM DTT for 30 min at 37°C (data not shown). These results indicate that MT proteins can be found heavily glutathionylated in cellular extracts as well as *in vitro* conditions. This might be an important finding in light of the fact that under physiological conditions MT S-glutathionylation may represent an antioxidant device that reduces the impact of oxidative stress (e.g., associated with ageing), but at the same time can modulate protein activity if critical protein thiols are involved.

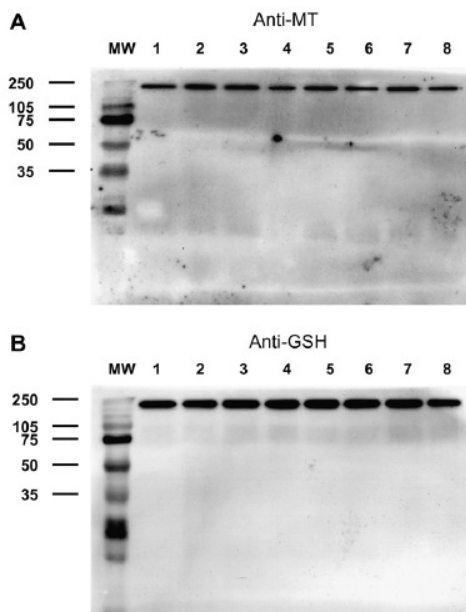


Figure 5. Western blot analysis of S-glutathionylation of MT in peripheral blood mononuclear cell extracts. Lysates of PBMC from voluntary healthy donors (lanes 1–8) were immunoprecipitated with a polyclonal antibody raised against MT, and immunoprecipitates were analyzed by non reducing Western blot using monoclonal antibodies against MT or GSH-Pro. Under our experimental conditions, MT is found to be highly aggregated (A) and heavily glutathionylated (B).

Levels of S-glutathionylated endogenous MTs in different age groups

In an attempt to correlate MT glutathionylation and the oxidative/nitrosative stress that can occur in the ageing process, we investigated whether endogenous MT S-glutathionylation levels might show an alteration upon ageing. To this purpose, we tested PBMC isolated from individuals of different age groups. We screened PBMC obtained by more than 180 individuals processing total cellular protein extracts for MT glutathionylation analysis, as described above. Briefly, total protein extracts were immunoprecipitated with an anti-MT antibody; immunoprecipitates were loaded in polyacrilammide gels and subjected to non-reducing SDS-PAGE, followed by probing membranes with an anti-GSPro antibody.

Obtained results were then processed and statistically analysed: the histogram in Fig. 6 shows that endogenous MT *S*-glutathionylation levels are high in younger subjects and dramatically fall in the 80-85 years old group, whereas individuals older than 85 years tend to behave like the younger ones. These results can be rationalized suggesting that *S*-glutathionylation could represent a mechanism that protects critical protein cysteines from the irreversible oxidation, thus preventing permanent loss of function as a consequence of oxidative insults usually occurring upon ageing. However, under chronic stress conditions probably *S*-glutathionylation is not able to protect protein against irreversible post-translational modifications. Furthermore, we analysed endogenous *S*-glutathionylated MT levels in individuals belonging to different age groups subjected to supplementation with zinc, whose antioxidant properties have been demonstrated (Powell, 2000). Unexpectedly, we found that zinc supplementation did not appreciably modify endogenous *S*-glutathionylated MT level (data not shown). These results can be explained if we assumed that, in order to be effective, the zinc protective mechanisms must occur in the young age, when oxidative/nitrosative stress conditions are usually transient.

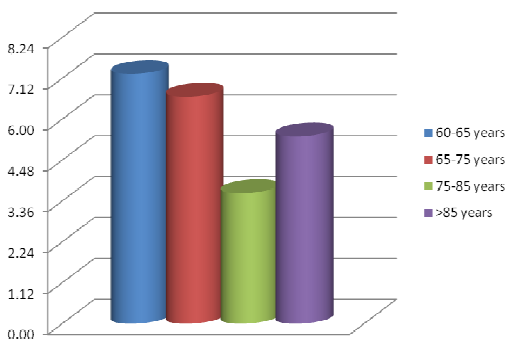


Figure 6. Levels of *S*-glutathionylated endogenous MTs in different age groups. PBMC isolated from individuals of different age groups were lysed and subjected to immunoprecipitation with polyclonal anti-MT antibody. Immunoprecipitated samples were analyzed by non reducing Western blot using monoclonal antibodies against MT or GSH-Pro. Results has been analysed by densitometry.

S-glutathionylation of endogenous Sp-1 in PBMC

The DNA binding region of the transcription factor Sp-1 is made up of three zinc-finger domains in which a zinc ion is coordinated by two Cys and two His residues (Oka *et al.*, 2004). Analysis of the structure of the three zinc-fingers domains (Fig. 7) reveals the conservation of a Lys residue (Lys546, Lys576, and Lys604, in domains 1, 2 and 3, respectively) in the vicinity of one of the zinc coordinating cysteine residues (Cys544, Cys574, and Cys602, in domains 1, 2 and 3, respectively). Interestingly, it has been demonstrated that Sp-1 is *S*-nitrosylated and that NO affects the DNA binding activity of Sp-1 (Jeong *et al.*, 2004). The latter effect may be mediated by *S*-nitrosylation of the above mentioned cysteine residues, facilitated by electrostatic interactions with the Lys residues, which would concomitantly lead to zinc release and impairment of the DNA binding ability of Sp-1. On the basis of these considerations, it is conceivable that the same “activated” cysteine residues are potential *S*-glutathionylation sites of the Sp-1 transcription factor.

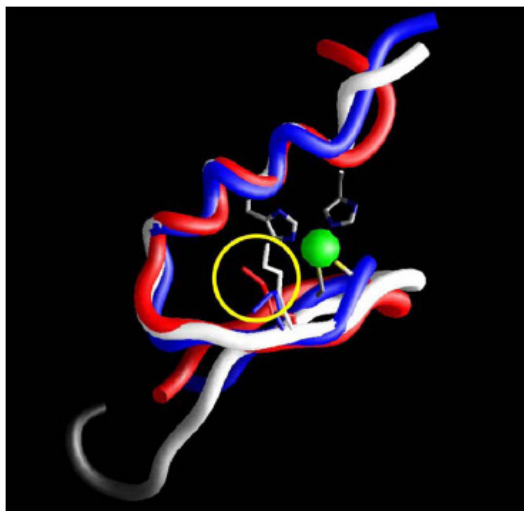


Figure 7. Structural superposition of the three DNA binding domains of the transcription factor Sp-1 (PDB codes 1VA1, 1VA2, 1VA3, Oka *et al.*, 2004). The backbone of domains 1, 2 and 3, and the corresponding Lys residues (Lys546, Lys576, and Lys604), enclosed in a yellow circle, are colored white, red and blue, respectively. The green sphere represents the zinc ion. For clarity, only the zinc binding site of domain 1 is shown. This and the following figure have been made with Grasp (Nicholls *et al.*, 1991).

In order to evaluate *S*-glutathionylation of Sp-1 under oxidative/nitrosative stress conditions, we exposed PBMC and PBMC protein extracts to the oxidizing agent diamide in the presence of 1 mM GSH, for 30 min at 37°C. Note that Sp-1 binding activity has been demonstrated to be sensitive to oxidation by diamide. As reported in Fig. 8, a GSPro-immunoreactive band corresponding to Sp1 protein was present in extracts of both cells and proteins diamide-treated samples, thus indicating that Sp-1 is heavily *S*-glutathionylated under our experimental conditions. Furthermore, when protein extracts were treated with 10 mM DTT following diamide exposure, the effect of diamide was reversed, thereby demonstrating the involvement of thiol groups (data not shown).

These findings, although preliminary, lead us to hypothesize, as for MT, a role for glutathionylation in protecting Sp-1 from irreversible oxidation or in modulating its DNA-binding activity, as it has been extensively demonstrated for other posttranslational modifications. Clearly, further studies are required, using specific inhibitors, to clarify the roles of Sp-1 glutathionylation in oxidative/nitrosative stress conditions.

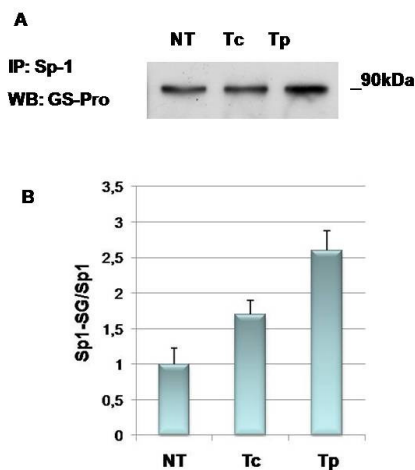


Figure 8. Effect of diamide on Sp-1 protein thiols. PBMC and PBMC protein extracts were exposed to 1 mM diamide (in the presence of 1 mM GSH only for protein extracts treatment). Samples were immunoprecipitated with anti-Sp-1 antibody and Western blot performed with anti-GSPro monoclonal antibody (A) and anti-Sp1 antibody (not shown). (B) Estimates of the amounts of glutathionylated Sp1 (Sp1-SG) have been obtained by dividing the optical density of the area of the relative band with the same area of the Sp1 protein band (not shown). (NT: non treated cells; Tc: treatment on cells; Tp: treatment on protein extracts)

3. NO signalling through NOS interaction with target proteins

As widely described in the first section of this work, NO exerts a broad spectrum of functions in several systems. The regulatory actions of NO are currently explained by the physiological intracellular concentrations of NO and by some well known NO-dependent cell signalling and regulatory pathways. Given that NO is a free radical gas with a half-life of a few seconds, physical interactions of each of the major NOS isoforms with heterologous proteins have emerged as a mechanism by which the activity, spatial distribution, and proximity of the NOS isoforms to regulatory proteins and intended targets are governed. Recent studies have emphasized the role of physical association of NOS isoforms with a variety of regulatory and structural proteins. These interactions may be categorized as constitutive interactions and inducible or signal-dependent interactions.

Interaction of neuronal NOS (nNOS or NOS-I) with mitochondrial cytochrome *c* oxidase (CcOX) plays a crucial role in mediating the inhibitory action of NO on CcOX activity, this effect being reversible and in competition with molecular oxygen (Brown and Cooper, 1994; Cooper and Giulivi, 2007). It is noteworthy that nNOS is unique among NOS isoforms, since its N-terminus contains a PDZ (post-synaptic density protein-95, discs-large, ZO-1) domain that allows interactions of this enzyme with different proteins in specific regions of the cell. Besides its involvement in the formation of active nNOS dimers, the nNOS PDZ domain is apparently responsible for the interaction of nNOS with the PDZ motif of alpha-1-syntrophin in skeletal muscle (Adams *et al.*, 1993; Adams *et al.*, 1995), and the second PDZ binding module of the postsynaptic density protein 95 (PSD)-95, a member of the membrane-associated guanylate kinase that are heavily localized at the post synaptic density of neuronal cells (Brenman *et al.*, 1996) and the related protein, PSD-93 (Brenman *et al.*, 1996).

Notably, the nNOS PDZ domain recognizes and binds short amino acid sequences at the C-terminal of protein targets. In particular, a comprehensive study in which 13 billions distinct peptides were screened for their ability to tightly bind to the PDZ domain of nNOS demonstrated preferential binding of peptides ending with Asp-X-Val-COOH (Stricker *et al.*, 1997), a consensus sequence observed in various proteins that binds nNOS (Jaffrey *et al.*, 1998; Tochio *et al.*, 1999; Riefler and Firestein, 2001).

3.1 Mitochondrial NOS interaction with CcOX

Mitochondria are major cellular sources of superoxide anion, whose reaction with NO yields peroxynitrite, which irreversibly modifies susceptible targets within mitochondria and induces oxidative/nitrosative stress. Consequently, mitochondria are important targets of NO and contribute to several of the biological functions of NO (Ghafourifar and Colton, 2003).

Many studies have reported the presence in mitochondria of a constitutively active mtNOS whose activity were reported first in mitochondria from rat liver (Ghafourifar and Richter, 1997). Although the occurrence and functions of mtNOS have been confirmed in many tissues, organs and cells, the isozyme of NOS that accounts for mtNOS is still a matter of debate. However the similarity between mtNOS and nNOS has been observed in several studies (Kanai et al., 2001; Riobo et al., 2002; Kanai et al., 2004). And in 2002, the mtNOS from rat liver mitochondria has been sequenced and identified as an inner membrane protein and as the transcript of nNOS, splice variant alpha (Elfering *et al.*, 2002).

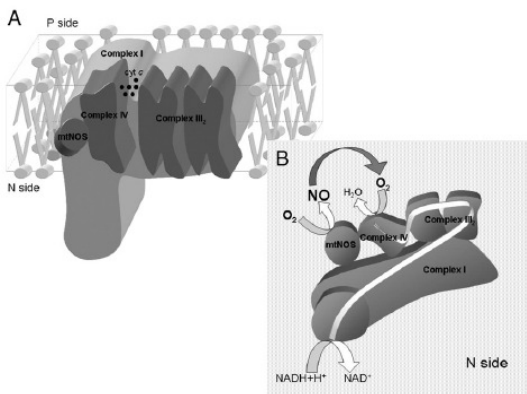


Figure 1. Solid state model of mtNOS and the supercomplexes of the mitochondrial respiratory chain. Nitric oxide, product of mtNOS activity, regulates mitochondrial respiration by the reversible and O₂-competitive inhibition of cytochrome c oxidase. The physiological activity of mtNOS is regulated by innermembrane potential and by the electron transfer through NADH-dehydrogenase. The supercomplex complex I-complex IV-mtNOS constitutes a hydrophobic domain that is sensitive to the mitochondrial oxidative damage (from Navarro and Boveris, 2008).

Crossed immunoprecipitation and kinetic evidence suggest that mtNOS is structurally attached to both complex I (NADH-ubiquinone reductase) and to complex IV (cytochrome c oxidase) (Persichini *et al.*, 2005; Franco *et al.*, 2006) (Fig. 1). The structural vicinity of mtNOS and cytochrome oxidase add to the consideration of a physiological regulatory role of NO in cell respiration.

The first evidence of a physical association between mtNOS and CcOX has been reported in our laboratory, as demonstrated by experimental and molecular modeling studies (Persichini *et al.*, 2005). In particular, careful analysis of linear sequences of all thirteen CcOX subunits, demonstrated that the last eight amino acids of subunit Va of CcOX are Glu-Glu-Leu-Gly-Leu-Asp-Lys-Val-COOH, the last three residues representing the consensus sequence for nNOS PDZ binding, previously unreported in the enzyme and similar to the one found in the melatonin receptor and CtBP.

In order to verify the structural compatibility between the C-terminal tail of subunit Va of CcOX and the PDZ domain of mtNOS, we built a molecular model of the complex formed by the last nine amino acids of CcOX subunit Va and the PDZ domain of nNOS (Fig. 2), by using the structure of the nNOS PDZ domain in complex with a synthetic heptapeptide derived from the C-terminal domain of the melatonin receptor (melR-PDZ) (Persichini *et al.*, 2005). In detail, all the relevant interactions characterizing the complexes of nNOS PDZ domain with peptides displaying the Asp-X-Val-COOH consensus sequence are conserved in the model complex. In particular, the terminal carboxyl group of the peptide is involved in a strong electrostatic interaction with the ammonium group of Lys16, while the Asp residue in position -2 preserves the interactions with Tyr77 and Arg79. The Leu-Gly-Leu sequence is easily accommodated in a hydrophobic pocket of the PDZ domain, mainly formed by Val32, Val41 and Leu72, stabilizing further the complex through the formation of a "mini hydrophobic core". In addition, an electrostatic interaction occurs between the Glu residue in position -7 of the peptide and Lys27 of the PDZ domain. Noteworthy, neither the latter interaction nor the "mini hydrophobic core" are observed in the melR-PDZ complex. Thus, the binding between nNOS and CcOX appears to involve stereochemical interactions tighter than those regulating the association of nNOS with the melatonin receptor, consistent with the biological relevance of the nNOS/CcOX interaction.

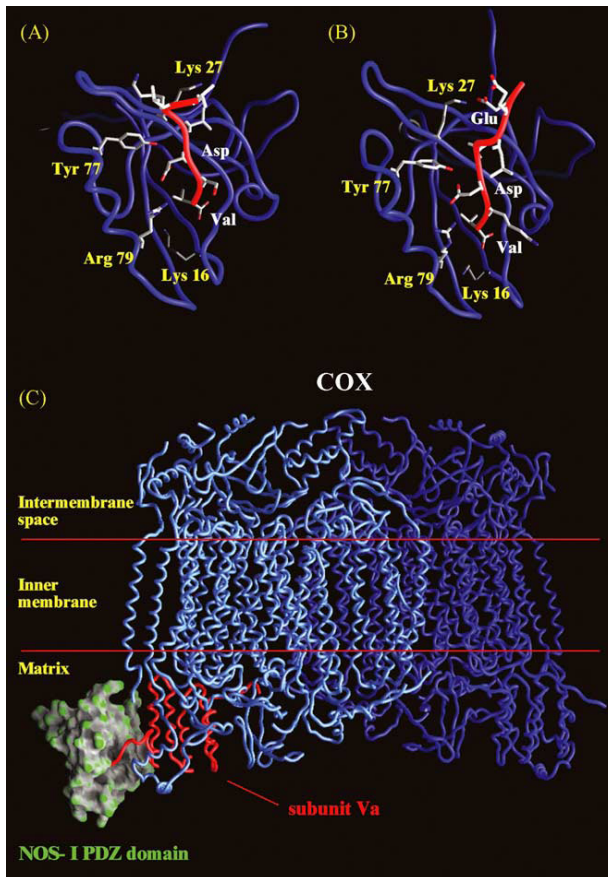


Fig. 2. Molecular modeling of the complex mtNOS-I-CcOX. Panel A: Schematic representation of the NOS-I PDZ domain in complex with a synthetic heptapeptide derived from the C-terminal domain of the melatonin receptor (melR-PDZ, PDB code 1B8Q) (Tochio *et al.*, 1999). Panel B: Three-dimensional model of the complex between the last nine amino acids of CcOX subunit Va and the PDZ domain of NOS-I (for details see text). Panel C: Model of the NOS-I-CcOX interaction complex; the subunit Va of CcOX is shown in red, the NOS-I PDZ domain is shown as molecular surface. The figure was made with Grasp (Nicholls *et al.*, 1991). (from Persichini *et al.*, 2005)

An issue that deserves attention is whether and how the interaction between the PDZ domain of mtNOS and the C-terminal tail of subunit Va of COX is regulated. In other PDZ domain-mediated interactions, control of the interaction is accomplished by phosphorylation of the Ser residue in position -2 in the C-terminal tail of the interacting protein. This is, for instance, the case of the Kir2.3 potassium channel-PSD-95 interaction (Cohen *et al.*, 1996). The C-terminal peptide consensus sequence of subunit Va of CcOX appears different from the above example in that the Ser residue in position -2 is substituted by an Asp residue. However, a scan of the PDZ domain of nNOS against the PROSITE database (Hulo *et al.*, 2004) revealed a tyrosine protein kinase substrate consensus sequence centred around Tyr77. Phosphorylation of Tyr77 would abolish the ability of this residue to interact with the Asp residue located in position -2 in the target peptide. Thus the regulation of the nNOS/CcOX interaction might rely on a mechanism similar to that described for the Kir2.3/PSD-95 interaction, dependent, however, on the modification or substitution of a different amino acid.

3.2 Aim of the work

As mentioned above, NO production by NOS is finely regulated to dictate specificity of its signalling and to limit toxicity to other cellular components. Physical interactions of NOS isoforms with heterologous proteins have emerged as a mechanism for governing the activity, spatial distribution, and proximity of the NOS isoforms to regulatory proteins and intended targets.

Moreover it is well established that the interaction of NO with CcOX plays crucial roles in mediating the physiological effects of this short-lived messenger.

This study aimed at verifying whether modifications of nNOS PDZ domain are able to regulate its interaction with the target protein CcOX. In particular, we wondered if Tyr77 in the nNOS PDZ domain may constitute a critical aminoacidic residue through which the interaction between mtNOS and the C-terminal tail of Va subunit of CcOX can be regulated.

3.3 RESULTS AND DISCUSSION

hnNOS alpha cloning and mutagenesis

In order to obtain direct evidence for nNOS PDZ Tyr77 implication in nNOS-CcOX interaction, we decided to overexpress, in mammalian cells, the nNOS and the nNOS(Y77E) mutant where a tyrosine (Tyr77) was mutated to glutamic acid. Indeed we hypothesized that this mutation might prevent the binding between nNOS PDZ domain and the C-terminal portion of CcOX Va subunit.

To this purpose, we first constructed two plasmids: one for nNOS wt and one for nNOS(Y77E) mutant expression in mammalian cells. Briefly, nNOS alpha cDNA has been purified from SH-SY5Y human neuroblastoma cells following total RNA extraction and subsequent retrotranscription. Full-length cDNA has been then amplified by using specific primers displaying *EcoRI/KpnI* restriction sites allowing the cloning in the expression vector pcDNA3.1. To improve the efficiency of ligation of the PCR product into the expression plasmid, we performed a pGEM-T Easy Vector step before the cDNA insertion in pcDNA3.1. Then, standard DNA cloning and manipulation techniques have been used to obtain wt and Y77E mutant of nNOS. Site-directed mutagenesis for the substitution of Tyr 77 with a residue of glutamic acid has been carried out using commercial kits and verified by DNA sequencing.

Over-expression of wild type and mutant nNOS in PC12 cells

The functionality of the nNOS mutant with respect to the interaction with CcOX has been evaluated *in vivo*, using PC12 cells as a neuronal model system. Due to the very high similarity between human and rat NOS, efficient protein-protein recognition in the system formed by human NOS and rat CcOX can be reasonably expected.

PC12 cells were transfected with the vectors containing the full-length nNOS cDNA, which have been projected to guide the wt and the Y77E mutant nNOS expression in mammalian cells under control of the constitutive CMV promoter.

To evaluate the efficiency of cell transfection and verify the levels of expression of the recombinant proteins, we performed PCR (data not shown), immunofluorescence and Western blot analysis with an antibody specific for the nNOS.

Immunofluorescence assay, carried out 24 hours after PC12 transfection, provided an indication of its efficiency. A representative image of confocal analysis reported in Fig. 1A show that lipofectamine transfection efficiency is not very high, however in non transfected cells nNOS signal appear slower than the one observed in transfected cells.

For Western blot experiment, 24 hours after transfection total proteins were harvested, lysed and then analyzed using a polyclonal antibody against nNOS. As shown in Fig. 1B, an nNOS-immunoreactive band of 160 kDa / a 160 kDa band corresponding to nNOS was present both in wt and mutant cells, whereas in non transfected PC12 cells endogenous nNOS is expressed at low levels if compared to the transfected ones. Indicating that this model system is suitable for our experiments.

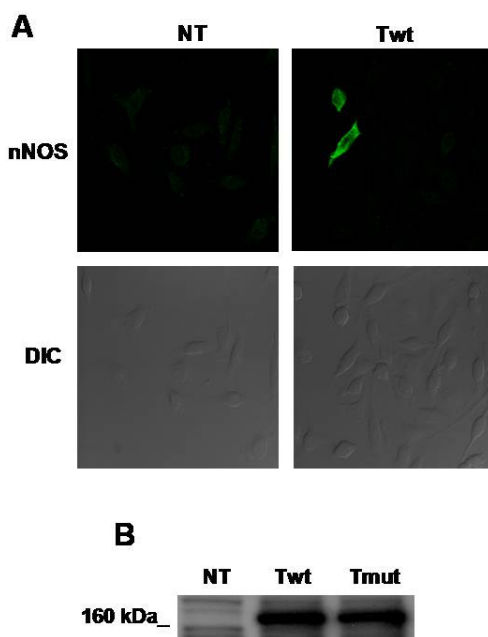


Figure 1. Expression of recombinant nNOS in PC12 cells. (A) PC12 non transfected (left panel) and wt nNOS transfected (right panel) cells were fixed in 4% paraformaldehyde-PBS at room temperature, permeabilized with 0.1% triton and then stained with α -nNOS (green). Labeled cells were then observed with a confocal laser-scanning microscope. (B) Western blot analysis of total cellular lysates of non transfected and transfected cells, performed using α -nNOS. (NT: non transfected cells; Twt: nNOS wt transfected cells; Tmut: nNOS(Y77E) mutant cells).

Further confirmation of the nNOS over expression has been obtained by testing non transfected and nNOS transfected cells for NO production. NO levels have been assessed by the DAF-2/DA detection system. Non transfected and transiently transfected PC12 cells were double-stained through an incubation with the fluorescent mitochondrial label Mito Tracker Red CM-H2Xros and the DAF-2/DA probe (green) as described in Materials and Methods section. Then, cells were processed for confocal microscopy analysis. As shown in Fig. 2, a basal level of DAF-2/DA fluorescence (in green) was observed in non transfected cells, probably due to low levels of NO produced by the constitutive forms of NOS or by other sources. By contrast, in transfected cells we detected a considerable higher NO-fluorescence signal. Additional evidence is provided by the observation of the superimposed images. As shown in Fig. 2 (merge panels), in nNOS transfected cells, the formation of the DAF-2-derived fluorescent product seems to be localized also in mitochondria (labeled with Mito Tracker Red), providing a first indication relative to nNOS mitochondrial localization.

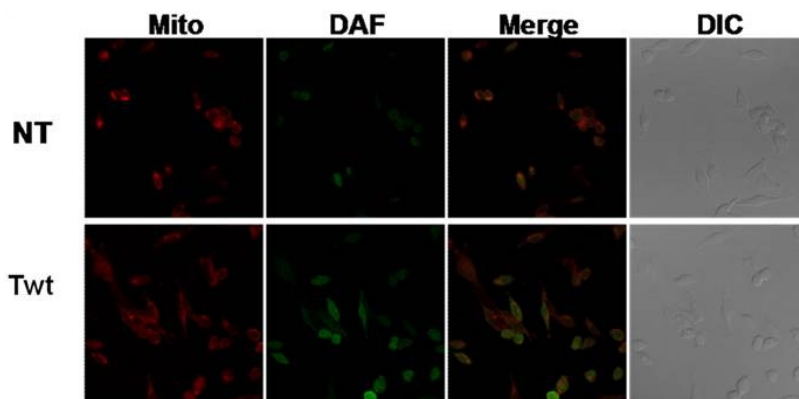


Figure 2. NO production in PC12 transfected cells. PC12 non transfected (upper panel) and wt nNOS transfected (lower panel) cells were grown overnight in 35-mm dishes, and then double-labeled with Mito Tracker Red CM-H2Xros and the DAF-2/DA probe (green). The cells were washed with PBS and then fixed in 4% paraformaldehyde-PBS at room temperature. Fixed cells were observed with a confocal laser-scanning microscope.

Mitochondrial Localization of nNOS and co-localization with CcOX

In an attempt to determine whether Y77E mutation inserted in nNOS cDNA could influence the physical interaction between this protein and the Va subunit of CcOX, we examined transiently transfected PC12 cells through double immunofluorescence staining and confocal analysis of the two proteins.

At first, we verified the specificity of the anti-CcOX Va antibody performing a double staining of PC12 cells, with Mitotracker Red and the antibody against CcOX Va subunit revealed by fluorescein-conjugated secondary antibody (Fig. 3). As expected, confocal images report a good overimposition of the two fluorescence signals, in according with the mitochondrial localization of CcOX.

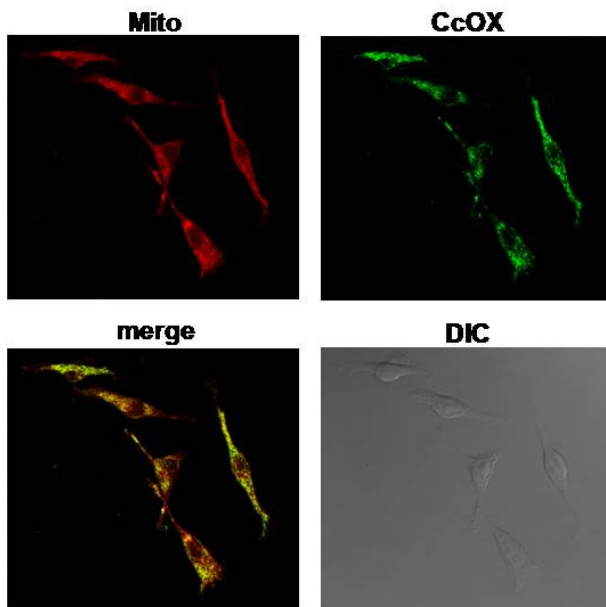


Figure 3. CcOX co-localizes with Mitochondria in PC12 cells. Confocal laser scanning microscopy of double immunofluorescence staining performed on PC12 cells. Fixed cells were labelled with Mitotracker Red (red) and a-CcOX (green).

Having established that the anti-CcOX antibody herein utilized exhibited an high specificity for the mitochondrial protein, experiments were performed to establish whether nNOS co-localized with CcOX. Moreover possible differences in localization between the wild-type and the mutant nNOS were investigated under identical conditions. To this end, transiently transfected PC12 cells were analyzed through double immunofluorescence staining and confocal analysis of the two proteins.

In particular, non transfected cells (data not shown) as well as nNOS wt and nNOS(Y77E) mutant transfected cells were fixed, permeabilized and incubated with a polyclonal anti-nNOS antibody and a monoclonal antibody raised against the Va subunit of CcOX. A representative image is reported in Figure 4. In both cell types, the CcOX fluorescence (shown in red) appeared with the rod shaped feature that is typical of mitochondrial morphology. In contrast, nNOS (visualized in green) presents a more wide distribution, showing, as expected, the maximum of fluorescence in the cytoplasm, but also an intense mitochondrial staining. Unexpectedly, the merged images shows that the nNOS co-localizes with CcOX (orange-yellow fluorescence) both in nNOS wt and in nNOS(Y77E) mutant cells.

To better investigate this issue, we further analyzed the interaction between nNOS and CcOX through co-immunoprecipitation analysis. Non transfected and transiently transfected cells were lysed and total protein extracts were immunoprecipitated with anti-CcOX Va antibody. Subsequent Western blot analysis performed with an antibody against nNOS revealed a strong signal in both wt and mutant transfectants, thus showing nNOS and CcOX interaction despite the inserted mutation (Fig. 5A). To further validate this interaction, we performed the reverse immunoprecipitation experiment. As shown in Figure 5B, immunoprecipitating the same extracts for nNOS and probing the filter with the antibody against CcOX Va, we clearly found its association with CcOX both in wt and mutant cells.

Altogether these unexpected results seemed to contrast with our hypothesis, suggesting that Tyr77 substitution with a glutamic acid residue actually was not sufficient to inhibit nNOS-CcOX interaction in mitochondria.

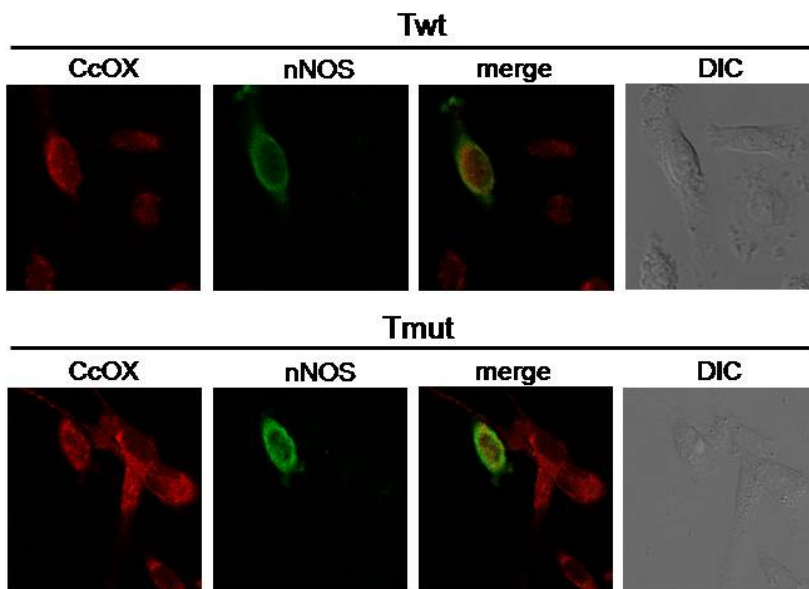


Figure 4. nNOS co-localizes with CcOX in PC12 transfected cells. PC12 cells transfected with the nNOS wt or the nNOS(Y77E) mutant plasmid were grown overnight in 35-mm dishes, and then fixed in 4% paraformaldehyde-PBS at room temperature. Fixed cells, after being permeabilized with 0.1% triton were stained with α -nNOS (green) and α -CcOX Va (red) and observed with a confocal laser-scanning microscope. (Twt: nNOS wt transfected cells; Tmut: nNOS(Y77E) mutant cells)

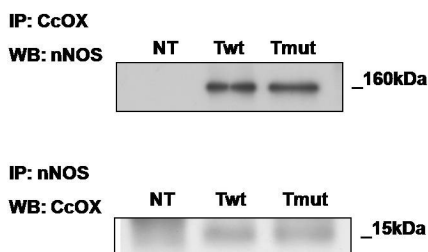


Figure 5. Western blot analysis of nNOS and CcOX immunoprecipitation. Immunoprecipitation and Western blot analysis on PC12 non transfected and transfected cells. (A) nNOS western blot on CcOX Va immunoprecipitation. (B) CcOX Va western blot on nNOS immunoprecipitation. (NT: non transfected cells; Twt: nNOS wt transfected cells; Tmut: nNOS(Y77E) mutant cells)

Molecular modeling

To deeply investigate the involvement of Tyr77 in nNOS-CcOX interaction, we initially examined a three-dimensional model of the complex between the last nine amino acids of CcOX subunit Va and the PDZ domain of nNOS (Fig. 6). As shown in panel 6B, the aminoacidic substitution in PDZ-Tyr77Glu leads to a modest shift of the peptide representing the C-terminal tail of CcOX Va subunit. Shift that probably is not sufficient to inhibit the binding, as the peptide is still in contact with the PDZ binding pocket.

In an attempt to find a possible mechanism through which the interaction between nNOS and the complex IV of the mitochondrial respiratory chain might be regulated, we focused on a possible tyrosine phosphorylation considering the vast literature that report the phosphorylation-dependent modulation of nNOS signalling. Indeed, it has been recently reported that trafficking and activation of nNOS is regulated by tyrosine phosphorylation through a mechanism involving pituitary adenylate cyclase activating polypeptide (PACAP) and NMDA receptor (Ohnishi *et al.*, 2008). The importance of phosphorylation/dephosphorylation in regulating NOS activity and, more recently, mitochondrial processes has been recognized and several protein kinases and phosphatases have been identified in mitochondria. Noteworthy, phosphorylation in mitochondria not only involves serine/threonine but also tyrosine and histidine residues. In particular, the presence of members of Src family of protein tyrosine kinases (SFK) has been recently shown in mitochondria of rat brain. Interestingly, the tyrosine kinase activity is mainly located at the surface of the inner membrane of brain mitochondria (Salvi *et al.*, 2002; Salvi *et al.*, 2005). Moreover, it has been recently reported the existence of a signal transduction pathway from plasma membrane to mitochondria, resulting in increasing Src-dependent mitochondrial tyrosine phosphorylation (Tibaldi *et al.*, 2008). As an example the epidermal growth factor receptor, a typical plasma membrane receptor with intrinsic tyrosine kinase activity, is targeted to mitochondria and interacts with the CcOX subunit II (Boerner *et al.*, 2004), suggesting how mitochondrial functions could be regulated. As a final note, Src activation has been demonstrated as an early intermediate in the signalling pathway induced by NGF in neurons (Tucker *et al.*, 2008).

In the light of these evidences, we wondered if a phosphorylation event at level of the PDZ binding pocket could influence the interaction with the peptide. To investigate this possibility, we performed molecular modeling simulation of the complex between the last nine amino acids of CcOX subunit Va and the PDZ domain of nNOS in presence of a phosphate group bound to the PDZ-Tyr77 and deriving from an hypothetical phosphorylation

reaction (Fig. 6C). In this case the peptide is subjected to a considerable shift, losing every interaction with the PDZ pocket.

On the basis of these molecular modeling simulation, we hypothesized that modification of the PDZ domain of nNOS likely results in modifications of the ability of the enzyme to physically interact with target proteins, in particular nNOS-CcOX binding could be affected by phosphorylation modifications.

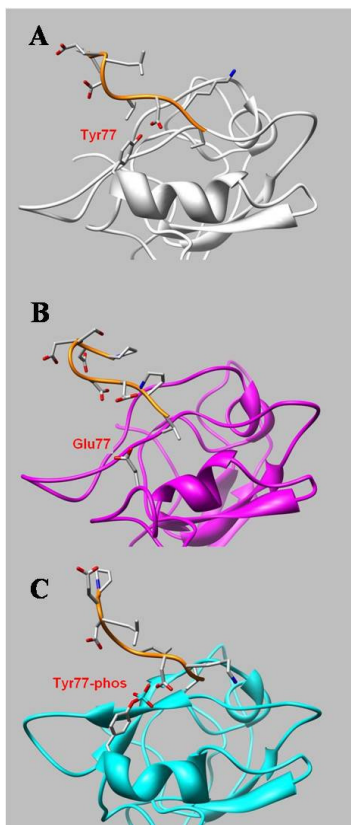


Figure 6. Molecular modeling of the complex nNOS-PDZ/CcOX. (A) Three-dimensional model of the complex between the last nine amino acids of CcOX subunit Va and the PDZ domain of nNOS. Tyr77 is shown. The subunit Va of CcOX is shown in orange, the nNOS PDZ domain is shown as molecular surface. (B) Model of the complex in presence of substitution of Tyr77 with Glu77. (C) Model complex in presence of phosphorylated Tyr77.

Analysis of nNOS/CcOX interaction in transfected cells after NGF treatment

Thus, we performed experiments for the evaluation of tyrosine phosphorylation effects on NOS/COX interaction in wt and mutant NOS-transfected cells. To this purpose, cells were treated with the growth factor NGF, and the expected increase of tyrosine phosphorylation has been assessed by double immunofluorescence staining and confocal analysis of the two proteins.

nNOS wt and nNOS(Y77E) mutant transiently transfected cells were incubated with 100 ng/ml NGF for 15 min at 37°C and subsequently processed for confocal immunofluorescence analysis. Briefly, after paraformaldehyde-fixation and triton-permeabilization, cells were incubated with the antibodies specific for nNOS and the Va subunit of CcOX, revealed by fluorescein- and rodamine-conjugated secondary antibody respectively. Confocal images illustrated in Figure 7 shown a reduction in nNOS (visualized in green) and CcOX (in red) co-localization after treatment with NGF of nNOS wt transfected cells. Whereas nNOS mutant transfected cells seemed not to be affected by the growth factor treatment.

Furthermore, to verify confocal analysis results, we performed co-immunoprecipitation experiments on total protein extracts from non transfected PC12 and wt and mutant nNOS-transfected cells, untreated or treated with 100 ng/ml NGF. Western blot assay (Fig. 8A) performed on the immunoprecipitates and densitometric analysis (Fig. 8B) clearly indicated a decrease in nNOS/CcOX interaction subsequent to NGF exposure in nNOS wt transfected cells (Fig. 8). Note that also nNOS(Y77E) mutant cells showed a slight interaction in absence of NGF, if compared with the non transfected cells. However, as expected, this interaction is not affected by NGF treatment, indicating that nNOS mutant cells can be considered as control samples in our experiments. These results suggest a likely modulation of nNOS/CcOX interaction by phosphorylation of the Tyr77 in PDZ binding domain of nNOS.

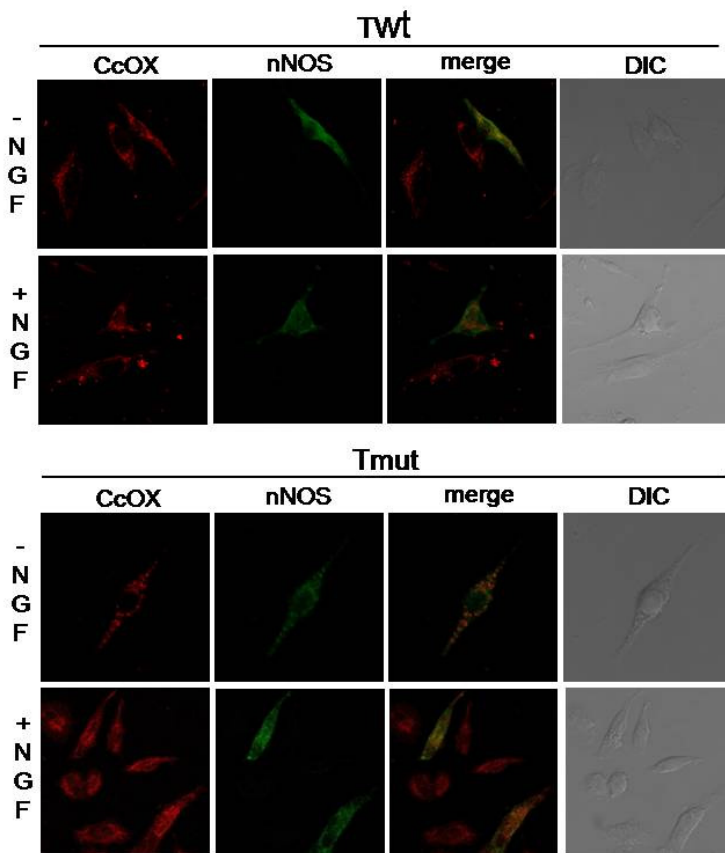


Figure 7. NGF treatment reduces nNOS/CcOX co-localization in PC12 nNOS wt transfected cells. PC12 cells transfected with the nNOS wt or the nNOS(Y77E) mutant plasmid have been treated (+NGF) or not (-NGF) with 100ng/ml NGF for 15 min. After being fixed and permeabilized, cells were stained with α -nNOS (green) and α -CcOX Va (red) and observed with a confocal laser-scanning microscope. (Twt: nNOS wt transfected cells; Tmut: nNOS(Y77E) mutant cells)

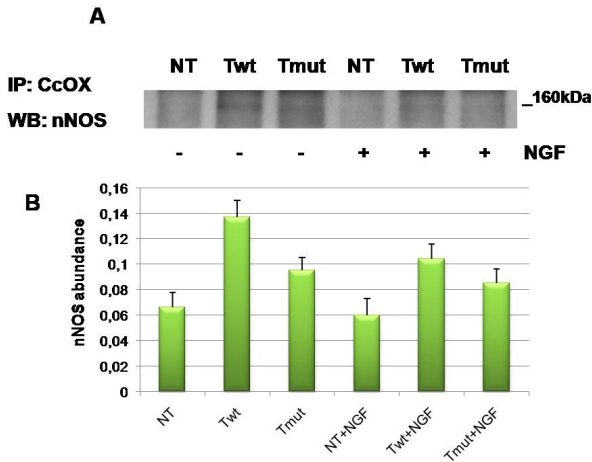


Figure 8. Treatment with NGF reduces nNOS/CcOX interaction in in PC12 nNOS wt transfected cells. Immunoprecipitation and Western blot analysis on PC12 non transfected and transfected cells performed on untreated (-NGF) or treated (+NGF) cells with 100ng/ml NGF. (A) nNOS western blot on CcOX Va immunoprecipitation. (B) Densitometric analysis. (NT: non transfected cells; Twt: nNOS wt transfected cells; Tmut: nNOS(Y77E) mutant cells)

To determine whether the differences observed in nNOS/CcOX interaction between NGF-treated and untreated cells were actually due to tyrosine phosphorylation, we carried out a series of experiments by using different inhibitors.

We initially tested the effects of the generic protein tyrosine kinases inhibitor genistein on transfected cells. To this purpose, transfected cells were pre-incubated with 50 μ M Genistein for 30 min and, then, treated with NGF as described above. Visualization of the NGF-treated cells with the confocal microscope (Fig. 9) revealed that genistein treatment restored co-localization of the nNOS and CcOX proteins at mitochondria level, thus suggesting an involvement of protein tyrosine phosphorylation in nNOS/CcOX interaction

The effect of the protein tyrosine phosphatase inhibitor PTP I was also tested. The results (data not shown) further validated those observed up to here, indeed in presence of the inhibitor co-localization between nNOS and CcOX is conserved.

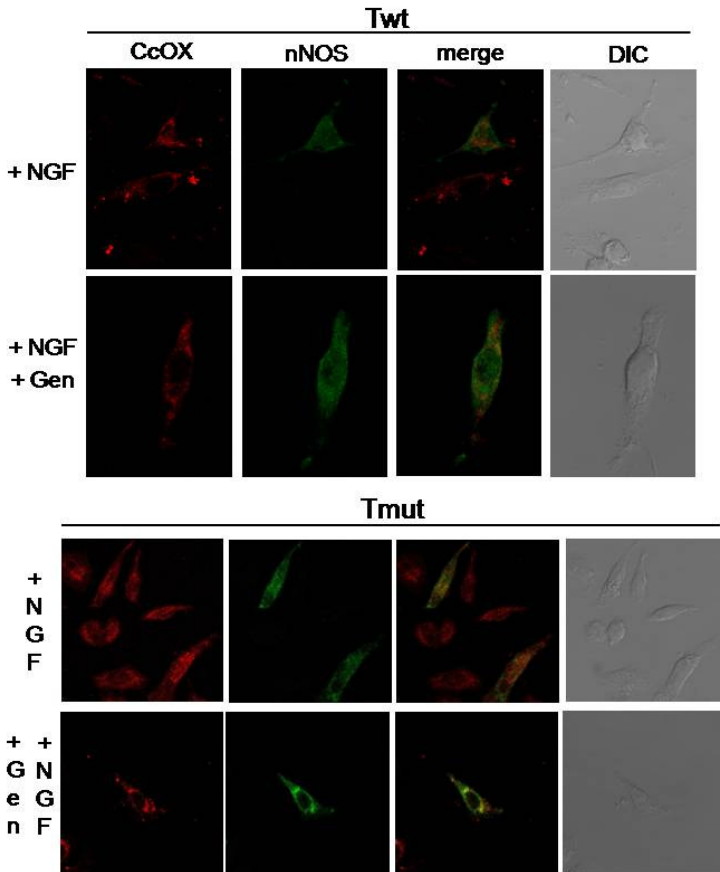


Figure 9. Genistein exposure of NGF-treated cells restore nNOS/CcOX colocalization in PC12 nNOS wt transfected cells. PC12 cells transfected with the nNOS wt or the nNOS(Y77E) mutant plasmid have been pre-incubated with 50 μ M Genistein and then treated with 100ng/ml NGF for 15 min. After fixation and permeabilization, cells were stained with α -nNOS (green) and α -CcOX Va (red) and observed with a confocal laser-scanning microscope. (Twt: nNOS wt transfected cells; Tmut: nNOS(Y77E) mutant cells)

In an attempt to determine which was the kinase likely implicated in modulation of nNOS/CcOX interaction, we examined whether selective inhibitors of the src family of tyrosine kinases could alter NGF treatment effects in nNOS transfected PC12.

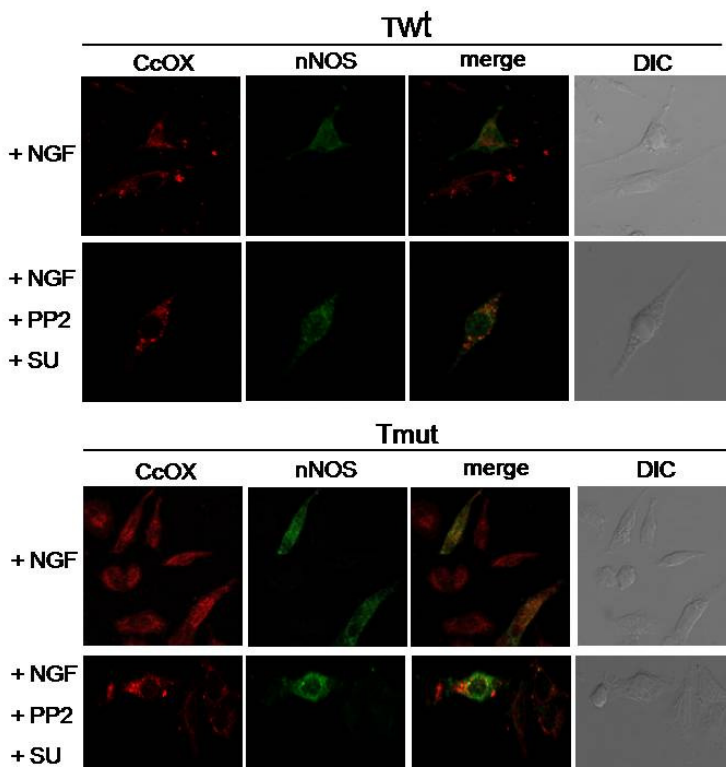


Figure 10. Src tyrosine kinase inhibitors exposure of NGF-treated cells restore nNOS/CcOX co-localization in PC12 nNOS wt transfected cells. PC12 cells transfected with the nNOS wt or the nNOS(Y77E) mutant plasmid have been pre-incubated with 1 μ M PP2 plus 1 μ M SU66 and then treated with 100ng/ml NGF for 15 min. After being fixed and permeabilized, cells were stained with α -nNOS (green) and α -CcOX Va (red) and observed with a confocal laser-scanning microscope. (Twt: nNOS wt transfected cells; Tmut: nNOS(Y77E) mutant cells)

Briefly, transfected cells were pre-incubated with 1 μ M PP2 plus 1 μ M SU66 for 30 min and, then, treated with NGF as described above. Fixed cells were subsequently permeabilized and incubated with a polyclonal anti-nNOS antibody and a monoclonal antibody raised against the Va subunit of CcOX. A representative image is reported in Figure 10. Treatment with the inhibitors consistently reduces the nNOS and CcOX co-localization, indicating that src are the kinases likely involved in this regulation pathway.

To further investigate about the kinase involved in this pathway we carried out co-immunoprecipitation experiments on cells treated with the selective inhibitors of the src family of tyrosine kinases PP2 and SU66. Briefly, transfected cells after pre-incubation with the two inhibitor were treated with NGF and then processed for co-immunoprecipitation analysis. The immunoprecipitates samples for nNOS were loaded in a polyacrilammide gel and filters were probed with an antibody raised against CcOX Va. Results shown in Fig. 11 clearly indicate that treatment with NGF abolishes the nNOS/CcOX interaction in nNOS wt transfected cells, whereas the binding appear reversed by PP2 and SU66, which specifically inhibit src tyrosine kinases. Providing a demonstration of the likely involvement of src tyrosine kinases in modulation of nNOS/CcOX interaction mediated by phosphorilatyon.



Figure 11. Treatment with specific src tyrosine kinase inhibitors restores nNOS/CcOX interaction in NGF-treated cells. Immunoprecipitation with α -nNOS and Western blot analysis with α -CcOX on PC12 non transfected and transfected cells. Analysis were performed on untreated or treated cells with 100ng/ml NGF. With or without a pre-incubation with PP2 and SU66 src inhibitors. (NT: non transfected cells; Twt: nNOS wt transfected cells; Tmut: nNOS(Y77E) mutant cells)

4. CONCLUSIONS

The regulatory actions of NO are currently explained both by the physiological intracellular concentrations of NO, in the range of 10 to 200 nM, and by at least three well known NO-dependent cell signalling and regulatory pathways. A first signalling pathway is the NO-activated cytosolic and soluble guanylate cyclase that yields cGMP as second messenger and results in signal amplification and activation of cGMP-dependent protein kinases (Ignarro, 2000; Jurado *et al.*, 2005; Schlossmann and Hofmann, 2005). The second regulatory pathway is the reversible and molecular oxygen competitive inhibition of mitochondrial cytochrome oxidase (Brown and Cooper, 1994; Cooper and Giulivi, 2007). The third pathway is the NO-dependent protein modifications, mainly *S*-nitrosylation of cysteine residues (Derakhshan *et al.*, 2007; Martinez-Ruiz and Lamas, 2007) and nitration of tyrosine residues (Radi, 2004). It has been observed that when the cellular NO concentration exceeds the physiological levels, as in the case of induction of astrocyte iNOS upon pro-inflammatory responses, NO-derived molecules of higher chemical reactivity (reactive nitrogen species, RNS) are generated and impair protein function (Davis *et al.*, 2001).

Our results give a further contribute to the comprehension of NO signalling pathways in regulating some cellular functions.

NO signalling involving protein post-translational modifications has been investigated in the first part of this work. The role of NO in this signalling route consists in its ability to produce covalent protein post-translational modifications in conjunction with other reactive oxygen and nitrogen species. Among these, the modification of cysteine residues has been shown to be of particular importance due to the functional relevance of many of them. In this study, we focused on the modification of the cysteine thiol by incorporation of a glutathione moiety (*S*-glutathionylation), a modification that, as *S*-nitrosylation, is produced by different reactions induced by NO-related species.

In particular, we found that both MT and Sp1, two proteins whose activity results impaired in oxidative/nitrosative stress conditions, can be reversibly glutathionylated under *in vitro* conditions.

In recent years increasing attention has been paid to *S*-glutathionylation as a modification associated with the stabilization of extracellular proteins, protection of proteins against irreversible oxidation of critical cysteine

residues, and regulation of enzyme functions and transcription. In view of this finding, *S*-glutathionylation of MT and Sp1 proteins, given its reversibility, would provide protection from irreversible oxidation of Cys residues, thus representing a mechanism of high potential biological relevance. In fact, while *S*-nitrosylation does not prevent further and irreversible oxidation of Cys residues, *S*-glutathionylation can be reversed provided that GSH/GSSG ratio is high or in conditions in which the oxidative stress within the cell has not reached a critical level. This process would regenerate the free thiol group of Cys residues thus restoring the metal binding ability and hence the biological function of MT and of Sp1. Altogether our results provided strong evidence that MTs and Sp1 can be glutathionylated under oxidative/nitrosative stress conditions. But there still remains the question of physiological relevance; whether the results obtained by treating proteins or cells with oxidizing agents represent changes that occur within the physiological range of oxidative stress. As discussed earlier, these reactions, although clearly possible under experimental conditions, may be effectively quenched by antioxidants in tissues under normal physiological states.

In the second part of this work, we focused on another NO-dependent regulatory pathway: the NO-dependent cytochrome c oxidase regulation.

NO has been known for many years to bind cytochrome c oxidase, the terminal enzyme in the mitochondrial electron transport chain. However, the potential biological relevance of this action only became apparent after the discovery that NO inhibits respiration in mammalian cells. The finding that low nanomolar concentrations of NO inhibit cytochrome c oxidase reversibly and competitively with molecular oxygen indicated that these interactions had a potential physiological role in the control of cell respiration, and also that the inhibitory effect might be involved in the inception of pathology.

Recently, we reported that mtNOS is physically associated with CcOX, and that this binding is mediated by the PDZ motif of mtNOS. In order to further analyze the role played by NO in CcOX activity regulation, we investigated whether phosphorylation of a specific tyrosine residue (Tyr77) in the nNOS PDZ domain could be implicated in the modulation of this interaction.

The analysis of the obtained results suggests a likely mechanism of modulation of the mtNOS-CcOX interaction mediated by phosphorylation, likely through the action of some src tyrosine kinases. However, additional studies will be required to obtain a detailed understanding of the specific

protein tyrosine kinases involved. This finding would give an important contribution to a deeper comprehension of NO regulation of CcOX activity and, more in general, would improve our understanding of the basics of NO signalling and its biological effects in mitochondria physiology.

A fine regulation of mtNOS-CcOX coupling might be extremely important especially in view of the pathological consequences of high concentrations of NO on mitochondrial metabolism. Extensive evidence indicates that high concentrations of NO lead to persistent inhibition of several mitochondrial enzymes including complexes I and II in the respiratory chain as well as enzymes in the glycolytic pathway and Krebs cycle, accompanied by increased generation of reactive oxygen species, ultimately leading to metabolic imbalance and cell death (Stadler *et al.*, 1991; Brunori *et al.*, 1999; Clementi *et al.*, 1999). Interaction between mtNOS-I and CcOX might be one of the cellular events designed to limit these adverse consequences of NO. Indeed, a regulated interaction between mtNOS-I and CcOX might contribute considerably to increase the functional coupling between the mitochondrial NO-generating and the sensing systems, leading, in turn, to fine-tuning of CcOX activity by NO in the absence of overall increases in the cellular NO levels. Furthermore, the tight regulation of NO production in close proximity to CcOX might also optimize the protective actions mediated by physiological concentrations of NO through this enzyme, such as the maintenance of the mitochondrial membrane potential, that delays the onset of apoptosis (Beltran, 2000) and the redistribution of oxygen, that limits the adverse consequences of hypoxia (Jaffrey *et al.*, 1998).

Apart from the implications in mtNOS-CcOX interaction modulation, we can postulate that phosphorylation of Tyr77 in the nNOS PDZ domain may represent a wide mechanism by which the interaction with NOS-binding partners can be regulated. Although protein-protein contacts involving nNOS PDZ domain have been described and in part deciphered in many tissues, their regulation in the control of the physio-pathological action of NO has not been fully explored, and, in many cases, yet to be addressed.

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APPENDIX

Papers published during Ph.D.:

Casadei M, Persichini T, Polticelli F, Musci G, Colasanti M. S-glutathionylation of metallothioneins by nitrosative/oxidative stress. *Exp Gerontol.* 2008; 43:415-22.

Marcocci L, **Casadei M**, Faso C, Antoccia A, Stano P, Leone S, Mondovì B, Federico R, Tavladoraki P. Inducible expression of maize polyamine oxidase in the nucleus of MCF-7 human breast cancer cells confers sensitivity to etoposide. *Amino Acids.* 2008;34:403-12.

Musci G, Persichini T, **Casadei M**, Mazzone V, Venturini G, Polticelli F, Colasanti M. Nitrosative/oxidative modifications and ageing. *Mech Ageing Dev.* 2006;127:544-51.