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EFFECT OF PROTEIN GLUTATHIONYLATION ON NEURONAL CYTOSKELETON: A POTENTIAL LINK TO NEURODEGENERATION

B. CARLETTI,[a] C. PASSARELLI,[a] M. SPARACO,[b] G. TOZZI,[a] A. PASTORE,[c] E. BERTINI[a] AND F. PIEMONTE[a]*

[a]*Unit of Molecular Medicine for Neuromuscular and Neurodegenerative Diseases, Children's Hospital and Research Institute "Bambino Gesù", Piazza S. Onofrio 4, 00165, Rome, Italy*

[b]*Division of Neurology, Department of Neurosciences, Azienda Ospedaliera, "G. Rummo," Via Pacevecchia 53, 82100 Benevento, Italy*

[c]*Biochemistry Laboratory, Children's Hospital and Research Institute "Bambino Gesù", Piazza S. Onofrio 4, 00165, Rome, Italy*

Abstract—Neurons are highly susceptible to oxidative stress and oxidation of cytoskeletal proteins is considered one of the first steps of neurodegeneration. Protein glutathionylation is a key event in the redox regulation of protein function and constitutes a sensor of tissue oxidative stress in pathophysiological conditions. In this study, we analyzed for the first time tubulin glutathionylation and its relation to neurites degeneration. For this purpose, we exposed motoneuronal cells to the physiological oxidant glutathione disulfide (GSSG) and we analyzed the extent and morphology of axonal changes caused by protein glutathionylation in these cells. Then we studied the effect of glutathionylation on the distribution of stable and dynamic microtubules in the same cells. Our results indicate that oxidative stress conditions determined by an increased intracellular level of oxidized glutathione may cause an alteration of the cytoskeleton organization and function leading to axon degeneration. These findings might contribute to understand the sequence of pathogenic events involved in the axonal degeneration that characterizes many diseases of the nervous system associated with oxidative stress. **© 2011 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: motor neurons, NSC34 cells, glutathionylated proteins, cytoskeleton, oxidative stress, dying-back neurodegeneration.

The role of oxidative stress in the pathogenesis of many neurodegenerative diseases such as Parkinson's disease (PD) (Shadrina et al., 2010), Huntington's disease (HD) (Browne and Beal, 2006), Amyotrophic lateral sclerosis (ALS) (Carrí et al., 2003; Cozzolino et al., 2008; Barber and Shaw, 2010), Friedreich's ataxia (FRDA) (Santos et al., 2010), Hereditary spastic paraplegia (HSP) (Casari et al., 1998; Albers and Beal, 2000; Barnham et al., 2004) and Alzheimer's disease (AD) (Querfurth and LaFerla, 2010) has been largely proved. However, in most of these diseases the molecular mechanism responsible for the dying-back type of axonal degeneration that frequently precedes neuronal death still remains poorly characterized.

The dying-back process is determined by a progressive axonal degeneration which begins distally and spreads proximally to the cell body according to the axonal retraction model (Gillingwater and Ribchester, 2003). Previous *in vitro* studies with microtubule depolymerizing agents, have suggested that the main mechanism involved in the process of axonal retraction-degeneration is represented by cytoskeleton disassembly (Yamada et al., 1970; Solomon and Magendantz, 1981; Baas and Ahmad, 2001). This hypothesis is also supported by a growing part of the literature which evidenced as in many neurodegenerative diseases axonopathy is frequently associated with cytoskeletal disruption (Vickers et al., 2009). In this study, we analyzed for the first time the role of the physiological oxidant glutathione disulfide (GSSG) in the genesis of the neuronal oxidative stress process leading to cytoskeleton disassembly and axonal degeneration.

Glutathione is the major intracellular non protein thiol compound and it plays a crucial role in the process of cellular detoxification of reactive oxygen species (ROS) (Maher, 2006). During conditions of oxidative stress, intracellular protein thiol groups can be modified by the reaction of protein glutathionylation, consisting in the formation of mixed disulfides between glutathione and protein cysteine residues. Protein glutathionylation normally occurs in the human CNS (Sparaco et al., 2006) and is considered a sensitive redox-marker of tissue oxidative stress (Giustarini et al., 2004). Cytoskeletal proteins are particularly susceptible to oxidation because their supra-molecular organization depends on the presence of exposed sulfhydryl residues (Dalle-Donne et al., 2007). Therefore, neuronal cytoskeleton may represent a primary target for post-translational modification by GSSG. Moving from the results of previous biochemical *in vitro* studies showing oxidation of purified tubulin by GSSG (Banerjee et al., 1985; Landino et al., 2004), we hypothesized that in oxidative stress conditions the increase of intracellular GSSG level may cause a cytoskeletal dysfunction and eventually an axonal degeneration via protein glutathionylation. In order to address this issue, we analyzed the effects of the oxidative damage mediated by GSSG on cultured motor neuronal cells. For this purpose, we incubated NSC34 cells, a neuroblastoma-spinal cord hybrid cell line displaying motor neuron-like phenotype, with a membrane-permeable form of GSSG. We first analyzed the extent and morphology of axonal changes caused by protein glutathionylation and then we

*Corresponding author. Tel: +0039-06-68592102.
E-mail address: fiorella.piemonte@opbg.net (F. Piemonte).
Abbreviations: AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; FRDA, Friedreich's ataxia; GSH, reduced glutathione; GSSG, glutathione disulfide; GSSGme, GSSG methyl ester; MT, microtubule; PD, Parkinson's disease; ROS, reactive oxygen species.

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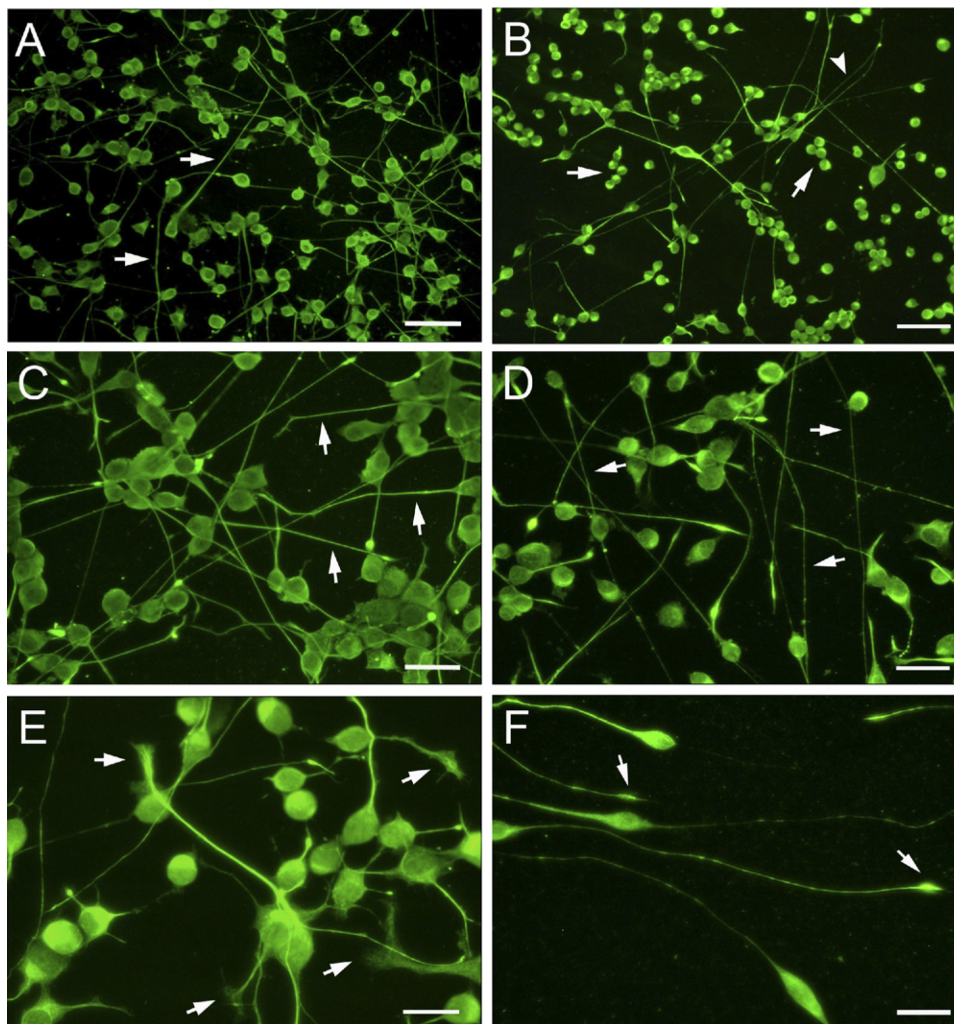

Fig. 1. Differentiated NSC34 cells immunostained for NF-H. (A) Control experimental condition (untreated). Numerous NF-H positive NSC34 cells presented long axon-like processes (arrows). (B) Cells treated with 100 μM GSSGme for 20 min; arrows indicate the presence of cells devoid of neurites whereas arrowheads point to the withdrawing remaining neuronal processes. (C, D) show the difference in neurites calibre between control (arrows in C) and treated cells (arrows in D). The high magnification picture (E) illustrates the abundant growth cones which characterized axon-like neurites in control condition. Arrows in (F) indicate the formation of retraction bulbs at neurites ending after GSSGme treatment. Bars: (A–B)=77 μm, (C–D)=37 μm and (E–F)=24 μm.

studied the effect of glutathionylation on the distribution of stable and dynamic microtubules in the same cells.

EXPERIMENTAL PROCEDURES

Cell culture

NSC34 cells (originally donated by Neil Cashman) were maintained in DMEM (Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Invitrogen), 1% penicillin-streptomycin (20 U/ml; Invitrogen) and 1% glutamine (2 mM; Invitrogen). For the experiments the cells were differentiated as described previously (Eggett et al., 2000) with some modifications. Briefly, cells were cultured at a density of 2000 cell/cm^2 on tissue culture glass cover slips coated with 100 μg/ml of poly-D-lysine (Sigma, St. Louis, MO, USA). The cells were maintained for 6–7 days *in vitro* (d.i.v.) at 37 °C in a humidified atmosphere with 5% CO_2, in DMEM/F12 (Invitrogen), supplemented with glutamine (2 mM; Invitrogen), penicillin-streptomycin (20 U/ml; Invitrogen), 1% fetal bovine serum (Invitrogen) and 1% non essential amminoacids (Sigma). Under this culture condition about 35% of the cells showed a clear neuronal morphology with long neurites radiating out of the perikaryon. NSC34 cells with a mature neuronal appearance were immunopositive for the neurofilament heavy chain subunit (NF-H) (Fig. 1A, arrows) and exhibited the morphological features already described for this cell population (Cashman et al., 1992; Durham et al., 1993). Somata were usually multipolar and all neurites showed an axon-like structure. It's known that the NSC34 neuronal processes do not express the dendritic marker MAP2 and they are all positive for the axonal marker SMI34 (data not shown). Thus, considering the axonal properties common to all NSC34 neuronal processes, we referred to them in the text indifferently both as neurites and axon-like processes. They exceeded generally 200 μm in length and sometimes presented lateral ramifications. Beside these differentiated cells, there was a second population of small NF-H positive cells with an undifferentiated morphology characterized by very short processes showing irregular expansion with a tuft shape. We focused our study on the first group of cells. Time lapse recordings were performed on tissue culture dishes at room temperature in normal air. Cells were

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