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DYSREGULATION OF IRON PROTEIN EXPRESSION IN THE G93A MODEL OF AMYOTROPHIC LATERAL SCLEROSIS

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Abstract—Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder characterized by selective loss of motor neurons which leads to progressive paralysis and death by respiratory failure. Although the cause of sporadic ALS is still unknown, oxidative stress is suggested to play a major role in the pathogenesis of this disease and of the rare familial form, which often exhibits mutations of the superoxide dismutase 1 (SOD1) gene. Since enhanced iron levels are discussed to participate in oxidative stress and neuronal death, we analyzed the expression levels of Fe-related mRNAs in a cell culture ALS model with the G93A mutation of SOD1.

We observed an increased total iron content in G93A-SOD1 SH-SY5Y neuroblastoma cells compared to wild-type (WT)-SOD1 cells. mRNA expression for transferrin receptor 1 (TfR1) and divalent metal transporter 1 was increased in G93A-SOD1 cells, which was in accordance with higher iron uptake. Experiments with the iron chelator deferoxamine revealed a normal reaction of WT and mutant cells to cytoplasmic iron depletion, i.e. TfR1 upregulation, suggesting a basically conserved function of the iron-responsive element/iron regulatory protein (IRE/IRP) pathway, designed to adapt gene expression to iron levels. Expression levels of mitoferrin 1 and 2, frataxin, and iron–sulfur cluster scaffold protein were also significantly increased in G93A-SOD1 cells, suggesting higher mitochondrial iron import and utilization in biosynthetic pathways within the mitochondria. Moreover, expression of these transcripts was further enhanced, if G93A-SOD1 cells were differentiated by retinoic acid (RA). Since RA treatment increased cytoplasmic reactive oxygen species (ROS) levels in these cells, an IRE/IRP independent, ROS-mediated mechanism may account for dysregulation of iron-related genes.

Key words: amyotrophic lateral sclerosis, iron proteins.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder characterized by selective loss of motor neurons in the spinal cord, brainstem and cerebral cortex. The rapid destruction of motor neurons soon begins to affect even the basic movements, requiring the use of a wheel chair. The most common cause of death among ALS patients is respiratory failure due to damage of the nerves and muscles, which control breathing (Hardiman, 2011). Normally, ALS is characterized by adult onset, and occurs with an incidence of about 1/100,000, and leads usually to death within 2–3 years (Carri et al., 2003). Two forms of ALS have been identified: familial (5–10%) and sporadic (90–95%), both of which show similar clinical and neuropathological features. Missense point mutations in the superoxide dismutase 1 (SOD1) gene, found in approximately 20% of the familial ALS cases (Rosen et al., 1993) helped generating different transgenic models for studying the disease (Julien and Kriz, 2006). More than 150 SOD1 mutations have meanwhile been associated with ALS (Milani et al., 2011). Among the most intensely studied ones is the G93A mutation. Copper–zinc SOD (SOD1), an abundantly expressed cytoplasmic enzyme, plays a primary role in the detoxification of superoxide radicals (O_2^-), the by-products of the mitochondrial electron transport chain (ETC). SOD1 catalyzes the dismutation of O_2^- to hydrogen peroxide (H_2O_2) and molecular oxygen (O_2). Although the precise mechanisms are still debated, the G93A mutation generates enhanced reactive oxygen species (ROS) production in cell cultures (Beretta et al., 2003), an increase of the oxidative stress marker malondialdehyde (MDA) in the spinal cords of mice

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Abbreviations: ALS, amyotrophic lateral sclerosis; β2MG, β2-microglobulin; Ct, cycle threshold; DCF, dichlorodihydrofluorescein; DFO, deferoxamine; DCFH-DA, dihydrodichlorofluorescein diacetate; DMT1, divalent metal transporter 1; DMEM, Dulbecco's-modified Eagle's medium; EDTA, ethylene diamine tetraacetic acid; ETC, electron transport chain; FAC, ferric ammonium citrate; FCS, fetal calf serum; Fxn, frataxin; H_2O_2, hydrogen peroxide; Hepes, hydroxyethyl piperazineethanesulfonic acid; IRE/IRP, iron-responsive element/iron-regulatory protein; IscU, iron–sulfur cluster scaffold protein; MDA, malondialdehyde; Mfrn1, mitoferrin 1; PBS, phosphate-buffered saline; ROS, reactive oxygen species; RA, retinoic acid; SDS, sodium dodecyl sulfate; SOD1, superoxide dismutase 1; TBST, Tris buffered saline with Tween-20; Tf, transferrin; TfR1, transferrin receptor 1; WT, wild type.

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(Wang et al., 2011) and an increased ROS production in isolated mitochondria from the rat brain and spinal cord (Panov et al., 2011), thereby modeling oxidative stress, which is discussed as a major mediator of sporadic ALS (reviewed in Barber and Shaw, 2009).

Iron overload is discussed to play an additional role in oxidative stress, due to the ability of iron to react with H_2O_2 via the Fenton reaction, thereby producing highly reactive hydroxyl radicals (OH•). Moderately enhanced iron levels along the corticospinal tracts (Langkammer et al., 2010), and transferrin receptor-containing deposits, called bunina bodies (Mizuno et al., 2006) have been described in sporadic ALS. These findings suggest a more generalized role of iron dysregulation in ALS and implicate the need to learn more details about altered iron homeostasis.

Iron homeostasis is regulated at both, systemic and cellular levels. Transferrin (Tf) allows iron internalization into cells via receptor-mediated endocytosis by binding to transferrin receptor 1 (TfR1), while ferritin is the main intracellular iron-binding protein in nonhematopoietic tissues. The expression levels of the latter two proteins are tightly regulated in response to the intracellular iron concentration via the *iron-responsive element/iron-regulatory protein* (IRE/IRP) system. IRPs 1 and 2 are cytosolic RNA-binding proteins that bind to IREs in the 3′- and 5′-untranslated regions of mRNAs, allowing antipodal regulation of iron uptake (TfR1) and storage (ferritin) (Richardson et al., 2009). Once internalized, the Tf–TfR1 complex is found in the endosome, where iron is released from Tf upon acidification, becomes reduced to the ferrous form (Fe^{2+}) and is thereafter transported to the cytoplasm, primarily by divalent metal transporter 1 (DMT1). The import of iron into mitochondria is an important prerequisite for its further usage, not only in mitochondrial but also in cytoplasmic or nucleic Fe–S proteins. This is the case, since the initial steps of iron–sulfur cluster scaffold protein (IscU) biogenesis take place in the mitochondria. Iron is imported into these organelles by mitoferrins, located in the inner mitochondrial membrane. The impact of mitoferrin 1 (Mfrn1) has been well established in erythrocyte precursors (Shaw et al., 2006), but Mfrn2 was shown to be an independent iron transporter (Paradkar et al., 2009).

Following uptake into mitochondria, iron is assembled into prosthetic groups, namely heme and IscUs. Biogenesis of the latter is assisted by protein complexes, which contain the iron-sulfur cluster scaffold protein (IscU). It has been suggested that IscU accepts the metal from the mitochondrial protein frataxin (Fxn), since both proteins interact physically and can be co-precipitated (Bridwell-Rabb et al., 2011; Schmucker et al., 2011). In addition, Fxn donates iron to ferrochelatase, the last enzyme of heme-biosynthesis (Bencze et al., 2007).

However, little is known about the above-mentioned iron-proteins and their regulation in ALS models. To address this issue, we studied transgenic neuroblastoma SH-SY5Y cells, which carried either the human wild-type (WT) or a mutated SOD1 gene (G93A).

EXPERIMENTAL PROCEDURES

Cell culture, treatment with chemicals and cell differentiation

Transgenic neuroblastoma SH-SY5Y cells, stably transfected with either the WT (further referred as WT-SOD1) or the mutated human SOD1 gene (G93A) (further referred as G93A-SOD1) (Carri et al., 1997) were received from Prof. M.T. Carri, University of Rome. The untransfected parental cell line was obtained from a commercial source (DSMZ, Braunschweig, Germany). The cells were continuously cultured in Dulbecco's-modified Eagle's medium (DMEM) with 20 mM glucose and 1 mM pyruvate (PAA Laboratories GmbH, Pasching, Austria), containing 10% fetal calf serum (FCS) and 1% penicillin–streptomycin (PAA).

For chemical treatments, cells were seeded in 12 well plates at a density of 200,000 cells/well. Six wells from each plate contained untreated cells, serving as controls. Deferoxamine (DFO) treatment was performed with 50–100 μM DFO mesylate (Sigma–Aldrich, Steinheim, Germany) for 24–48 h, as indicated in the results. Ferric ammonium citrate (FAC, Sigma–Aldrich) was applied at 2 and 20 μM for 24 h each. H_2O_2 was applied as a single bolus of 500 μM (prepared from a 30% (w/w) solution; Sigma–Aldrich) and RNA was isolated 24 h after treatment.

For retinoic acid (RA)-induced differentiation, cells were seeded in 12-well plates at a density of 100,000 cells/well (for subsequent real time PCR analysis) or in the same density in 175 cm^2 flasks (for subsequent ROS measurement) and further cultured for 24 h. Then DMEM was exchanged with neurobasal medium (Life Technologies GmbH, Darmstadt, Germany), containing B27 supplement without antioxidants (Invitrogen, Germany), 10 μM RA (Invitrogen), 1% penicillin–streptomycin (PAA) and 2 mM L-glutamine (PAA). Cells were cultured for 4 days, which was the period needed for them to stop dividing and become post-mitotic. On each 12-well plate, six wells were seeded with cells grown in normal DMEM as controls.

Fluorimetric DCF assay

Cells at a density of 20,000 ml^{-1} were resuspended in phosphate-buffered saline (PBS) with 20 mM glucose and then loaded with 2 μM dihydrodichlorofluorescein diacetate (DCFH-DA) for 15 min at 37°C. DCFH-DA becomes hydrolyzed to DCFH, which is a fluorescent probe for ROS detection (excitation at 548 nm and emission at 520 nm), allowing time-dependent monitoring of intracellular ROS generation at 25°C in a microplate reader fluorimeter (Tecan Austria GmbH, Salzburg, Austria). Known H_2O_2 concentrations served as standards. The ROS production levels were normalized to cellular protein, determined by the BCA Protein assay kit (Pierce, Rockford, IL).

Isolation of mitochondria

To isolate mitochondria, 2×10^7 cells were suspended in 0.8 ml of ice-cold sucrose-based medium (250 mM sucrose, 5 mM Hepes, 0.1 mM EDTA, pH 7.2) and homogenized using a Dounce homogenizer. After adding 0.8 ml sucrose-based medium, the mixture was centrifuged (700×*g*, 10 min, 4°C). The resulting supernatant was again centrifuged (12,000×*g*, 15 min, 4°C), the mitochondrial pellet was washed once in a sucrose-based medium and resuspended in 0.5 ml of the same medium. The protein concentration of this suspension, used to normalize the iron content of mitochondria, was measured using a commercial Protein Determination Kit (Pierce).