Mitochondrial redox signalling by p66Shc mediates ALS-like disease through Rac1 inactivation

Maria Grazia Pesaresi1,5, Ilaria Amori1, Carlotta Giorgi2, Alberto Ferri3, Paolo Fiorenzo1, Francesca Gabanella3, Anna Maria Salvatore3, Marco Giorgio4, Pier Giuseppe Pelicci4, Paolo Pinton2, Maria Teresa Carri1,5,† and Mauro Cozzolino1,∗

1Laboratory of Neurochemistry, Fondazione S. Lucia IRCCS, Rome, Italy, 2Section of General Pathology, Department of Experimental and Diagnostic Medicine, Interdisciplinary Center for the Study of Inflammation (ICSI) and LTTPA Center, University of Ferrara, Ferrara, Italy, 3Institute of Cell Biology and Neurobiology, Rome, Italy, 4IFOM-IEO Campus, Milan, Italy and 5Department of Biology, University of Rome Tor Vergata, Via della Ricerca Scientifica, Rome, Italy

Received June 15, 2011; Revised and Accepted August 4, 2011

Increased oxidative stress and mitochondrial damage are among the mechanisms whereby mutant SOD1 (mutSOD1) associated with familial forms of amyotrophic lateral sclerosis (ALS) induces motoneuronal death. The 66 kDa isoform of the growth factor adapter Shc (p66Shc) is known to be central in the control of mitochondria-dependent oxidative balance. Here we report that expression of mutSOD1s induces the activation of p66Shc in neuronal cells and that the overexpression of inactive p66Shc mutants protects cells from mutSOD1-induced mitochondrial damage. Most importantly, deletion of p66Shc ameliorates mitochondrial function, delays onset, improves motor performance and prolongs survival in transgenic mice modelling ALS. We also show that p66Shc activation by mutSOD1 causes a strong decrease in the activity of the small GTPase Rac1 through a redox-sensitive regulation. Our results provide new insight into the potential mechanisms of mutSOD1-mediated mitochondrial dysfunction.

INTRODUCTION

Damage to mitochondria is emerging as a central feature that contributes to the degeneration of motor neurons in amyotrophic lateral sclerosis (ALS). Recent evidence indicates that mitochondria are one of the primary location of damage inside motor neurons (1), but also astrocytes and muscle cells, which both have been involved in the disease, show deficits in mitochondrial metabolism (2–4). Dysfunction of mitochondria is observed early in patients (and in experimental models for ALS) and causes the death of neurons, which underlies onset of paralysis and death of patients. A large body of studies in cells and mice overexpressing mutSOD1s, which model many characteristics of the disease, have addressed different aspects of mitochondrial dysfunction occurring in ALS, ranging from altered morphology (swelling and fragmentation) (5–8) to impaired activity of respiratory chain complexes (9,10), weakened calcium buffering capacity (11) and mitochondria-dependent execution of apoptosis (12–14). Moreover, recent investigations have drawn attention to the presence of a generalized energetic imbalance both in patients and in mice, suggesting that ubiquitous defects in mitochondrial physiology might contribute to the disease process (15,16).

Compelling evidence has accumulated that uncontrolled association of mutSOD1 with mitochondria may be directly responsible for mitochondrial impairment (6,17,18), either by decreasing protein import selectively in spinal cord mitochondria (19) or by binding and inactivating specific mitochondrial targets such as the voltage-dependent anion channel 1 (20), the anti-apoptotic protein Bcl-2 (21) or the mitochondrial form of lysyl-tRNA synthetase (22). However, there is also indication that mitochondrial localization might not be necessary for mutant SOD1 (mutSOD1) to damage...
mitochondria (23), suggesting that cytosolic signals, yet to be identified, mediate the transfer of the inherent toxic properties of mutSOD1 to mitochondria.

A novel, mitochondria-related signalling mechanism involving the 66 kDa isoform of the growth factor adapter Shc (p66Shc), which is operative in conditions of oxidative stress, has been recently identified. The protein p66Shc is an alternatively spliced isoform of a growth factor adapter that is phosphorylated upon oxidative stress (24). In this form, a fraction of p66Shc localizes to mitochondria, where it binds to cytochrome c and acts as an oxidoreductase, generating ROS and leading to organelle dysfunction and cell death (25). p66Shc−/− mice exhibit a 30% extended lifespan, reduced H2O2 levels and an enhanced resistance against oxidative stress (26), indicating that p66Shc acts as a key molecular sentinel that controls cellular stress responses and mammalian lifespan.

On the basis of these considerations, we have investigated the role of p66Shc in mutSOD1-induced cell toxicity, as well as the functional consequences of p66Shc ablation in transgenic mice overexpressing exogenous mutSOD1s, endogenous mouse SOD1 in control cells is not evident in this exposure.

RESULTS

Apoptosis induced by mutSOD1 is accompanied by phosphorylation of p66Shc at serine 36

p66Shc is phosphorylated on a serine residue in position 36 in response to cellular stress induced by various stimuli, and this event is crucial to p66Shc-mediated oxidative stress and apoptosis (26,27). As an initial step to investigate the role of p66Shc in mutSOD1-induced cell stress, human wild-type or G93A-SOD1, the most studied SOD1 mutation associated with familial amyotrophic lateral sclerosis (fALS), was transiently overexpressed in SH-SY5Y human neuroblastoma cells by adenoviral infection. In these conditions, G93A-SOD1 induces the sequential activation of caspase-3, cleavage of PARP and accumulation of fragmented nuclei, all suggestive of an ongoing apoptotic process (Fig. 1). Similar results were obtained when SH-SY5Y cells were infected with the fALS mutant H80R-SOD1 (Fig. 7 and not shown), indicating that the pro-apoptotic effect is common to fALS-linked SOD1s. In contrast, infection with adenoviruses expressing wtSOD1 or a control GFP protein (not shown) has no evident effects. To determine whether p66Shc is activated by mutSOD1 through phosphorylation on Ser36, SH-SY5Y cells stably overexpressing a serine 36-to-alanine (and therefore non-phosphorylatable) mutant of p66Shc (S36A; Fig. 2B) were infected with adenoviruses coding for wild-type SOD1 or G93A mutSOD1 and analysed after 72 h for apoptotic markers. As shown in Figure 2B, the number of apoptotic cells generated by mutSOD1, but not of wtSOD1, induces p66Shc phosphorylation at Ser36 (Fig. 2A), whereas the expression levels of all Shc protein isoforms (p46, p52 and p66) are not affected.

Overexpression of dominant-negative, functionally inactive p66Shc proteins protects cells against mutSOD1-induced mitochondrial damage and apoptosis

To investigate the functional relevance of the phosphorylation of p66Shc at Ser36 induced by mutSOD1, SH-SY5Y cells stably overexpressing a serine 36-to-alanine and therefore non-phosphorylatable mutant of p66Shc (S36A; Fig. 2B) were infected with adenoviruses coding for wild-type SOD1 or G93A mutSOD1 and analysed after 72 h for apoptotic markers. As shown in Figure 2B, the number of apoptotic cells generated by mutSOD1 is reduced by ~90% in cells expressing the S36A mutant of p66Shc, and a similar decrease

Figure 1. mutSOD1 induces apoptosis in SH-SY5Y cells. (A) SH-SY5Y cells were infected with adenoviruses coding for wild-type SOD1 (wtSOD1) or the fALS G93A-SOD1 mutant. After 72 h, nuclei of cells were stained with Hoechst 33342, and apoptotic nuclei were quantified as described in Materials and Methods. (B) Cells were infected as in (A) and analysed after 48 h by indirect immunofluorescence analysis with antibodies against SOD1 (green) or the active form of caspase-3 (activeC3, red). Cell nuclei are in blue. (C) After 72 h of expression of the indicated SOD1s, nuclear and cytosolic fractions from cells were isolated. Nuclei were analysed in western blot with antibodies recognizing the cleaved form of PARP1. The nuclear protein Lamin B was analysed as a standard for equal protein loading. Cytosolic fractions were also controlled for the expression of SOD1. Owing to the high levels of overexpressed exogenous mutSOD1s, endogenous mouse SOD1 in control cells is not evident in this exposure.
in caspase-3 activity is observed (Fig. 2C). These data thus indicate that the phosphorylation of p66Shc plays a role in mutSOD1-induced cell death.

The pro-oxidant, pro-apoptotic activities of p66Shc depend upon two defined regions of the protein: two glutamic residues at position 132 and 133, the site where the redox activity of p66Shc has been mapped (25), and a cysteine at position 59, a regulatory disulphide/thiol site mediating a reversible dimer–tetramer transition, which has been proposed to control the protein’s apoptosis-inducing activity (28). When overexpressed in SH-SY5Y cells, both the E132Q/E133Q (EEQQ) and the C59S mutant p66Shc proteins are able to inhibit the pro-apoptotic activity of G93A mutSOD1, as measured by PARP cleavage, similar to what is observed with the S36A mutant (Fig. 2D). On the contrary, overexpression of wild-type p66Shc enhances the mutSOD1-induced apoptosis. Overall, these observations strongly indicate that p66Shc mediates the toxic effects exerted by mutSOD1.

It has been shown that mitochondrial Ca$^{2+}$ responsiveness, which is a highly sensitive readout of mitochondrial state (27), is dramatically compromised after p66Shc activation. Therefore, using the aequorin technology to monitor mitochondrial Ca$^{2+}$ signalling (29), we analysed the effects of both wild-type and mutSOD1 proteins on SH-SY5Y cells displaying different background for p66Shc, i.e. in wild-type SH-SY5Y cells, in SH-SY5Y cells overexpressing the mutant S36A-p66Shc, in SH-SY5Y cells overexpressing the mutant EEQQ-p66Shc and in SH-SY5Y cells overexpressing wild-type p66Shc.

In SH-SY5Y cells, application of carbachol, an extracellular agonist acting on a Gq-coupled receptor, causes the production of inositol 1,4,5-trisphosphate and thus the release of Ca$^{2+}$ from the endoplasmic reticulum and the transient increase of cytosolic and mitochondrial [Ca$^{2+}$] (29).

In wild-type SH-SY5Y cells (Fig. 3A), the overexpression of wild-type SOD1 causes an increased mitochondrial [Ca$^{2+}$] ([Ca$^{2+}$]$_{m}$) response after agonist stimulation. On the contrary, the overexpression of two different mutSOD1s causes a drastic reduction in the Ca$^{2+}$ spike evoked by agonist stimulation, as an early consequence of mitochondrial damage as previously reported after p66Shc activation during oxidative stress (27).
The presence of p66Shc mutant proteins (Fig. 3C and D) confers mitochondrial insensitiveness to mutSOD1, since the mitochondrial Ca²⁺ response after agonist stimulation is almost unaffected by the presence of mutSOD1. On the contrary, the alteration of mitochondrial responsiveness induced by mutSOD1 is maintained in SH-SY5Y cells overexpressing wild-type p66Shc (Fig. 3B).

We have recently shown that mutSOD1 induces alterations in mitochondrial bioenergetics and morphology of SH-SY5Y cells (5). To test whether these effects arise as a consequence of p66Shc activation, ATP production and mitochondrial morphology were assayed in cells overexpressing wild-type or mutSOD1 together with p66Shc mutant proteins. To analyse the mitochondrial capability for energy production, ATP concentration in SH-SY5Y cells exposed to mutSOD1s was measured. An [ATP] decrease was evident in cells overexpressing G93A or H80R mutSOD1 (Fig. 3E); in contrast, cells co-expressing functionally inactive p66Shc show a physiological ATP concentration.

As observed by immunofluorescence analysis of SH-SY5Y cells using antibodies anti-SOD1 and SOD2, a mitochondrial matrix protein (Fig. 4, left), cells expressing the G93A mutSOD1 show a significant alteration of the filamentous mitochondrial network which characterizes most of the untransfected or wild-type SOD1-transfected cells, with mitochondria appearing fragmented and swollen. Similarly, the filamentous network is essentially lost when cells are challenged with H₂O₂. On the contrary, in cells expressing the p66Shc EEQQ mutant (Fig. 4, right), the overall filamentous network is maintained either in the presence of mutSOD1 or after H₂O₂ treatment. Similar results were obtained with the S36A mutant p66Shc (not shown).

On the whole, these data clearly indicate that p66Shc is the effector downstream of mutSOD1 responsible for the alterations of mitochondrial physiology that eventually lead to cell death.

**Ablation of p66Shc significantly ameliorates ALS phenotype in mice**

To learn whether transgenic G93A-SOD1 mice, an accepted model for ALS linked to mutSOD1, would be rescued by genetic removal of p66Shc, we crossed these mice with p66Shc+/− mice (26) and determined several behavioural and biochemical parameters.

As shown in Figure 5B, p66Shc is highly expressed in the spinal cord of control mice, as well as wtSOD1 or G93A-SOD1 transgenic mice, and only to a lower extent in the brain and muscle. As expected, ablation of p66Shc does not affect the levels of expression of transgenic SOD1 in the spinal cord. As shown in Figure 5A, G93A-SOD1/p66Shc+/− mice show significantly delayed onset of the disease (118.96 ± 10.98 versus 98.07 ± 8.9 days, P < 0.0001), improved motor performance as measured by rotarod test and increased survival (141.26 ± 14.42 versus 157.50 ± 9.11 days, P < 0.0001) with respect to G93A-SOD1 mice. Such striking effects are paralleled by improved mitochondrial function specifically in the spinal cord, where the activity of complex IV is restored (Fig. 5C) together with the ratio between reduced and oxidized glutathione (GSH/GSSG) in mitochondria, which is an indicator of the redox state of the cell (Fig. 5D).

**p66Shc mediates the toxicity of mutSOD1 through Rac1 inactivation**

To learn more on the molecular mechanisms of mutSOD1-induced p66Shc toxicity, and to attempt the dissection of such mechanisms *in vitro*, we focused on Rac1, a member of the Rho family of small GTPases, which controls many intracellular processes, including ROS production and cytoskeletal dynamics, and whose activity has been linked to the activity of both SOD1 and p66Shc (30,31).
Overexpression of both G93A and H80R mutant proteins decreases the levels of active, GTP-bound form of Rac1 in SH-SY5Y cells, compared with control and wtSOD1-expressing cells, as measured by a GST-PAK1 pull-down assay (Fig. 6A and B). The effect is specific for Rac1, since mutSOD1s do not significantly affect the activity of either RhoA or Cdc42, two small GTPases functionally related to Rac1 (Fig. 6A and B). Moreover, the overexpression by adenoviral infection of a constitutive active (V12) form of Rac1 completely protects SH-SY5Y cells from mutSOD1-induced apoptosis, whereas the expression of a dominant-negative, inactive (N17) mutant of Rac1 has no effect (Fig. 7). Significantly, N17 Rac1 is able to induce apoptosis in control cells, and to re-establish the apoptotic phenotype in cells where apoptosis induced by mutSOD1s overexpression has been hampered by co-expression of functional inactive mutants of p66Shc (Fig. 8A and B), suggesting that Rac1 acts downstream of p66Shc. More importantly, in cells expressing the dominant-negative mutant p66Shc-S36A, Rac1 activity is dramatically compromised by mutSOD1s (Fig. 9A); on the contrary, when a wild-type p66Shc protein is overexpressed in cells, Rac1 activity is dramatically compromised by mutSOD1s (Fig. 9B). Altogether, these data thus clearly indicate that p66Shc activation is an obligatory step for mutSOD1 to inhibit Rac1, which in turn leads to cell death.

**p66Shc downregulates Rac1 activity through a redox-dependent mechanism**

It has been recently shown that the activity of Rac1 is physiologically controlled by the redox environment of the cell, according to a mechanism where SOD1 itself exerts a primary role (30). To test whether a redox-dependent mechanism could account for the inhibition of Rac1 activity following p66Shc activation, control SH-SY5Y cells or cells overexpressing a functional inactive mutant of p66Shc (S36A) were treated with H2O2 and the amount of GTP-Rac1 was assayed accordingly. Indeed, increasing concentration of H2O2 induces a proportional decrease in Rac1 activity, and this effect is associated with a strong decrease in cell viability (Fig. 10A and B). The expression of an S36A p66Shc mutant almost completely prevents H2O2-induced cell death, and in this condition the decrease in the activity of Rac1 is precluded (Fig. 10B and C). These results prompted us to investigate whether the activity of Rac1 could be affected by factors controlling the intracellular pool of GSH, which is the major determinant of the cellular redox state (32). As shown in Figure 10D, treatment of cells overexpressing mutSOD1 with the cell-permeable ethyl ester form of reduced GSH (GEE), which results in an increase in intracellular GSH, significantly restores Rac1 activity, whereas depletion of...
cytoplasmic GSH with l-buthionine-(S,R)-sulfoximine (BSO) has a striking inhibitory effect on the activity of Rac1.

**DISCUSSION**

In the present study, we show that inhibition of mitochondrial redox signalling by p66Shc is able to rescue viability in cell models and to improve survival in the mouse model for fALS linked to mutSOD1.

Indeed, it has been shown, both in models and patients, that the pathological phenotypes of ALS well correlate with alterations in all the processes related to or controlling mitochondrial function, i.e. morphology and bioenergetics, transportation and clearance, apoptosis and calcium buffering (33), thus sustaining a direct role of mitochondrial dysfunction in motor neuron degeneration in ALS pathogenesis.

However, evidence linking mitochondrial abnormalities to ALS is yet incomplete. Most of all, it is not entirely clear whether, and to what extent, mitochondrial preservation could be beneficial to the disease. To answer these questions, we decided to focus on p66Shc as a central regulator of mitochondrial ROS metabolism and the mitochondrial apoptosis pathway (24). p66Shc responds to a variety of stimuli by increasing ROS levels in the mitochondrial intermembrane space through an ROS-producing activity (25). The physiological role of this process is currently unknown, although it is clear that it might participate in the control of intracellular redox-based signal transduction pathways (34). These mechanisms are indeed relevant for the process of apoptosis, since p66Shc-mediated formation of ROS triggers the initiation of the mitochondrial apoptosis pathway, and more generally for the regulation of lifespan (26) and energy metabolism (35,36), which are intimately connected. Moreover, p66Shc has a function in various pathological conditions where oxidative stress plays a role, such as arteriosclerosis (37) and endothelial dysfunctions (38).

Although it has been initially suggested that other, non-mitochondrial activities of p66Shc might be needed to exert its pro-apoptotic function (25), activation of the p66Shc pathway has emerged as a clear readout of mitochondrial damage in cells (39), and data presented in this work clearly point to the intrinsic, mitochondrial redox activity of p66Shc in mediating the toxicity exerted by mutSOD1. Different lines of evidence support this conclusion: (i) the accumulation in cells expressing mutSOD1 of p66Shc species phosphorylated on serine 36, a critical regulatory site for the mitochondrial pro-apoptotic activity of p66Shc (27), and the rescuing effect on cell viability of the non-phosphorylatable S36A p66Shc mutant; (ii) the inhibitory effect over the toxic action of mutSOD1s of the EEQQ and C59S p66Shcs, two...
The relationship between the activation of p66Shc by mutSOD1 and overexpression of mutSOD1s, we analysed the functional activation is responsible for neuronal cell damage induced in this process. Mutations in alsin, which are responsible for a recessive form of juvenile-onset ALS, are predicted to affect the guanine nucleotide exchange factor (GEF) activity of this Rac1, a prominent member of the Rho family of small GTPases. Rac1 is an intracellular transducer known to regulate multiple signalling pathways that control the organization of cytoskeleton, gene expression and cell proliferation. Rac1 and its relatives are also essential regulators of cytoskeleton, gene expression and cell proliferation. Moreover, Rac1 inhibition in the process of neurodegeneration in ALS.

Figure 6. MutSOD1 proteins decrease Rac1-GTP levels in SH-SY5Y cells. (A) SH-SY5Y cells were infected with adenoviruses expressing wild-type or the G93A and H80R mutSOD1s. After 72 h, GTP-bound Rac1 and Cdc42 were pulled-down by a GST-PAK protein, whereas GTP-RhoA was pulled-down with a GST-Rhotekin protein, both conjugated to GSH–sepharose. The GTP-bound (active) as well as the total (input) amounts of GTPases were detected in western blot using the indicated antibodies. (B) Quantification of active Rho GTPases in cells treated as in (A). The amounts of active GTPases were normalized to inputs and are expressed as mean ± SD of arbitrary densitometric units relative to control, non-infected cells. Values significantly different from relative controls are indicated with an asterisk when P < 0.01 (n = 4).

Rac1, a prominent member of the Rho family of small GTPases. Rac1 is an intracellular transducer known to regulate multiple signalling pathways that control the organization of cytoskeleton, gene expression and cell proliferation. Moreover, Rac1 inhibition in the process of neurodegeneration in ALS. p66Shc activation is clearly responsible for Rac1 inhibition. However, we found that active Rac1 and cell viability are directly linked in neuronal cells, and that cell death induced by mutSOD1s is achieved through a p66Shc-dependent inhibition of Rac1, whereas it has been clearly shown that the pro-inflammatory activity of mutSOD1-expressing microglial cells relies on an uncontrollable, constitutive activation of Rac1. Further, p66Shc was proposed to promote Rac1 activation, thereby triggering ROS production by NOX, whereas in our cell model, p66Shc activation is clearly responsible for Rac1 inhibition. The reasons for these apparent discrepancies can be partially explained by differences in the cellular milieu (neurons versus glia) and by the fact that the activity of Rho family GTPases is strictly dependent on their subcellular distribution and compartmentalization, which again can be profoundly different between neuronal and glial cells.

Nonetheless, different lines of evidence support a role of Rac1 inhibition in the process of neurodegeneration in ALS. Mutations in alsin, which are responsible for a recessive form of juvenile-onset ALS, are predicted to affect the guanine nucleotide exchange factor (GEF) activity of this Rac1.
protein (43,44). Alsin knockdown inhibits axon growth and induces cell death in cultured motoneurons. Notably, these cellular phenotypes are mimicked by expression of a dominant-negative Rac1 mutant and are completely blocked by expression of a constitutively active Rac1 mutant (45). Overexpression of alsin protects NSC34 cells from mutSOD1-induced apoptosis, and this neuroprotective activity is completely inhibited by knocking down the endogenous Rac1 expression with siRNA for Rac1 (46). On the whole, these observations clearly point to disruption of Rac1 GTPase function as a causative event of motor neuron degeneration. This conclusion is further supported by recent results obtained in neuronal cells depleted of the 43 kDa TAR DNA-binding protein (TDP-43), a major component of the ubiquitinated inclusions characteristic of ALS and frontotemporal lobar degeneration with ubiquitin-positive inclusions, whose mutations have been causally linked to familial ALS. The knockdown of TDP-43 in differentiated Neuro-2a cells inhibits neurite outgrowth and induces cell death through the inactivation of Rho family members RhoA and Rac1, and Cdc42 GTPases. These effects are associated with the inhibition of protein geranylgeranylation, a key post-translational modification for Rho family activity and intracellular localization (47). Our results point to a direct role of ROS in Rac1 inactivation, as suggested by the observation that the decrease of Rac1 activity by both hydrogen peroxide or mutSOD1 is prevented by inhibition of p66Shc-dependent mitochondrial redox signalling or by increased intracellular concentration of GSH. This is in line with recent findings indicating that GTPases may also be directly controlled by redox agents (48), a mechanism of regulation that may be particularly relevant in pathological conditions, such as ALS, where ROS are generated and the cellular redox balance altered.

In conclusion, our data emphasize that mitochondrial redox signalling, p66Shc and Rac1 are linked by an intimate connection in neurons in vitro and in vivo, in adult mice. That such connections are altered in a genetic context mimicking part of ALS patients strongly supports the concept that molecules involved in this signalling play an important role in this disease.
stable expression of wild-type or mutant p66Shc, cells were co-transfected with plasmids coding for different p66She proteins in the presence of 1:20 of a plasmid coding for hygromycin resistance, using Lipofectamine Plus reagent (Invitrogen). After selection with 400 mg/ml hygromycin (Invitrogen), about 20 clones for each construct were isolated independently and analysed in western blot with anti-Shc antibodies. At least three clones for each plasmid were chosen for equivalent expression of p66Shc proteins and used for further analysis. All the clones analysed gave consistent results and data from one clone are shown.

Construction of recombinant adenoviruses expressing wild-type SOD1, as well as G93A and H80R mutSOD1, was carried out by inserting cDNAs coding for the different SOD1s into the pShuttle2 and BD Adeno-X viral DNA (BD Bioscience). Transient transfections of HEK293 cells were performed using Lipofectamine 2000 (Invitrogen). Viral titre was determined by dilution assay using HEK293 cells according to the manufacturer’s instructions. Adenoviruses were propagated in HEK293 cells as described in Latella et al. (49). Infection of SH-SY5Y cells was carried out for 1 h in OPTIMEM; after removal of the virus, cells were grown for the indicated period of times before being subjected to further experimental manipulations. To optimize the protocol for SH-SY5Y infection, cells were infected with Ad-GFP and Ad-SOD1s, and expression was measured by immunoblotting and immunofluorescence with the antibody anti-SOD1. Efficiency of infection was ~90% at a multiplicity of infection (m.o.i.) of 1000 after infection for 48 h. V12 and N17 Rac1 adenoviruses were described in Cozzolino et al. (50) and used at an m.o.i. of 50.

Nuclei isolation and cleaved PARP1 analysis
On 60 mm Petri dishes, 1.5 × 10^6 SH-SY5Y cells were plated. After the indicated treatments, cells were rinsed in ice-cold PBS and lysed in 150 ml of low-salt buffer (10 mM Hepes, pH 7.4, 42 mM KCl, 5 mM MgCl2, 0.5 % CHAPS, 1 mM DTT, 1 mM PMSF, 1 mg/ml leupeptin). After centrifugation at 2000g, nuclei were resuspended in 50 ml of high-salt buffer (50 mM Tris–HCl, pH 7.5, 400 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% Nonidet-P40, 10% glycerol, 2 mM DTT, 1 mM PMSF, protease inhibitor cocktail). After 30 min on ice, lysates were centrifuged at 20 000g, supernatants were collected and analysed as nuclear fractions in western blot with antibodies anti-cleaved PARP1 (Cell Signal) and anti-Lamin B (Santa Cruz Biotechnology).

Assessment of apoptosis and cell viability
Quantification of apoptotic cells was obtained by direct visual counting after nuclear staining of 4% paraformaldehyde-fixed cells with the fluorescent probe Hoechst 33342 (1 mg/ml) (Sigma-Aldrich). One hundred cells were examined for each field at a magnification of 200× and eight randomly chosen fields for each experimental condition were counted. Only the cells containing clearly picnotic or fragmented nuclei were considered apoptotic. Caspase 3 activity was measured with a TruePoint Caspase 3 assay kit (PerkinElmer), according to the manufacturer’s instructions.

**MATERIALS AND METHODS**

**Plasmid construction**

Human cDNA coding for p66She (accession number U73377) was cloned by reverse transcription-PCR from human SH-SY5Y neuroblastoma cells cDNA, using the forward primer 5’ AAA AAG CTT ATG GAT CTC CTG CCC CCC 3’ and the reverse primer 5’ TTT CTC GAG TCA CAG TTA GAG TCA GAG TTA CAG TTT CCG CTC CAC 3’. The resulting PCR fragment was inserted into HindIII/XhoI restriction sites of pcDNA3 (Invitrogen). For the mutagenesis of p66She, PCR site-directed mutagenesis was performed using pcDNA3/p66She as template, followed by digestion with DpnI. All the plasmid constructions were verified by automated sequencing.

**Cell culture, plasmid transfection and adenoviral infection**

Human neuroblastoma cells, SH-SY5Y, were purchased from the European Collection of Cell Culture and grown in DMEM (Euroclone), at 37°C in an atmosphere of 5% CO2 in air. For stable expression of wild-type or mutant p66She, cells were

---

Figure 10. Redox regulation of Rac1 activity by mutSOD1. (A) SH-SY5Y cells were treated with the indicated amounts of H2O2. After 24 h, active Rac1 was measured by a GST-PAK pull-down assay. (B) The viability of control SH-SY5Y cells or SH-SY5Y cells expressing the S36A mutant p66She and treated with 50 µM H2O2 was calculated after 24 h through an MTS assay. (C) The activity of Rac1 was measured in cells treated as in (B). (D) SH-SY5Y cells were infected with adenoviruses expressing wtSOD1 or the H80R mutSOD1, in the absence or in the presence of 5 mM GEE. Control cells were also treated with 10 mM BSO. After 72 h, cell lysates were subjected to GST-PAK pull-down assay for the assessment of Rac1 activity. The numbers represent fold activity above normalized activity in control uninfected cells (referred to as 1.0). The data are representative of n = 3 independent experiments.
Cell viability was assessed by a colorimetric assay using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (CellTiter 96 Aqueous One Solution Assay, Promega), according to the manufacturer’s instructions. Absorbance at 490 nm was measured in a multilabel counter (Victor3-V, PerkinElmer Life Sciences).

Mitochondrial Ca$^{2+}$ measurements

Cells were seeded before transfection onto 13 mm glass cover slips and allowed to grow to 50% confluence. At this stage, the cells were infected with the adenovirus expressing a mitochondrial targeted aequorin chimera. Thirty-six hours after infection, the cover slips with the cells were incubated with 5 mM coelenterazine for 1–2 h in DMEM supplemented with 1% FCS, and then transferred to the perfusion chamber. All aequorin measurements were carried out in KRB (Krebs–Ringer modified buffer: 125 mM NaCl, 5 mM KCl, 1 mM Na$_2$PO$_4$, 1 mM MgSO$_4$, 5.5 mM glucose, 20 mM HEPES, pH 7.4, 37°C) supplemented with 1 mM CaCl$_2$. The agonist, 1 mM carbachol, was then added to the same medium. The experiments were terminated by lysing the cells with 100 μM digitonin in a hypotonic Ca$^{2+}$-rich solution (10 mM CaCl$_2$ in H$_2$O), thus discharging the remaining aequorin pool. The light signal was collected and calibrated into [Ca$^{2+}$] values by an algorithm based on the Ca$^{2+}$ response curve of aequorin at physiological conditions of pH, [Mg$^{2+}$], and ionic strength, as previously described (29). Statistical data are presented as mean ± SEM, significance was calculated by Student’s t-test and correlation analysis was done with the SigmaPlot 5.0 software (SPSS, Inc.).

Immunofluorescence analysis

For immunofluorescence analysis, cells cultured on poly-L-lysine-coated glass cover slip were washed in PBS and fixed with 4% paraformaldehyde in PBS for 10 min. Fixed cells were washed in PBS followed by permeabilization with 0.1% Triton X-100 in PBS for 5 min. Cells were blocked for 1 h in 2% horse serum in PBS and incubated for 1 h at 37°C with primary antibodies: mouse monoclonal anti-active casp3 (Cell Signaling), rabbit anti-SOD1 (Stressgen), rabbit anti-SOD2 (Stressgen), mouse monoclonal anti-SOD1 (clone SD-G6, Sigma-Aldrich). Cells were washed in blocking buffer and incubated for 1 h with an Alexa Fluor 488 goat anti-mouse (Invitrogen) and Cy3 goat anti-rabbit (Jackson Immunoresearch Laboratories) antibodies. After rinsing in PBS, cells were stained with 1 μg/ml Hoechst 33342 (Sigma-Aldrich) and examined under a Zeiss LSM 510 confocal microscopy. Fluorescence images were processed using Adobe Photoshop.

Measurement of cellular ATP

Measurement of cellular ATP was performed using the ATPlite Assay (Perkin Elmer-Cetus, Norwalk, CT, USA). In brief, cells seeded in 96-well microplates were resuspended in 50 μl of lysis buffer and mixed for 10 min. Forty microlitres of substrate solution (Luciferase/Luciferin) was added to each sample. The luminescence was measured using a luminescence plate reader (Victor3-V, PerkinElmer Life Sciences). The ATP concentration was normalized to total cellular protein concentration estimated by Bradford protein assay (Bio-Rad).

Rho family pull-down assay

The Rac-GTP and Cdc42-GTP pull-down assay was performed as previously described (50). Briefly, cells were lysed in a buffer containing 50 mM Tris, pH 7.2, 100 mM NaCl, 5 mM MgCl$_2$, 1 mM DTT, 10% glycerol, 1% Nonidet-P40, plus protease inhibitors. One-fifth of cell lysates were subjected to immunoblotting. Cell lysates were mixed with 10 μg of bacterially expressed GST-PAK (rat PAK amino acids 1–252) bound to GSH–sepharose and incubated at 4°C with tumbling for 30 min. Beads were collected by centrifugation and washed twice in lysis buffer before addition of Laemmli buffer and analysis by western blot with anti-Rac1 and anti-Cdc42 antibodies. For the RhoA-GTP pull-down assay, cell lysates were washed in 50 mM Tris–Cl, pH 7.2, 500 mM NaCl, 1% (v/v) Triton X-100, 5 mM MgCl$_2$, 1 mM DTT and protease inhibitors. Bacterially expressed GST-Rhotekin (murine amino acids 7–89) bound to GSH–sepharose was used in place of PAK.

Immunoprecipitation

After rinsing the cultures with ice-cold PBS, cell lysis was performed in RIPA buffer (50 mM Tris–HCl, 0.5% Triton X-100, 0.25% Na-deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, 5 mM MgCl$_2$) containing 1 mM PMSF and a protease inhibitor cocktail (Sigma-Aldrich). A clear supernatant was obtained by centrifugation and washed twice on washing buffer at 17 000g for 10 min. Protein content was determined using Bradford protein assay (Bio-Rad). Equal amounts of lysates were incubated at 4°C for 2 h with a rabbit polyclonal anti-Shc antibody (Cell Signal) and the immunocomplexes were collected by binding to protein A-Agarose beads (Roche), followed by three washes with lysis buffer.

Electrophoresis and western blot

Standard SDS–PAGE was performed as described (10). Western blot was performed onto nitrocellulose membranes (Amersham), except for RhoA, Rac1 and Cdc-42 that were blotted onto PVDF membranes (Millipore). After incubation in Tris-buffered saline (TBS) solution containing 0.1% Tween 20 and 5% non-fat milk, filters were incubated for 2 h at room temperature with the indicated antibodies diluted in a 2% non-fat milk, 0.1% Tween 20/TBS solution. Immunoreactive SOD1 was detected with a rabbit polyclonal anti-SOD1 antibody (Stressgen). p66Shc proteins were detected using a mouse monoclonal anti-Shc antibody which recognizes all the three isoforms of Shc (p46, p52 and p66). Phosphorylated p66Shc on serine 36 was detected using a mouse monoclonal specific antibody (Alexis). A goat antibody (Santa Cruz Biotechnology) was used to detect the cleaved form of PARP1. Antibodies anti-RhoA and Cdc42 were from Santa Cruz Biotechnology. Anti-Rac1 was from...
Millipore. β-Actin was detected using a mouse monoclonal antibody from Sigma. A goat antibody anti-Lamin B was from Santa Cruz Biotechnology.

Following extensive washing in 0.1% Tween 20/TBS solution, filters were incubated with the appropriate peroxidase-conjugated secondary antibodies, washed in 0.1% Tween 20/TBS solution and developed using the POD chemiluminescence detection system (Roche). Image analysis and quantifications were performed by Kodak Image Station (KDS IS440CF 1.1) with 1D Image Analysis software.

Animals
All animal procedures have been performed according to the European Guidelines for the use of animals in research (86/609/CEE) and the requirements of Italian laws (D.L. 116/92). The ethical procedure has been approved by the Animal Welfare Office, Department of Public Health and Veterinary, Nutrition and Food Safety, General Management of Animal Care and Veterinary Drugs of the Italian Ministry of Health.

At the indicated time, mice were anaesthetized with 500 mg/kg chloral hydrate, sacrificed and dissected for the different experiments. All efforts were made to minimize suffering. All animals have been raised and crossed in the indoor animal house in a 12 h light/dark cycle in a virus/antigen-free facility with controlled temperature and humidity and have been provided with water and food ad libitum.

Wild-type SOD1 mice B6.Cg-Tg(SOD1)2Gur/J and SOD1G93A mice B6.Cg-Tg(SOD1*G93A)1Gur/J were purchased from The Jackson Laboratory and were on C57BL/6J background. p66Shc−/− mice were also in the C57BL/6J background.

Mice compared in this study were all littermates and housed together to minimize environmental factors. Mice were genotyped using PCR protocols from The Jackson Laboratory.

Western blot analysis for p66Shc and SOD1 expression was carried out as described above on total protein extracts from various tissues obtained through homogenization in RIPA buffer.

Symptom onset, survival and rotarod analysis
Behavioural analysis was performed according to the standard operating procedures indicated by Ludolph et al. (51). Briefly, mice were considered terminally paralysed if they were unable to right themselves after 10 s of being placed on their side. To assess symptom onset, mice were subjected to grip test twice a week, starting at 70 days of age. Rotarod testing was performed using the accelerating rotarod apparatus (Ugo Basile 7650 model). The rod was accelerated at a constant rate of 4 r.p.m. starting from 3 r.p.m. for a maximum of 5 min. The time (seconds) at which the animal fell from the bar was recorded. Mice were tested twice a week for three trials, each starting at 80 days of age, until they were unable to remain on the rotarod for at least 20 s. The best trial per day was recorded and used for analysis. Each time point represents the mean ± SE of performance of all mice at each data point as previously described (52).

Determination of complex IV activity and GSH/GSSG ratio were carried out on purified mitochondria as previously described (10).

Statistical analysis
Statistical analysis of rodatar data was performed using ANOVA. Kaplan–Meier curves were compared using the log-rank test. Comparisons of protein expression levels, complex IV activity and values of GSH/GSSG ratio were performed using two-tailed unpaired Student’s t-test. P-values of <0.05 were considered significant.

ACKNOWLEDGEMENTS
We are grateful to Claudia Crosio and Ciro Iaccarino for help with construction of adenoviruses, to Cristiana Valle and Simona Rossi for help with animal motor tests and to Silvia Middei for statistical analysis.

Conflict of Interest statement. None declared.

FUNDING
This work was supported by Association Francaise contre les Myopathies (Project 14354 to M.C.), by Fondation Thierry Latran and Neuron EraNet (to M.T.C.) and by the Italian Association for Cancer Research (AIRC), Telethon (GGP09128), the Italian Ministry of Education, University and Research (COFIN) and Italian Ministry of Health (to P.P.).

REFERENCES


