

The inflammatory NADPH oxidase enzyme modulates motor neuron degeneration in amyotrophic lateral sclerosis mice

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ALS is a fatal paralytic disorder characterized by a progressive loss of spinal cord motor neurons. Herein, we show that NADPH oxidase, the main reactive oxygen species-producing enzyme during inflammation, is activated in spinal cords of ALS patients and in spinal cords in a genetic animal model of this disease. We demonstrate that inactivation of NADPH oxidase in ALS mice delays neurodegeneration and extends survival. We also show that NADPH oxidase-derived oxidant products damage proteins such as insulin-like growth factor 1 (IGF1) receptors, which are located on motor neurons. Our *in vivo* and *in vitro* data indicate that such an oxidative modification hinders the IGF1/Akt survival pathway in motor neurons. These findings suggest a non-cell-autonomous mechanism through which inflammation could hasten motor neuron death and contribute to the selective motor neuronal degeneration in ALS.

Akt | ALS | microglia | oxidation | non-cell autonomous

ALS is the most common adult-onset paralytic disease and is characterized by a loss of motor neurons in the cerebral cortex, brainstem, and spinal cord (1). It is invariably fatal, usually within 3–5 years after onset (1). Insights into its neurodegenerative mechanisms followed the discovery that dominant mutations in the gene for superoxide dismutase 1 (SOD1) cause familial ALS (2, 3). Overexpression of SOD1 mutants in rodents emulate clinical and pathological hallmarks of ALS through a toxic gain of function (4). Development of chimeric mice provided animals with a mixture of neuronal and nonneuronal cells expressing wild-type or mutant SOD1 (5); investigation of these animals suggested that nonneuronal cells influence the fate of spinal cord motor neurons (5). Corroborating this hypothesis is the demonstration that reduction of mutant SOD1, selectively in microglia, extended survival in transgenic SOD1^{G37R} mice (6). In light of the latter results, microglia are now thought to contribute to the non-cell-autonomous killing of motor neurons.

Among the microglia-derived mediators that could promote neurodegeneration are reactive oxygen species (ROS) produced by the enzyme NADPH oxidase complex (7). Although this ROS-generating multimeric oxidase is indispensable for protecting the host against invading microorganisms in infectious disorders (8), its inappropriate activation may be harmful in noninfectious neurodegenerative disorders. Such bystander cytotoxicity is thought to lead to the death of developing oligodendrocytes in periventricular leukomalacia (9), one of the most important causes of cerebral palsy. In light of these facts, we undertook the study of NADPH oxidase in both human ALS and one of its genetic models. Our results for both human and mouse postmortem tissues indicate that spinal cord microgliosis in ALS is accompanied with an up-regulation of NADPH oxidase. Furthermore, by using mutant deficient mice in functional NADPH oxidase as well as in neuron-like cell culture systems, we provide compelling evidence that supports the concept that this microglial ROS-generating enzymatic complex promotes spinal cord motor neuron degeneration by

a mechanism involving oxidative damage to critical survival signaling pathways.

Results

NADPH Oxidase Is Up-Regulated in Inflamed Spinal Cords of ALS Mice.

To determine the role of NADPH oxidase in motor neuron degeneration, we first evaluated its expression at different stages of the disease in transgenic mice expressing mutant human SOD1 with a substitution of glycine to alanine in position 93 (SOD1^{G93A}), the most widely studied model of ALS. Expression of NADPH oxidase in the spinal cord, which carries the brunt of the pathology in this ALS model, was determined by analyzing its catalytic subunit, gp91^{phox}. Both gp91^{phox} message and protein contents in whole-tissue extracts of spinal cord rose over time in transgenic SOD1^{G93A} mice (Fig. 1A, B, D, and E) in concert with the development of a glial response (Fig. 6, which is published as supporting information on the PNAS web site) and the progression of the paralytic phenotype. The levels of the p67^{phox} subunit that contains the NADPH-binding site of the NADPH oxidase complex (10) were increased in membrane fractions of spinal cord extracts from symptomatic transgenic SOD1^{G93A} mice (Fig. 1C), indicating that this cytosolic subunit did translocate to the plasma membrane in these animals. Conversely, none of these NADPH oxidase alterations were seen in age-matched nontransgenic littermates (Fig. 1A–E). Histological evaluation of the spinal cord of symptomatic transgenic SOD1^{G93A} mice showed numerous gp91^{phox}-positive cells, primarily in the gray matter of the anterior horn (Fig. 1G), whereas sparse staining was observed in the spinal cord of age-matched nontransgenic controls (Fig. 1F). Consistent with NADPH oxidase expression by professional phagocytes, confocal microscopy demonstrated the colocalization of the gp91^{phox} subunit with a microglial marker, the ricinus communis agglutinin lectin (Fig. 1H–J); no gp91^{phox} colocalization was detected with the motor neuron marker, nonphosphorylated neurofilament heavy chain, or with the astrocyte marker, glial fibrillary acid protein (data not shown).

NADPH Oxidase Causes Protein Oxidation in Transgenic SOD1^{G93A} Mice.

We further characterized the status of spinal cord NADPH oxidase in transgenic SOD1^{G93A} mice by probing for formation of ROS and evidence of protein oxidation. In nontransgenic mice, spinal cord production of ROS, evidenced by the fluorescence emitted by ethidium, the oxidation product of hydroethidine (11), was minimal (Fig. 1K). In contrast, in symptomatic transgenic

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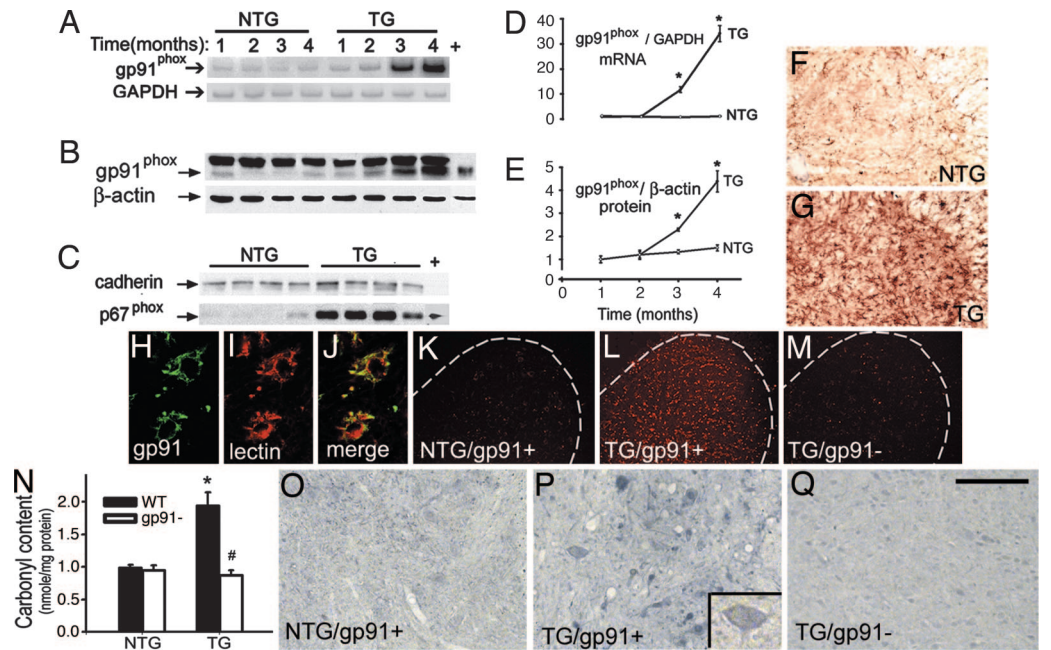
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Abbreviations: ROS, reactive oxygen species; SOD1, superoxide dismutase 1; IGF1, insulin-like growth factor 1.

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Fig. 1. Microglial NADPH oxidase stimulates carbonylation of spinal cord motor neurons in transgenic SOD1^{G93A} mice. (A–E) Spinal cord gp91^{phox} mRNA (A and D) and protein (B and E) in 1-month-old (asymptomatic) to 4-month-old (end-stage) transgenic SOD1^{G93A} (TG) and nontransgenic (NTG) mice. (C) Spinal cord p67^{phox} protein in 4-month-old TG SOD1^{G93A} and NTG littermates. +, positive control (mouse macrophage lysate). (F and G) Spinal cord gp91^{phox} immunohistochemistry in NTG (F) and TG (G) mice. (H–J) Confocal analysis of a TG spinal cord showing gp91^{phox} in green (H) and the microglial marker ricinus communis agglutinin lectin in red (I); merged image is shown in J. (K–M) Ethidium fluorescence (in red) in nontransgenic/gp91^{phox+} (NTG/gp91+) (K), transgenic SOD1^{G93A}/gp91^{phox+} (TG/gp91+) (L), and transgenic SOD1^{G93A}/gp91^{phox-} (TG/gp91-) (M) spinal cords. (N) HPLC quantification of protein carbonyls in spinal cords from all four genotypes. (O–Q) Immunohistochemistry for carbonyl adducts in NTG/gp91+ (O), TG/gp91+ (P), and TG/gp91- (Q) spinal cords by using an anti-dinitrophenylhydrazine antibody. (P Inset) Carbonylated motor neuron. Studies in F–Q are in 4-month-old mice. Values (means ± SEM; n = 4–8 mice per group) were compared by ANOVA followed by Newman–Keuls post hoc testing. *, P < 0.05, more than NTG mice; #, P < 0.05, lower than TG/gp91+ mice. (Scale bar in Q: F and G, 0.4 mm; H–J, 40 μm; K–M, 0.5 mm; O–Q, 0.15 mm.)



SOD1^{G93A} mice carrying the wild-type gp91^{phox} allele (SOD^{G93A}/gp91^{phox+}), spinal cord ethidium fluorescence was intense (Fig. 1L) and coincided anatomically with the areas of gp91^{phox} expression (Fig. 1G) and microglial activation (Fig. 6). In symptomatic transgenic SOD1^{G93A} mice carrying the nonfunctional mutant allele (SOD^{G93A}/gp91^{phox-}) (12), spinal cord ethidium fluorescence was negligible (Fig. 1M), as it was in the nontransgenic controls (Fig. 1K). A similar genotypic difference was found for the analysis of protein carbonylation (13), which is used as a general marker of protein oxidation (14). Symptomatic transgenic SOD1^{G93A}/gp91^{phox+} mice, but not age-matched SOD1^{G93A}/gp91^{phox-} mice, had increased levels of spinal cord protein carbonyl adducts compared with nontransgenic controls expressing either wild-type or null mutant gp91^{phox} (Fig. 1N). Immunohistochemically, the most robust labeling for protein carbonyl adducts occurred in spinal cord sections from SOD1^{G93A}/gp91^{phox+} mice at the level of cells with mixed morphology, including large motor neurons (Fig. 1O–Q).

NADPH Oxidase Induction and Neuronal Protein Carbonylation in Sporadic ALS. We then sought to determine whether the NADPH oxidase alterations identified in transgenic SOD1^{G93A} mice were also present in human sporadic ALS, the most common form of the disease (1). Consistent with the mouse data, gp91^{phox} content was low (Fig. 2A and B) and its immunoreactivity was faint in control postmortem spinal cords (Fig. 2D), whereas gp91^{phox} content was ≈3-fold higher and its immunoreactivity robust in sporadic ALS spinal cords (Fig. 2E). In the latter, gp91^{phox}-positive cells colocalized with the microglial-associated antigen CD68 (Fig. 2F) and were identified in all of the typical ALS loci of neurodegeneration, including the anterior horn and the lateral corticospinal track (Fig. 2C). Also comparable with the mouse data, there was a robust labeling for protein carbonyl adducts in postmortem spinal cord sections from sporadic ALS cases, which seemed to be mainly associated with large motor neurons (Fig. 2H). On average, 6–12 of 30 counted motor neurons were

positive for protein carbonyl adducts per lumbar spinal cord section in ALS patients, whereas no such immunoreactive motor neurons were seen in controls (Fig. 2G).

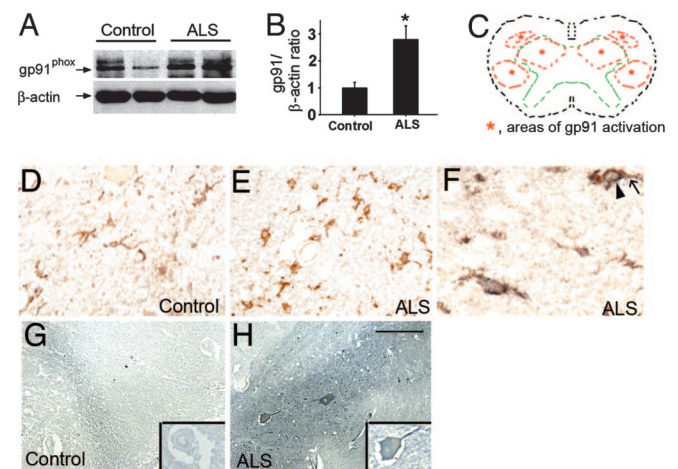


Fig. 2. NADPH oxidase is up-regulated and associated with motor neuron carbonylation in the spinal cord of sporadic ALS patients. (A and B) Immunoblots and bar graph for gp91^{phox} using spinal cord extracts from six normal controls and six age-matched ALS patients. Values in B are means ± SEM and were compared by Student's *t* test. *, P < 0.05, higher than normal controls. (C) Drawing of a spinal cord transversal plan showing the gray matter (area delineated by the green dashed line) and the loci of degeneration in ALS (areas delineated by the red dashed line). (D and E) Spinal cord gp91^{phox} immunohistochemistry in tissue sections from controls (D) and ALS patients (E). (F) In ALS patient samples, gp91^{phox}-positive cells (arrow) exhibit a brown membrane labeling, which colocalizes with the blue-gray cytosol labeling for the microglial marker CD68 (arrowhead). (G and H) Immunohistochemical detection of carbonyl adducts in spinal cord sections of normal controls and age-matched ALS patients obtained by using the same technique as in Fig. 1 O–Q. (Scale bar in H: D, E, G, and H, 0.2 mm; F, 0.5 mm.)

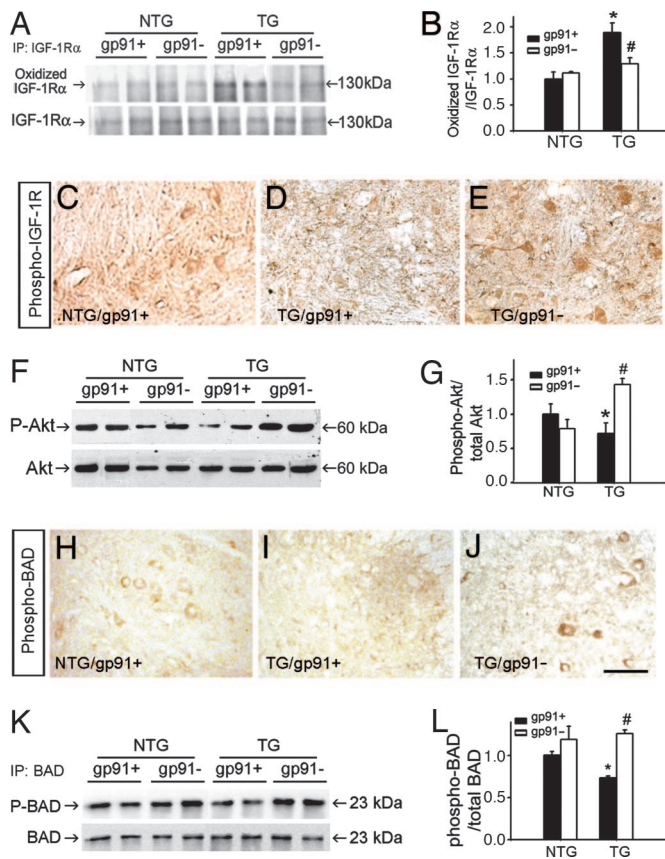


Fig. 4. Modulation of the IGF1/Akt pathway by NADPH oxidase-derived ROS. (A) Immunoprecipitation of IGF1 receptor α -chain followed by OxyBlot (upper blot) and immunoblot for spinal cord IGF1 receptor α -chain (lower blot). (B) Bar graph showing carbonylated/total α -chain ratios in the four genotypes shown in A. (C–E) Immunostaining for spinal cord phospho-IGF1 receptor in the different mouse groups. (F) Spinal cord phospho-Akt (P-Akt) (upper blot) and total Akt (lower blot) immunoblots. (G) Bar graph showing P-Akt/total Akt ratios in the four genotypes in F. (H–J) Immunostaining for spinal cord phospho-BAD in the different mouse groups. (K) Immunoprecipitation of spinal cord BAD followed by immunoblot for phospho-BAD (P-BAD) (upper blot) and total BAD (lower blot). (L) Bar graph showing P-BAD/total BAD ratios in the four genotypes in K. The mice were all 115 days old. In the quantifications, values (means \pm SEM; $n = 4$ –8 mice per group) were compared by ANOVA followed by Newman–Keuls post hoc testing. *, $P < 0.05$, different from nontransgenic controls; #, $P < 0.05$, different from transgenic SOD1^{G93A}/gp91^{phox+} mice. (Scale bar in J: C–E and H–J, 0.2 mm.)

mice compared with their age-matched SOD1^{G93A}/gp91^{phox-} counterparts. These data further support the idea that oxidative modification of IGF1 receptor in symptomatic transgenic SOD1^{G93A}/gp91^{phox+} mice is associated with a range of molecular perturbations.

Microglial-Derived ROS Recapitulate the IGF1/Akt Pathway Defect *In Vitro*. To test the idea that NADPH oxidase-derived ROS could impair IGF1 pathway function, an *in vitro* cell system using the neuron-like cell lines SH-SY5Y and IMR32 was used. First, cells were briefly incubated with 0.1–100 μ M human recombinant IGF1 in the presence of overnight-preconditioned serum-free medium supplemented with or without 0.1 milliunits/ml glucose oxidase to provide a constant flux of H₂O₂; IGF1 pathway responsiveness was monitored by Akt phosphorylation. Exposure to IGF1 caused a dose-dependent phosphorylation of Akt in SH-SY5Y cells (Fig. 5 A and B) and IMR32 cells (data not shown) in the presence of conditioned medium lacking glucose oxidase. Conversely, IGF1 barely increased Akt phosphorylation in the neuroblastoma cell

lines that were exposed to conditioned medium containing 0.1 milliunits/ml glucose oxidase, generating an average stable concentration of 75 μ M H₂O₂ (Fig. 5E); this effect was abolished by adding 1,000 units/ml catalase to the medium (data not shown). Next, SH-SY5Y cells were incubated with or without conditioned medium from LPS-activated BV2 microglial cells. Akin to the glucose oxidase experiments, brief exposure to LPS-activated microglial-conditioned medium, which contained increased levels of H₂O₂ (Fig. 5E), attenuated IGF1-mediated Akt phosphorylation in the neuroblastoma cell line (Fig. 5 C and D). Upon longer exposure to LPS-activated microglial-conditioned medium, the Akt phosphorylation response to the IGF1 recombinant remained depressed, and, at 72 h, a reduction of cell viability, indistinguishable from the condition without IGF1, was observed (Fig. 5F). However, both the alteration of IGF1-mediated Akt phosphorylation and the loss of cell viability mediated by LPS-activated microglia were counteracted by the specific NADPH oxidase inhibitor apocynin (Fig. 5 C, D, and F).

Discussion

Experimental evidence supports a model for ALS neurodegeneration in which nonneuronal cells such as microglia contribute to the demise of motor neurons (6, 16, 17). Germane to the molecular basis of this deleterious effect on motor neurons is our finding that virtually all spinal cord microglial cells express the gp91^{phox} subunit of the oxidant-producing enzyme NADPH oxidase (Fig. 1). Agreeing with the fact that in nonactivated phagocytes NADPH oxidase is quiescent (7), gp91^{phox}-positive cells in spinal cords from 1- to 4-month-old nontransgenic mice had a morphology of resting microglia and did not seem to produce ROS (Figs. 1 and 6). Conversely, in transgenic SOD1^{G93A} mice, paralleling the worsening of the ALS phenotype there was an intensification of spinal cord microgliosis accompanied by an up-regulation and activation of NADPH oxidase. These results suggest that over the course of the disease, spinal cord microglial cells become activated and acquire the capacity of oxidatively damaging nearby macromolecules and cells homed within inflamed ALS tissues. Corroborating this view are the levels of protein carbonyls, which were markedly elevated in spinal cord extracts of symptomatic transgenic SOD1^{G93A} mice, for the most part in a NADPH oxidase-dependent manner (Fig. 1). Evidence of microgliosis, NADPH oxidase up-regulation, and protein carbonylation was also found in postmortem spinal cords from human sporadic ALS cases (Fig. 2), supporting the conclusion that the occurrence of inflammation-mediated oxidative damage is not restricted to familial ALS caused by SOD1 mutations but is also a pathological hallmark of the prevalent nonfamilial, sporadic form of ALS.

Our results also show that abrogation of the gp91^{phox} subunit of NADPH oxidase in transgenic SOD1^{G93A} mice eliminates the production of microglial-derived ROS (Fig. 1M) and, importantly, prolongs survival and retards neurodegeneration in this ALS model (Fig. 3). Deletion of gp91^{phox} in transgenic SOD1^{G93A} mice did not alter the spinal cord microglial response or the expression of human SOD1 in transgenic SOD1^{G93A} mice (Fig. 7), which is a known determinant of disease severity in this ALS model (18). Consequently, the attenuated phenotype seen in transgenic SOD1^{G93A}/gp91^{phox-} mice is attributable to the lack of NADPH oxidase activity and not to either an impaired microglial response or expression of the human SOD1^{G93A} transgene. These data provide compelling evidence that NADPH oxidase contributes to the degeneration of motor neurons in ALS. They also underscore the significance of inflammatory-mediated oxidative stress in the pathogenesis of chronic, noninfectious pathological conditions such as ALS. However, the magnitude of benefit afforded by gp91^{phox} deletion in transgenic SOD1^{G93A} mice argues that targeting neuroinflammation by inhibiting just one of its mediators, such as NADPH oxidase, may not be sufficient to produce robust and lasting neuroprotection in ALS patients.

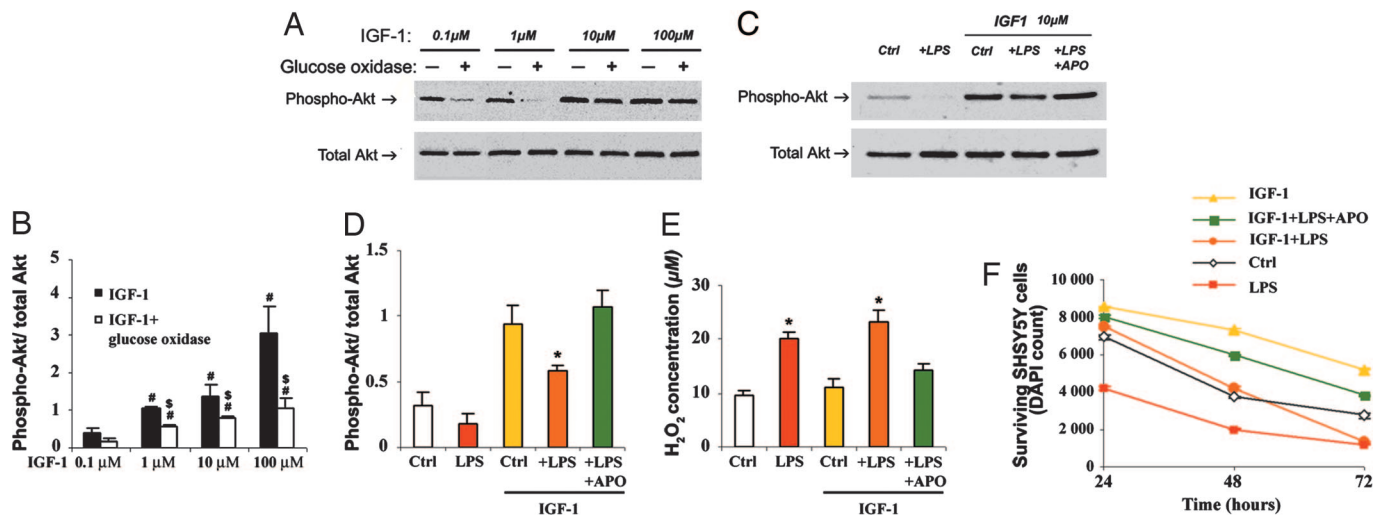


Fig. 5. Glucose oxidase- and microglial-derived ROS impair the IGF1 Akt pathway *in vitro*. (A) Phospho-Akt (upper blot) and total Akt (lower blot) immunoblots of IGF1-treated SH-SY5Y cells exposed or not exposed to 75 μ M glucose oxidase-generated H_2O_2 . (B) Bar graph showing phospho-Akt/total Akt ratios for the different IGF1 doses. #, $P < 0.05$, higher than 0.1 μ M IGF1; \$, $P < 0.05$, lower than the same IGF1 concentration without glucose oxidase. (C) Phospho-Akt (upper blot) and total Akt (lower blot) immunoblots of SH-SY5Y cells exposed to various BV2 serum-free conditioned media supplemented or not supplemented with 10 μ M IGF1. (D) Bar graph showing phospho-Akt/total Akt ratios. *, $P < 0.05$, lower than control and +LPS +APO in the IGF1-treated group. (E) Bar graph showing the H_2O_2 medium concentrations for the different conditions. *, $P < 0.05$, higher H_2O_2 concentration than controls and +LPS +APO. (F) SH-SY5Y survival kinetic over 72-h exposure to various BV2 serum-free conditioned media supplemented or not supplemented with 10 μ M IGF1, APO, apocynin. Cell survival was assessed by counting DAPI-labeled normal nuclei and confirmed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay (data not shown). Data are averages of three or more independent experiments and were compared by ANOVA or repeated-measures ANOVA for the survival kinetic, followed by Newman-Keuls post hoc testing. For the survival kinetic, only LPS and IGF1 + LPS within the 72-h time point are not significantly different from each other ($P = 0.386$).

All cells, not motor neurons only, which are located in the vicinity of activated microglia, may indiscriminately have their plasma membrane proteins and lipids damaged by NADPH oxidase-derived ROS. However, the chronic nature of ALS suggests that neuroinflammation is likely protracted and not as strong as in acute encephalitis. Accordingly, two hypotheses that are not mutually exclusive can be formulated to reconcile the expected nonselective nature of the oxidative stress with the selective demise of motor neurons in ALS. First, at that lower level of ROS production, oxidative stress may not kill cells but instead may accelerate the demise of those already compromised, as motor neurons probably are in ALS. Second, in this paralytic disease, microglial-derived oxidants may preferentially worsen motor neuron degeneration by damaging proteins that are specifically important for their survival. Relevant to the latter scenario are our results for IGF1, a trophic factor that is known to promote motor neuron survival (19–21).

In this study, we indeed found that receptors for IGF1 were primarily expressed on motor neurons in mouse spinal cords (Fig. 8) and that the IGF1 signaling pathway was impaired by a NADPH oxidase-dependent mechanism in symptomatic transgenic SOD1^{G93A} mice (Fig. 4). Although IGF1 *per se* did not seem to be damaged by inflammation, NADPH oxidase did stimulate the oxidative modification of IGF1 receptors (Fig. 4). The ligand-dependent kinase activation of IGF1 receptor relies on its arrangement into a heterotetrameric 2 α /2 β -chain complex (22). It may thus be predicted that oxidation of the IGF1 receptor main extracellular domains (i.e., the α -chains) could distort its proper assembly and transduction activity. Our *in vivo* and *in vitro* results (Figs. 4 and 5), in aggregate, show that several molecular events that are normally elicited by ligation of the IGF1 receptor, including autophosphorylation and Akt phosphorylation, were indeed abated by ROS in a microglial NADPH oxidase-dependent manner.

Our data also show that microglial NADPH oxidase, by impairing the IGF1 signaling pathway, renders SH-SY5Y cells in our *in vitro* system more prone to die upon exposure to a hostile environment such as that emulated by LPS-activated microglial-conditioned medium (Fig. 5). These results thus suggest that

reduction in vital surviving pathways may be one mechanism by which inflammation attenuates the capability of motor neurons to withstand the toxicity of etiologic agents such as mutant SOD1. Muscle-specific expression of IGF1 stabilizes neuromuscular junctions, reduces inflammation in the spinal cord, and enhances motor neuronal survival in transgenic SOD1^{G93A} mice (23). In our study, however, we did not find any evidence that the rescue of the IGF1 pathway by abrogating NADPH oxidase was associated with muscle hypertrophy (Fig. 3). Nevertheless, whether transgenic SOD1^{G93A} mice carrying the gp91^{phox} null mutation reach end-stage paralysis later and exhibit an attenuated neurodegenerative process because of some effects at the skeletal muscle level is an interesting possibility that cannot be excluded.

Injection of transgenic SOD1^{G93A} mice with an adeno-associated virus carrying an IGF1 gene prolongs survival in these animals (20, 24). Together with our data, these previous studies suggest that inflammation may not abrogate, but instead may blunt, the motor neuron survival response to IGF1 in ALS. Perhaps the modest change in ALS progression that is seen in patients treated with human recombinant IGF1 (25) may be related to the issue raised above. It may thus be argued that optimal therapeutic response to IGF1 in diseases such as ALS may rely on a concomitant administration of this trophic factor and an antiinflammatory agent.

Materials and Methods

Animals. Transgenic SOD1^{G93A} mice [C57BL/6J-Tg(SOD1-G93A)1Gur^{dl}] were crossed with gp91^{phox}-deficient mice (B6.129S6-Cybb^{tm1din}). Both lines were obtained from The Jackson Laboratory (Bar Harbor, ME) and maintained in the C57BL/6J strain. Progeny were genotyped by PCR as described in refs. 26 and 27. Refer to *Supporting Text*, which is published as supporting information on the PNAS web site, for details about the timeline of behavioral abnormalities in transgenic SOD1^{G93A} mice.

RNA Extraction and RT-PCR. Total RNA was extracted as described in ref. 28. Primer sequences for gp91^{phox}, glial fibrillary acidic protein, macrophage antigen complex 1, and GAPDH and PCR

