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Electrophilic nitro-fatty acids prevent astrocyte-mediated toxicity to motor neurons in a cell model of familial amyotrophic lateral sclerosis via nuclear factor erythroid 2-related factor activation

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Abstract

Nitro-fatty acids (NO₂-FA) are electrophilic signaling mediators formed in tissues during inflammation, which are able to induce pleiotropic cytoprotective and antioxidant pathways including up regulation of Nuclear factor erythroid 2-related factor 2 (Nrf2) responsive genes. Amyotrophic Lateral Sclerosis (ALS) is a fatal neurodegenerative disease characterized by the loss of motor neurons associated to an inflammatory process that usually aggravates the disease progression. In ALS animal models, the activation of the transcription factor Nrf2 in astrocytes confers protection to neighboring neurons. It is currently unknown whether NO₂-FA can exert protective activity in ALS through Nrf2 activation. Herein we demonstrate that nitro-arachidonic acid (NO₂-AA) or nitro-oleic acid (NO₂-OA) administered to astrocytes expressing the ALS-linked hSOD1^{G93A} induce antioxidant phase II enzyme expression through Nrf2 activation concomitant with increasing intracellular glutathione levels. Furthermore, treatment of hSOD1^{G93A}-expressing astrocytes with NO₂-FA prevented their toxicity to motor neurons. Transfection of siRNA targeted to Nrf2 mRNA supported the involvement of Nrf2 activation in NO₂-FA-mediated protective effects. Our results show for the first time that NO₂-FA induce a

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potent Nrf2-dependent antioxidant response in astrocytes capable of preventing motor neurons death in a culture model of ALS.

Keywords

ALS; astrocytes; motor neurons; heme oxygenase; Nrf2; nitro-fatty acids

INTRODUCTION

Nitro-fatty acids (NO₂-FA, nitroalkenes) are electrophilic products formed by the nitration of unsaturated fatty acids [1]. These species trigger signaling cascades *via* covalent and reversible post-translational modifications of susceptible nucleophilic amino acids in transcriptional regulatory proteins and enzymes. Moreover, NO₂-FA can activate heat shock [2] as well as antioxidant response pathways [3]. As electrophiles, nitroalkenes activate the nuclear factor-erythroid 2-related factor 2 (Nrf2) [2, 4]. Nrf2, a member of the cap “n” collar transcription factor family, represents a master regulator of the Antioxidant Response Element (ARE)-regulated genes. The binding of Nrf2 to the *cis*-acting DNA promoter sequence ARE allows transactivation of a group of cytoprotective genes that encode proteins known as phase-II enzymes. In normal conditions Nrf2 is bound to Kelch-like ECH-associated protein 1 (Keap1), and through a two-site interaction, the transcription factor is ubiquitinated by Cul3/Rbx1 and targeted for degradation [5, 6]. During oxidative stress the two-site interaction between Nrf2 and Keap1 is disrupted, allowing Nrf2 to evade Keap1-mediated ubiquitination and accumulate in the nucleus where it activates genes containing an ARE sequence within their promoters, leading to an induction of antioxidant response. Upregulation of the ARE-driven gene battery has a significant impact on the ability of the cell to withstand and survive inflammatory and metabolic stress [6]. Among phase-II enzymes, hemoxygenase-1 (HO-1) has attracted special attention because of its therapeutic effects in neurodegenerative disease models [7]. Hemoxygenase-1 oxidatively cleaves heme to biliverdin, forms CO and releases the chelated Fe²⁺. Bilirubin (a reduction product of biliverdin) also serves as a potent radical scavenger and protects neuronal cells against oxidative stress at nanomolar concentrations [8]. Two isoforms of heme oxygenase have been characterized: a constitutive isoform, HO-2, and the inducible enzyme, HO-1 [9]. The induction of HO-1 counteracts oxidative damage and confers cytoprotection [10, 11]. In the nervous system, HO-1 can be highly induced in glia by its substrate heme and by a variety of pro-oxidant, inflammatory stimuli and trophic factors [12–15]. In accordance, increased HO-1 expression is observed in neurodegenerative diseases involving glial activation, such as Alzheimer’s and Parkinson’s diseases and Amyotrophic Lateral Sclerosis (ALS) [7, 16].

Amyotrophic Lateral Sclerosis is the most common adult-onset motor neuron disease caused by the progressive degeneration of motor neurons in the spinal cord, brain stem and motor cortex [17]. Approximately 10–20% of familial ALS is caused by a toxic gain-of-function induced by mutations of the Cu/Zn-superoxide dismutase (SOD1) [18]. Rodents over-expressing mutated forms of hSOD1 generally develop an ALS-like phenotype [19, 20]. Although the molecular mechanism underlying this toxic gain-of-function remains unknown, toxicity to motor neurons requires mutant SOD1 expression in non-neuronal cells

as well as in motor neurons [21]. In ALS patients and rodent models, a strong glial reaction typically surrounds degenerating motor neurons [22]. Astrocytes isolated from hSOD1^{G93A} rats [23] or mice [24] are toxic to co-cultured motor neurons, suggesting a role of glial cells in motor neuron degeneration [22, 25–27].

Increased oxidative stress has been implicated in the pathogenesis of ALS and a variety of antioxidants have been tested [28]. Several studies have demonstrated that Nrf2 activation in astrocytes can improve antioxidant defenses and prevent the motor neuron loss induced by SOD1^{G93A} astrocytes [23, 29]. Consistently, it has been shown that ALS-mice with specific Nrf2 overexpression in astrocytes developed the onset of the disease later, increasing survival and exhibiting lower glial reactivity [30]. However, signaling by the Nrf2-ARE pathway may be initiated by several mechanisms not necessarily involving Nrf2 overexpression. In fact, there may be multiple approaches for targeting Nrf2 as therapeutic tool where exogenous and endogenous molecules have demonstrated an ability to activate Nrf2 and induce cytoprotective genes [31]. Furthermore, Nrf2 and HO-1 levels are increased and co-localized with reactive astrocytes in the degenerating lumbar spinal cord of hSOD1^{G93A} rats, suggesting this pathway counteracts accelerated disease progression [15]. On the other hand, astrocytic induction of Nrf2 by *tert*-butylhydroquinone (tBHQ), a prototypic inducer of Nrf2 activation, has also prevented motor neuron death induced by SOD1^{G93A} astrocytes *in vitro* through an increase in glutathione biosynthesis [23]. Considering that prophylactic activation of Nrf2 in astrocytes has shown to be a plausible strategy to ameliorate neuronal dysfunction and death, herein we explored the use of nitro-arachidonic acid (NO₂-AA) and nitro-oleic acid (NO₂-OA) [32, 33] as potential Nrf2 activators in astrocytes and their effects on motor neuron survival.

MATERIAL AND METHODS

Materials

Culture media and serum were obtained from Life Technologies, Inc. Primers were obtained from Integrated DNA Technologies, Inc. Antibody to HO-1 was from StressGen Biotech, antibodies to β -actin was from Sigma, antibodies against carboxyl and amino termini of Nrf2 and histone H1 were from Santa Cruz Biotechnology. Arachidonic acid (AA) and oleic acid (OA) were purchased from Nu-Check Prep (Elysian, MN). All other reagents were from Sigma unless otherwise specified.

Nitro-Fatty Acids

Synthesis and quantitation of NO₂-AA and NO₂-OA were performed as previously and analyzed for purity by 1H NMR and HPLC-MS [32, 33]. A mixture of NO₂-AA isomers was obtained: 12- and 15- NO₂-AA (23%), 9- NO₂-AA (55%) and 14- NO₂-AA (22%). No differences between batches were observed. Nitro-oleic acid is an equimolar mixture of 9- and 10-nitro-octadec-9-enoic acid.

Cell cultures

Primary astrocytes cultures were prepared from non-Tg or hemizygous rats and mice as indicated. Animals: Transgenic ALS Sprague-Dawley rats carrying the G93A mutated

human SOD1, strain NTac:SD-TgN(SOD1^{G93A})L26H, were obtained from Taconic (Hudson, NY; [20]) and were bred locally by crossing hemizygous male carriers to wild-type Sprague-Dawley female rats. Transgenic SOD1^{G93A} ALS mice, strain B6SJL-TgN(SOD1-G93A)1Gur [19], were obtained from Jackson Laboratories (Bar Harbor, ME, USA) and were bred locally by crossing hemizygous male carriers to B6SJLF1 female hybrids. The offspring was genotyped as previously described [34]. The animals were housed under controlled conditions with free access to food and drinking water. **Ethics Statement.** Procedures using laboratory animals were in accordance with the International Guiding Principles for Biomedical Research Involving Animals, as issued by the Council for the International Organizations of Medical Sciences and were approved by the Institutional Animal Committee resolution N° 66 (Exp. N° 071140-001465-10); Comisión honoraria de experimentación animal de la Universidad de la República (CHEA; <http://www.chea.udelar.edu.uy>). The offspring was genotyped at birth as described previously [35]. The non-transgenic littermates were used as controls. Primary cortical or spinal astrocyte cultures were prepared from 1–2 day-old rat pups according to the procedure of Saneto and De Vellis with minor modifications [36]. *Rat cortical astrocytes* were used for cell yield purposes and compared with findings in spinal cord rat o mice astrocytes. Previous reports indicate that astrocyte mediated-motor neuron toxicity is detected in astrocytes from spinal cord and cerebral cortex [34] and also from rats and mice SOD1^{G93A} transgenic rodent models of ALS [37]. Cells were plated at a density of 2×10^4 cells/cm² in 35-mm Petri dishes or 24-well plates and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, HEPES (3.6 g/l), penicillin (100 IU/ml), and streptomycin (100 µg/ml). Astrocyte monolayers were >98% pure as determined by GFAP immunoreactivity and were devoid of OX42-positive microglial cells. **Motor neuron cultures** were prepared from embryonic day 15 (E15) wild-type rat spinal cord by a combination of OptiPrep™ gradient centrifugation and immunopanning with the monoclonal antibody Ig192 against p75 neurotrophin receptor, as previously described [38]. Neurons were directly plated on astrocyte monolayers at a density of 300 cells/cm² and maintained for 72 h in L15 medium supplemented as previously described [36].

Cell Treatment and Transient Transfection

Confluent astrocyte monolayers from cortex and spinal cord were changed to Dulbecco's modified Eagle's medium supplemented with 2% fetal bovine serum prior to treatment. Astrocytes were treated for 24h with vehicle (MetOH, 1:400), AA, OA, NO₂-AA or NO₂-OA, homogenized and then proteins analyzed by Western blot. Transient transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Astrocytes were transfected with mammalian expression control vector (pEF) or dominant-negative mutant Nrf2 (Nrf2-DNM), kindly provided by Dr. Jawed Alam (Alton Ochsner Clinic Foundation, New Orleans, LA) [39]. Post-transfection astrocytes were treated with nitroalkenes as before and protein analyzed by Western blot.

siRNA transfection

Confluent spinal cord astrocyte monolayers were changed to Dulbecco's modified Eagle's medium supplemented with 2% fetal bovine serum prior to treatment. siRNA transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's

instructions. Astrocytes were transfected with 40 nM of predesigned dicer-substrate siRNAs (DsiRNAs) targeting Nrf2 mRNA (ID#RNC.RNAI.N031789.12.1, RNC.RNAI.N031789.12.2, RNC.RNAI.N031789.12.3, IDT) or negative control DsiRNA (DS NC1, IDT) 24 h before either total RNA isolation using TRIzol reagent (Invitrogen) for quantitative PCR, or treatment with vehicle or nitrated fatty acids for co-culture experiments.

Whole Cell and Nuclear Extracts

After treatment, astrocytes were washed with cold PBS and whole cell extracts were prepared in 50 mM HEPES, pH 7.5, 50 mM NaCl, 1% Triton X-100 and Complete protease inhibitor mixture (Roche) and sonicated 3 times for 3 seconds. Protein concentration was measured by the bicinchoninic acid method (Pierce). Nuclear cell extracts were prepared as described by Schreiber and cols. [40]. Briefly, cells were resuspended in ice-cold buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 g/ml aprotinin, and 1 g/ml leupeptin). The cells were allowed to swell on ice for 15 min, after which 0.5% Nonidet P-40 was added and the tube was vortex mixed for 10 s. The homogenate was centrifuged for 30 s and nuclear pellet was resuspended in ice-cold buffer C (20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM each of dithiothreitol, EDTA, EGTA, and phenylmethylsulfonyl fluoride, 1 g/ml aprotinin, and 1 g/ml leupeptin). The tube was mixed thoroughly and vigorously rocked at 4 °C for 15 min. The nuclear extract was centrifuged at 11,000 g for 5 min at 4 °C and the supernatant containing nuclear proteins was removed, quantified and stored in loading buffer for western blot.

Real-time PCR

Confluent astrocyte monolayers were changed to Dulbecco's modified Eagle's medium supplemented with 2% fetal bovine serum prior to treatment. Astrocytes were treated with vehicle, nitrated or non-nitrated fatty acids for 12h. Total RNA was isolated using TRIzol reagent (Invitrogen). 2 µg of RNA were randomly reverse transcribed using SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's protocol. PCRs were carried out in a 20 µl reaction with 1X SYBR Green PCR Master Mix Applied Biosystems containing 1 µl of cDNA and 20 pmoles of each specific primer in aStepOnePlus™ Real-Time PCR System (Life technologies). The cycling parameters were as follows: 95 °C, 10 s; 55 °C, 10 s; 72 °C, 15 s. Specific primers for Nrf2, HO-1, NQO1, GCLC, GCLM and β-Actin were used [30]. Primers for Sulfiredoxin-1 were:

Srxn1/5'GGCTTGGTTACTCTTGTTCCTCT

Srxn1/3'GGGTGCTTTGCTCGAATGTGTTG.

Glutathione measurement

Confluent astrocyte monolayers were changed to Dulbecco's modified Eagle's medium supplemented with 2% fetal bovine serum prior to treatment. Astrocytes were treated with vehicle as control or different concentrations of AA, NO₂-AA, OA or NO₂-OA for 24h. Cells were lysed with ice-cold 3% perchloric acid and total glutathione levels (GSH and GSSG) determined using the Tietze method as previously described [23]. Glutathione content was corrected by protein concentration determined as explained previously.

Co-culture experiments

Non-transgenic as well as SOD1^{G93A} spinal cord astrocyte monolayers were changed to L15 medium supplemented with 0.63 mg/ml sodium bicarbonate, 5 µg/ml insulin, 0.1 mg/ml conalbumin, 0.1 mM putrescine, 30 nM sodium selenite, 20 nM progesterone, 20 mM glucose, 100 IU/ml penicillin, 100 µg/ml streptomycin and 2% horse serum. Astrocytes were treated for 24 h with different concentrations of nitroalkenes. After washing twice with phosphate buffered saline (PBS) wild-type motor neurons were plated on top at a density of 350 cells/cm². Co-cultures were maintained in L15 medium supplemented with 0.63 mg/ml sodium bicarbonate, 5 µg/ml insulin, 0.1 mg/ml conalbumin, 0.1 mM putrescine, 30 nM sodium selenite, 20 nM progesterone, 20 mM glucose, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 2% horse serum for 48 hours. Motor neuron survival was assessed after fixing the cells and immunostained for p75NTR. Counts were performed over an area of 0.9 cm² in 24-well plates and by counting all cells displaying intact neurites longer than 4 cells in diameter [26].

Statistics

All statistics were performed using Sigmaplot 12 (Systat software, San Jose, CA, USA), GraphPad Prism 5.0 or GraphPad InStat software, version 3.06.

RESULTS

NO₂-FA induce Nrf2 activation-dependent HO-1 expression in astrocytes

Cultured non-transgenic (non-Tg) astrocytes were exposed to nitro-arachidonic acid (NO₂-AA) or arachidonic acid (AA) 5µM for 24 h. In these experimental conditions, NO₂-AA-but not AA-induced a significant accumulation of Nrf2 in the nucleus (Figure 1A). As a positive control *tert*-butyl hydroquinone (tBHQ), an electrophilic activator of Nrf2, was included (Figure 1A). In addition, this treatment determined a potent increase in hemoxygenase-1 (HO-1) protein levels (Figure 1B). As expected, HO-1 protein levels were not detected in untreated or non-nitrated fatty acid-treated cells. To further support the role of Nrf2 in HO-1 expression, transfection of astrocytes with a dominant negative Nrf2 plasmid partially abrogated NO₂-AA and tBHQ-induced HO-1 expression (Figure 1C). Furthermore, we compared this effect on SOD1^{G93A}-expressing astrocytes. As expected, NO₂-AA induced HO-1 expression in both non-Tg (Figure 1D) and SOD1^{G93A}-expressing ones (Figure 1E). This effect was not selective for NO₂-AA since the use of nitro-oleic acid (NO₂-OA) instead NO₂-AA produced similar induction of HO-1.

NO₂-FA induce Nrf2/ARE-related genes expression in non-transgenic and SOD1^{G93A} astrocytes

To confirm that the changes observed were not due to increased Nrf2 mRNA levels we performed real-time PCR on astrocytes cultures. Neither NO₂-AA nor NO₂-OA induced changes in the mRNA levels of Nrf2 in either non-Tg or SOD1^{G93A} astrocytes (Figure 2A). Since HO-1 expression depends almost exclusively on *de novo* gene transcription, we analyzed NO₂-FA effects on HO-1 mRNA levels. A 6-fold increase in HO-1 mRNA was observed after treatment with either NO₂-AA or NO₂-OA as compared with vehicle and

non-nitrated fatty acids conditions (Figure 2B). Similar results were observed when analyzing other Nrf2/ARE-related gene expression: NAD(P)H:quinone oxidoreductase 1 (NQO1) and Sulfiredoxin 1 (Srx1, Figures 2C and 2D).

NO₂-FA increase glutathione biosynthesis through the glutamate-cysteine ligase modulatory subunit

The increase in intracellular GSH levels is indicative of the activation of the Nrf2/ARE pathway. Transgenic SOD1^{G93A} astrocytes incubated with either NO₂-AA or NO₂-OA exhibited greater levels of GSH + GSSG compared to control AA and oleic acid (OA) treatments (Figure 3A). This was due to increased expression of the modulatory subunit of glutamate-cysteine ligase (GCLM, Figure 3B) without affecting the expression of the catalytic subunit (GCLC, Figure 3C). Similar results were observed in non-Tg cells (Figure 3).

NO₂-FA inhibit SOD1^{G93A} astrocyte-mediated motor neurons death

A feeder layer of SOD1^{G93A} astrocytes decreased the survival of non-Tg motor neurons by 50% compared with a feeder layer of non-Tg astrocytes (Figure 4A) as previously described [23]. Neuronal survival on top of a feeder layer of non-Tg astrocytes was considered 100% (dotted line in Figure 4A). Pre-treatment of SOD1^{G93A} astrocytes with either NO₂-AA or NO₂-OA before motor neuron plating, prevented motor neuron death induced by SOD1^{G93A} astrocytes (Figure 4A). Control studies showed no effects of NO₂-AA on motor neurons survival when added in the absence of astrocytes (not shown). To find out whether the protective effects of NO₂-FA on SOD1^{G93A} astrocyte-mediated motor neuron death were Nrf2-dependent, we used synthetic siRNAs to induce Nrf2 RNA interference (Figure 4B, C). Transfection of the astrocyte monolayers with Nrf2-siRNA prior to NO₂-FA treatment abolished the beneficial effect of the treatment on motor neuron survival (Figure 4C).

DISCUSSION

Nitro-fatty acids exert pleiotropic anti-inflammatory and adaptive signaling actions, including activation of HO-1 expression in activated macrophages as well as down regulation of nitric oxide synthase 2 (NOS2) expression and inhibition of pro-inflammatory cytokines secretion [32, 41, 42]. Significant inhibition of NADPH oxidase assembly and superoxide production by activated macrophages also occurs [43]. Moreover, NO₂-AA is a non-competitive inhibitor of inducible prostaglandin endoperoxide H synthase (PGHS-2) which, in addition to the suppression of NOS2 expression after inflammatory stimulus can also contribute to the limitation of inflammatory responses [44, 45]. The mechanisms of NO₂-FA incorporation into cells are currently unknown, while fatty acid binding proteins (FABPs) may be involved (unpublished results). Once into cells, NO₂-FA could activate Nrf2 through electrophilic-mediated reversible nitroalkylation reactions. Nrf2 activity is principally governed by Kelch-like ECH-associating protein 1 (Keap1) a protein with elevated cysteine content, which renders it highly reactive to electrophiles. Villacorta et al. demonstrated a direct reaction of NO₂-FA with Keap1 impairing Keap1-mediated inhibition of Nrf2/ARE signaling [3].

It is currently unknown whether NO₂-FA exert actions in neurodegenerative diseases. Herein, we demonstrate a potent protective role of NO₂-FA on astrocytes expressing the ALS-linked SOD1^{G93A} mutation-mediated toxicity to motor neurons. The effects of NO₂-FA on motor neuron degeneration induced by astrocytes revealed that NO₂-FA administration to cultured astrocytes caused a) Nrf2 activation and antioxidant phase II enzymes induction and b) an increase in total glutathione levels. These effects were independent of changes in Nrf2 mRNA levels. Previous work has shown that NO₂-OA exerted potent antioxidant and anti-inflammatory potential through Nrf2 activation [2–4, 42, 46]. Herein, the involvement of Nrf2 in astrocyte activation was demonstrated by the observed increase in Nrf2 translocation to the nucleus in the presence of NO₂-FA as well as by a decrease in HO-1 expression when cells were transfected with a negative dominant plasmid and then exposed to NO₂-AA or NO₂-OA.

Approximately 10%–20% of familial ALS is caused by a toxic gain-of-function induced by mutations of SOD1 [18]. Over-expression of mutated forms of hSOD1 in rodents resulted in animal models of the disease, e.g. hSOD1^{G93A} rats [20] or mice [19]. Toxicity to motor neurons requires mutant SOD1 expression in non-neuronal cells as well as in motor neurons [21]. Increased motor neurons HO-1 expression occurs in the spinal cord from ALS patients [47]. We have previously shown that both Nrf2 and HO-1 levels were increased and co-localized with reactive astrocytes in the degenerating lumbar spinal cord of hSOD1^{G93A} rats [15]. Herein, we show that HO-1 expression in isolated cultured astrocytes from both transgenic and non-transgenic cells increased following exposure to NO₂-FA. NQO1 and Srxn1, two other Nrf2-driven genes, were also induced by nitroalkenes in cultured astrocytes. Primary spinal cord astrocyte monolayers support the survival of purified embryonic motor neurons in the absence of added trophic factors [36], where ~50% of motor neurons die when co-cultured with transgenic astrocytes [36]. Thus, the influence of NO₂-AA or NO₂-OA applied to astrocytes bearing the SOD1^{G93A} mutation on astrocyte-mediated motor neurons death in co-culture conditions was explored. Pre-treatment of SOD1^{G93A} astrocytes with either NO₂-AA or NO₂-OA significantly reduced motor neurons loss at low micromolar levels. This effect was prevented by transfecting astrocytes with a Nrf2-siRNA before NO₂-FA treatment, further supporting that Nrf2 activation is mediating the protective effect of NO₂-FA. Herein, we also demonstrate that an increase in GSH levels in astrocytes may account for the observed protection of motor neurons death. In fact, when SOD1^{G93A} astrocytes were incubated with either NO₂-AA or NO₂-OA, GSH + GSSG levels increased concomitant with an induction of the modulatory subunit of the glutamate-cysteine ligase which catalyzes GSH synthesis.

Besides providing structural and functional support to neurons, neighboring astrocytes also collaborate during the progression of neurological disease displaying high antioxidant capacity [48]. These properties might be due in part to the metabolic interaction between astrocytes and neurons affecting glutathione metabolism. In fact, *de novo* neuronal biosynthesis of GSH depends on the supply of GSH precursors from astrocytes [48]. Increased production and secretion of glutathione by astrocytes is known to protect cocultured neurons from oxidative insults [49]. Moreover, astrocytes are enriched with antioxidant enzymes, such as the ARE-regulated gene HO-1 [50], whose upregulation could protect surrounding neuronal cells from oxidative stress. In addition, enhancing

mitochondrial antioxidants defenses in SOD1^{G93A} astrocytes reverts astrocyte-mediated toxicity [34]. Thus the increase in antioxidant defenses induced by the NO₂-FA treatment could potentially improve mitochondrial function in astrocytes and be partially responsible for the protection observed. These mechanisms may play an important role in NO₂-FA-triggered astrocyte-mediated increase in motor neuron survival.

Although the molecular mechanism underlying the selective death of motor neurons in ALS remains unknown, there is strong evidence that the mechanism is non-cell-autonomous, as the expression of mutant SOD1 in neurons affects disease onset, but glial cells, and in particular astrocytes, play a fundamental role in modulating disease progression [51, 52]. Herein, we show that NO₂-FA induces ARE-driven gene expression as well as astrocytic GSH production, and has a significant protective effect against astrocyte-mediated motor neuron death. However, direct stimulatory effects of NO₂-FA on Nrf2 signaling in motor neurons cannot be discarded with the data presented here and needs further experimentation.

Our results show for the first time that NO₂-FA induce a potent antioxidant response in astrocytes which is dependent on Nrf2 activation and prevents motor neurons death in a culture model of ALS. Overall, our data not only propose NO₂-FA as potential novel therapeutic agents in ALS but also support the role of astrocyte antioxidant defenses in determining motor neuron fate. Considering that the central nervous system is abundant in polyunsaturated fatty acids, it is possible that NO₂-FA being generate as adaptive response during inflammatory conditions to protect motor neurons by the mechanisms reported here. Current work is focusing on demonstrate the neuroprotective role of NO₂-FA by their ability to cross the brain blood barrier and extend the survival of ALS-linked mutant SOD1^{G93A} mice.

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List of Abbreviations

NO₂-FA	Nitro-fatty acids
SOD1	Cu/Zn superoxide dismutase
ALS	Amyotrophic Lateral Sclerosis
NO₂-OA	Nitro-oleic acid
NO₂-AA	Nitro-arachidonic acid
Nrf2	Nuclear factor-erythroid 2-related factor 2
ARE	Antioxidant Response Element
Keap1	Kelch-like ECH-associated protein 1
HO-1	Hemoxygenase-1
tBHQ	<i>tert</i> -butylhydroquinone

AA	Arachidonic acid
OA	Oleic acid
NQO1	NAD(P)H:quinone oxidoreductase 1
Srxn1	Sulfiredoxin 1
GCL	Glutamate-cysteine ligase

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Figure 1. NO₂-FA induce HO-1 expression in astrocytes via Nrf2

(A) Nrf2 activation was determined by western blot of a nuclear extract from astrocytes treated with NO₂-AA for 6h. Controls with AA or tBHQ were included. (B) Confluent astrocyte monolayers were treated with MetOH (vehicle), AA (5 and 10 μM), NO₂-AA (5 and 10 μM) or tBHQ (40 μM). After 24h, HO-1 protein levels were analyzed by western blot. (C) The Nrf2-dependent HO-1 expression was determined by treatment with AA (5μM), NO₂-AA (5 μM) or tBHQ (40 μM) of astrocytes transfected with a dominant-negative mutant Nrf2 form (Nrf2-DNM). As in figure (B), after 24h HO-1 expression was analyzed by western blot. Controls were performed by transfecting the cells with an empty vector (pEF). Band intensities were determined and related to the condition with NO₂-AA. (D) Non-transgenic (non-Tg) and (E) SOD1^{G93A} astrocyte monolayers were treated with MetOH (vehicle), AA (5μM), NO₂-AA (5μM), OA (5μM), NO₂-OA (5μM) as in figure (A). After 24h, HO-1 protein level was determined by western blot. Band intensities were determined and related to the response elicited by tBHQ (A, B, C) or NO₂-AA (D, E). Results shown are representative of at least three independent experiments.

Figure 2. Phase II antioxidant enzymes are induced by NO₂-FA in SOD1^{G93A} astrocytes
(A) Nrf2 mRNA levels in both non-Tg and SOD1^{G93A} astrocytes were determined by real-time PCR, in the absence or presence of 5μM NO₂-AA or NO₂-OA. Controls with the non-nitrated AA or OA were included. **(B–D)** Activation of the Nrf2 pathway by nitro-fatty acids was analyzed by real-time PCR and the mRNA levels of HO-1 (A), NQO1 (B) and Srxn1 (C) are shown. Non-Tg and SOD1^{G93A} cells were incubated with the 5 μM nitrated or non-nitrated fatty acids, as in (A). Results are expressed as the mean ± SD of the fold increase of mRNA respect to vehicle condition, n=5. *: Significantly different from AA and vehicle (p<0.05); #: significantly different from OA and vehicle (p<0.05).

Figure 3. NO₂-FA increase glutathione content in SOD1^{G93A} astrocytes by increasing the Glutamate-cysteine ligase (GCL) modulatory subunit

Non-Tg and SOD1^{G93A} astrocytes were exposed to NO₂-FA as before. (A) GSH + GSSG content in the cells after 24 h of incubation were analyzed. The mRNA levels of both the modulatory (B) and catalytic (C) subunits of the GCL were analyzed by RT-PCR. Vehicle and non-nitrated fatty acids were included as controls. In all cases, data are expressed as the mean $\pm$ SD, n=5. *: Significantly different from AA and vehicle (p<0.05); #: significantly different from OA and vehicle (p<0.05).

Figure 4. NO₂-FA prevent motor neurons death

(A) Purified Non-Tg motor neurons were plated on top of non-Tg or SOD1^{G93A} astrocytes pre-treated as described above. Treatment of non-Tg astrocytes with NO₂-AA, NO₂-OA or tBHQ had no significant effects on motor neuron survival (100%, dotted line). As expected, SOD1^{G93A} astrocytes induced a 50% reduction in motor neuron death respect to non-Tg ones in basal conditions. Motor neuron loss observed in co-culture with SOD1^{G93A} astrocytes was significantly reduced when astrocytes were treated with NO₂-AA or NO₂-OA for 24h before motor neuron plating. Controls with the non-nitrated fatty acids were included without any effect in the survival of the motor neuron cells. *: Significantly different from vehicle and AA (p<0.05); #: significantly different from vehicle and OA (p<0.05). (B) Nrf2 mRNA levels in astrocytes treated with Nrf2-siRNA were determined by real-time PCR. A siRNA that does not target any sequence in the transcriptome was included

as negative control (NC siRNA). *: Significantly different from vehicle and NC siRNA ($p < 0.05$). (C) Motor neuron survival on a feeder layer of SOD1^{G93A} astrocytes treated with NO₂-FA following a pre-treatment with Nrf2-siRNA or NC-siRNA (negative control). Nrf2-siRNA pre-treatment abolished the beneficial effects of NO₂-FA on motor neuron survival. *: Significantly different from vehicle and NO₂-AA+Nrf2-siRNA ($p < 0.05$); #: significantly different from vehicle and NO₂-OA+Nrf2-siRNA ($p < 0.05$). Data are expressed as the mean $\pm$ SEM of at least three independent experiments.