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Cyclo (His-Pro) Protects SOD1G93A Microglial Cells from Paraquat–Induced Toxicity

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Abstract

Cyclo (His-Pro), an endogenous cyclic dipeptide produced by the cleavage of the hypothalamic thyrotropin releasing hormone, crosses the blood brain barrier and improves recovery in models of traumatic injury to the brain and LPS-induced neuroinflammation. The protective effects are sustained by the ability of the cyclic dipeptide to interfere with the Nrf2–NF-κB signalling systems, the former governing the antioxidant and the latter the pro-inflammatory cellular response. Amyotrophic lateral sclerosis is a fatal disease which affects motor neurons and causes death of the patient from respiratory failure within a few years following diagnosis. Most patients suffer from sporadic amyotrophic lateral sclerosis, but about 5–10% of all amyotrophic lateral sclerosis cases can be attributed to familial forms, which are caused by mutations in the gene encoding for superoxide dismutase1. Transgenic mice overexpressing the human gene encoding for superoxide dismutase1 mutated in Gly93-Ala recapitulate several aspects of the disease. By exposing microglial cells overexpressing the mutated human gene superoxide dismutase1 to paraquat, we investigated whether cyclo (His-Pro) is able to alleviate the oxidative stress in a pathological environment. We found that cyclo (His-Pro) was effective in triggering, through Nrf2 activation, the antioxidant response which resulted primarily in the elevation of the intracellular glutathione levels. Intriguingly, we also found that cyclo (His-Pro) acts as a neurotrophic agent by inducing neuronal differentiation in PC12 cells.

Keywords: Amyotrophic lateral sclerosis; Nrf2; NF-κB; MAP kinases; Bdnf

Abbreviations

Akt: protein kinase B; ALS: Amyotrophic Lateral Sclerosis; Bdnf: Brain Derived Neurotrophic Factor; CCM: Cell Conditioned Medium; ERK 1/2: Extracellular-Signal-Regulated Kinases; Gapdh: Glyceraldehyde-3-phosphate-dehydrogenase; Gclc: Glutamate cysteine ligase catalytic subunit; Gclm: Glutamate cysteine ligase modifier subunit; Gdnf: Glial cell line Derived Neurotrophic Factor; gp91phox: Heme-binding membrane glycoprotein gp91phox; GSH: Reduced Glutathione; JNK: C-Jun N-terminal Kinase; LPS: lipopolysaccaride; NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells; Ngf: Nerve Growth Factor; Nqo1: NAD (P)H: quinone oxidoreductase 1; Nrf2: Nuclear factor (erythroid-derived 2)-like 2; PQ: Paraquat; Prdx1: Peroxiredoxin 1; ROS: Reactive Oxygen Species; SOD1: Superoxide Dismutase 1; Tnfα: Tumor necrosis factor alpha; xCT: Cystine/glutamate Transporter.

Introduction

Amyotrophic Lateral Sclerosis (ALS) is a devastating disease affecting motor neurons in the motor cortex, brain stem, and spinal cord, leading to axon degeneration, muscle atrophy, paralysis, and death of the patient from respiratory failure within a few years following diagnosis. Most patients suffer from sporadic ALS, but about 5–10% of all ALS cases can be attributed to familial forms, which are caused by mutations in the gene encoding for Superoxide Dismutase 1 (SOD1) [1]. Transgenic mice overexpressing the human gene encoding for SOD1 mutated in Gly93-Ala (SOD1G93A) recapitulate several aspects of the disease and provide a powerful model system to identify pathophysiological mechanisms associated with ALS and to screen potential therapeutics [2,3]. The mutant SOD1 proteins can either be/become misfolded and consequently oligomerize into increasingly high-molecular-weight species that ultimately lead to the death of motor neurons (oligomerization hypothesis), or catalyze oxidative reactions that damage substrates critical for viability of the affected cells (oxidative damage hypothesis) [4]. Indeed, the pathological mechanism of the disorder likely involves protein aggregation, oxidative stress, excitotoxicity, and mitochondrial dysfunction [1]. These factors eventually lead to loss of neuromuscular junction integrity, retrograde axonal degeneration, and motoneuronal cell death. It is widely accepted that motor neuron death in ALS is not cell autonomous but depends upon active and passive roles for ambient glial cells. Glial cells in the vicinity of motor neurons contribute importantly to ALS pathophysiology [5]. Indeed, spinal astrocytosis and microgliosis accompany the onset of clinical symptoms in transgenic ALS mice [6,7]. Pro-inflammatory cytokines, chemokines, prostaglandins and oxygen radicals accumulate in tandem with reactive gliosis in spinal cords of symptomatic mice [8-10], leading to proposed secreted glial factors being pathogenic in ALS. Cyclo (His-Pro) (CHP), an endogenous cyclic dipeptide produced by the cleavage of the hypothalamic thyrotropin releasing hormone, crosses the blood brain barrier and improves recovery in models of traumatic injury to the brain and LPS-induced neuroinflammation [11-13]. Cyclo (His-Pro) protective effects are

sustained by the ability of the cyclic dipeptide to interfere with the Nrf2–NF-κB signalling systems, the former governing the antioxidant and the latter the pro-inflammatory cellular response [14-16]. Cyclo (His-Pro) can exert cytoprotection against oxidative insults by activating Nrf2- leading to the up-regulation of antioxidant cellular defence [17]. Therefore, a potential therapeutic use of cyclo (His-Pro) against oxidative stress-related disease has already been proposed [12]. Paraquat (PQ) is a redox cycling agent which, by interfering with electron transfer in the mitochondria, produces destructive Reactive Oxygen Species (ROS) and oxidative stress [15,16,18].

Therefore, by exposing microglial cells overexpressing the mutated human gene SOD1G93A to a pro-oxidant agent such as paraquat, we aimed to determine whether cyclo (His-Pro) is able to alleviate the oxidative stress in a SOD1G93A environment.

Materials and Methods

Materials

Cyclo (His-Pro) was synthesized as described elsewhere [19]. All the reagents, unless otherwise stated, were from Sigma-Aldrich (St. Louis, MO). All the antibodies, unless otherwise stated, were from Santa Cruz Biotech (Santa Cruz, CA). Cell culture reagents were from Life Technologies (GibcoBRL, Gaithersburg, MD).

Immortalized microglia

Immortalized microglial cells, obtained from embryonic (E14) cortices from hSOD1G93A mice according to Righi and colleagues [20], were a kind gift of Dr. G. Pietrini (Università di Milano). Microglia were then characterized by Western blot and immunofluorescence for the presence of selective markers (colony stimulating factor 1, CSF-1) and the absence of astrocyte-specific molecules (i.e., glial fibrillary acidic protein, GFAP).

Cell cultures and viability

hSOD1G93A microglial cells were cultured in DMEM F12 supplemented with 5% Foetal Bovine Serum (FBS), glutamine (4 mM), penicillin (50 U/ml), and streptomycin (50 mg/ml) at 37°C in a humidified 5% CO_2 environment. After 24 h subculture, cells were incubated for 24 h with 50 μM cyclo (His-Pro) and then exposed to Paraquat (PQ) for various time. Inhibitors, dicumarol (NAD (P)H: quinone oxidoreductase 1) and apocynin (NADPH oxidase), were added to hSOD1G93A microglial cells, either in the presence or in the absence of 50 μM cyclo (His-Pro), 1h prior to 25 μM PQ exposure. PC12 cells were purchased from ATCC (Manassas, VA) and cultured as described by Minelli et al. [15,16]. After 24 h subculture, cells were exposed to cell conditioned medium (CCM) from hSOD1G93A microglial cells treated for 24 h with 25 μM PQ, either in the presence or in the absence of 50 μM cyclo (His-Pro). Viable cells, stained with Trypan blue, were counted using a hemocytometer. Results were expressed as the percentages of viable cells assuming the viability of control cells as 100%. Cell redox activity was measured using the 3-[4,5-dimethylthiazol-2-yl]-2,5-dephenyl tetrazolium bromide (MTT) reduction assay. The dark blue formazan crystals formed in intact cells were solubilized with lysis buffer (10% sodium dodecylsulfate, 0.01 M HCl) and the absorbance at 550 nm was measured with a microplate reader (Seac, Florence, Italy). Results were expressed as reduced MTT assuming the absorbance of control cells as 100%.

Glutathione determination

hSOD1G93A microglial cells (3×10^5), seeded in 6-well plates, were treated with 50 μM cyclo (His-Pro) for 24h and then exposed to PQ (25 μM) for the indicated time. The concentration of glutathione (GSH) was determined in whole cell lysate after perchloric acid precipitation using the dithionitrobenzoic acid (DTNB) method, measuring the absorbance at 412 nm (molar extinction coefficient 13.6 $mmol^{-1}cm^{-1}$) [21]. GSH levels were expressed as nmoles/mg protein.

Measurement of intracellular fluorescence

The 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) method was used to detect the levels of intracellular ROS [22]. DCFH-DA diffuses into cells, where it is hydrolyzed by intracellular esterase to polar 2′,7′-dichlorodihydrofluorescein. This non-fluorescent fluorescein analogue gets trapped inside the cells and is oxidized by intracellular oxidants to a highly fluorescent, 2′,7′-dichlorofluorescein, and fluorescence intensity is proportional to the amount of oxidant species produced by the cells. hSOD1G93A microglial cells (1×10^4), seeded in 96-well plates, were loaded with DCFH-DA (10 μM) for 30 min at 37°C. The fluorescence of 2′,7′-dichlorofluorescein was detected at 485 nm excitation and at 535 nm emission, using a microplate reader Titertek Fluoroscan II (Flow Laboratories, McLean, VA, USA). Results, expressed as percentage of the control DCF fluorescence, were normalized to cell viability.

Apoptosis determination by flow cytometry

hSOD1G93A microglial cells were incubated with 50μM cyclo (His-Pro) for 24 h and then exposed to PQ for a further 24h prior to propidium iodide (PI) (50 μg/ml in 0.1% sodium citrate plus 0.1% triton X-100) addition. The PI fluorescence of individual nuclei was measured by flow cytometry using standard FACScan equipment (Becton Dickinson, Franklin Lakes, NJ). The data were recorded in a Hewlett Packard (H9 9000, model 310, Palo Alto, CA) computer. The percentage of apoptotic cell nuclei (sub-diploid DNA peak in the DNA fluorescence histogram) was calculated with specific FACScan research software (Lysis II). At least 10,000 events were analysed in each sample.

Real time PCR

Total RNA was isolated with TRIZOL Reagent (Invitrogen Ltd, Paisley, UK) according to the manufacturer's instructions and cDNA was synthesised using iScript cDNA synthesis kit (Bio-Rad Lab, Hercules, CA.). Real time PCR was performed using the iCycler iQ detection system (Bio-Rad) and SYBR Green chemistry. Primer sequences are listed in Table 1. SYBR Green RT-PCR amplifications were carried out in a 96-well plate in a 25 μl reaction volume that contained 12.5 μl of 2x iQ™ SYBR® Green JumpStart™ Taq ReadyMix™, 400 nM forward and reverse primers, and 5 to 40 ng of cDNA. In each assay, no-template controls were included and each sample was run in triplicates. The thermal profile consisted of incubation at 95°C 3 min, followed by 40 cycles of denaturation for 10 s at 95°C and an annealing/extension step of 30 s at 62°C. Mean of Ct values of the stimulated sample was compared to the untreated control sample. ΔCt is the difference in Ct values derived from the target gene (in each assayed sample) and Gapdh, while ΔΔ Ct represents the difference between the paired samples. The n-fold differential ratio was expressed as $2^{-\Delta\Delta\ Ct}$.

Gene name	Gene symbol	Primer sequences (F: forward; R: reverse)
Glyceraldehyde-3-phosphate-dehydrogenase	Gapdh	F. GCCAAATTCAACGGCACAGT
		R. AGATGGTGATGGGCTTCCC
Glutamate cysteine ligase catalytic subunit	Gclc	F. GGCGATGTTCTTGAGACTCTGC
		R. TTCCTTCGATCATGTAACTCCC
Glutamate cysteine ligase modifier subunit	Gclm	F. CACAGGTAAAACCCAATAGTAACCAAGT
		R. GTGAGTCAGTAGCTGTATGTCAAATTGTT
Heme-binding membrane glycoprotein gp91phox	gp91phox	F. TCACCACTAGTACCAGCATCACCA
		R. ACTCTGTCTTGCATTTCTGGATGCC
Cystine/glutamate transporter	xCT	F. CCTGGCATTTGGACGCTACAT
		R. TCAGAATTGCTGTGAGCTTGCA
Peroxiredoxin 1	Prdx1	F. TTGGCGCTTCTGTGGATTCT
		R. GGTGCGCTTGGGATCTGATA
Tumor necrosis factor alpha	Tnfα	F. GCCCACGTCGTAGCAAACCAC
		R. GGCTGGCACCACTAGTTGGTTGT
Nerve growth factor	Ngf	F. GAGCGCATCGAGTTTTGGC
		R. CCTCACTGCGGCCAGTATAG
Glial cell line derived neurotrophic factor	Gdnf	F. GTCTGGCAGCCAACAAACAG
		R. TAGCAGCCACAAAGGGAGTG
Brain derived neurotrophic factor	Bdnf	F. ACTGCAGTGGACATGTCTGG
		R. CTGCAGCCTTCCTTGGTGTA
NAD (P)H: quinone oxidoreductase 1	Nqo1	F. GGCTGGTTTGAGAGAGTGCT
		R. TCTGGAAAGGACCGTTGTCG

Table 1: Primer sequences

Western Blotting analyses

hSOD1G93A total microglial cell lysate was obtained using boiling Laemmli Sample Buffer. hSOD1G93A microglial cells nuclear extract was obtained using NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Rockford, IL) according to manufacturer's instruction. Extracts were loaded on SDS-polyacrylamide gel, transferred on nitrocellulose membrane and immunoblotted with phospho-NF-κB p65 (Ser536) antibody (1:1000), phospho-Akt (Ser 473) antibody (1:1000), Akt antibody (1:1000), phospho-p38 MAPK (Thr 180/Tyr 182) antibody (1:1000), p38 MAPK antibody (1:1000), phospho-p44/42 MAPK (ERK 1/2) (Thr 202/Tyr 204) antibody (1:1000), p44/42 MAPK (ERK 1/2) antibody (1:10000), phospho-SAPK/JNK (Thr 183/Tyr 185) antibody (1:1000), SAPK/JNK antibody (1:1000) (Cell Signalling Technology,Danvers, MA), Nrf2 (C-20) antibody (1:200), and horseradish peroxidase-conjugated -anti IgG antibody (1:5000). Lamin B (C-20) (1:200) and GAPDH (6-C5) (1:500) antibodies were used as marker proteins for nuclear/total extracts. Immunocomplexes were visualized with an enhanced chemiluminescence kit (ECL, Pierce Biotechnology, Rockford, IL).

Actin Labeling

Phalloidin was used to detect filamentous actin (F-actin) content on hSOD1G93A microglial and PC12 cells. Cells, seeded on glass coverslips, were fixed with 4% paraformaldehyde for 20 minutes at room temperature and F-actin was stained with tetramethylrhodamine (TRITC)-labeled phalloidin (1:250) for 30 minutes at room temperature. Cells were washed with PBS and cell nuclei were counter-stained with 4',6-diamidino-2-phenylindole (DAPI). After mounting, the cells were viewed on a DM Rb epifluorescence microscope (Leica, Wetzlar, Germany) equipped with a digital camera.
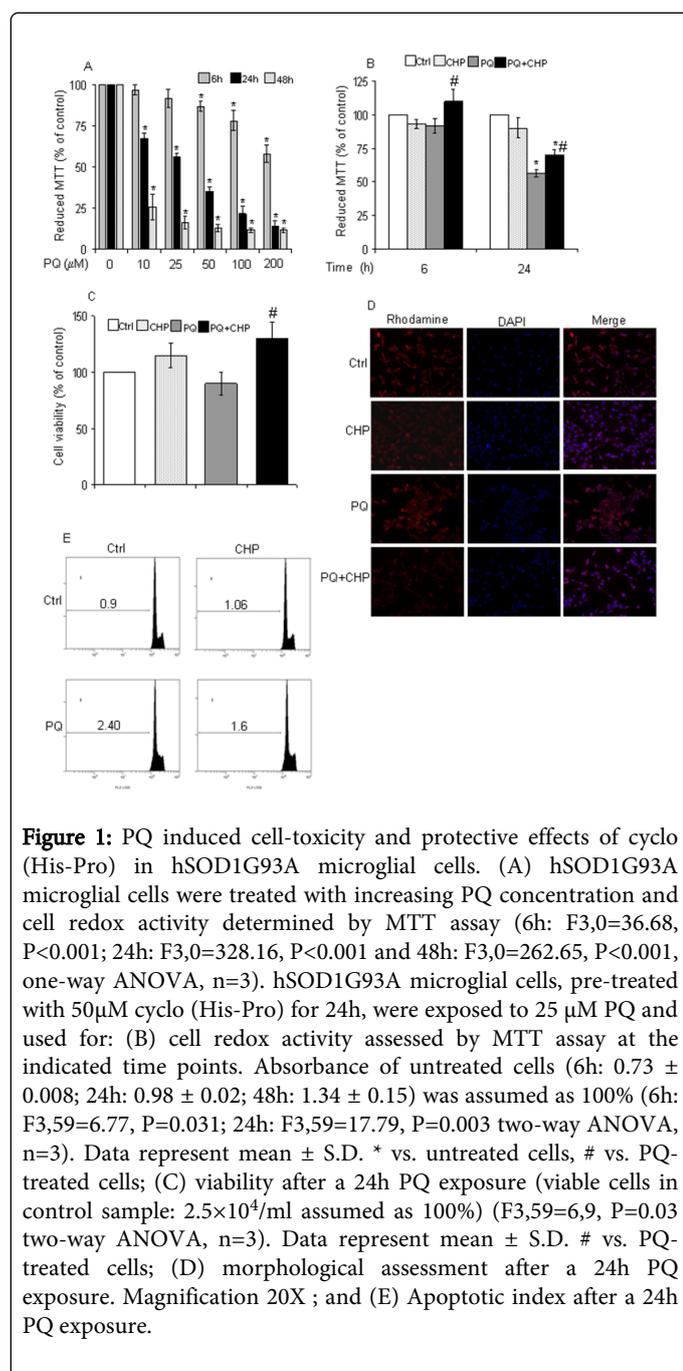
Statistical analysis

All results, confirmed in at least 3 separate experiments, were subjected to one-way or two-way Analyses of Variance (ANOVA). Post-hoc comparisons were performed using Dunnett's tests ($p < 0.05$).
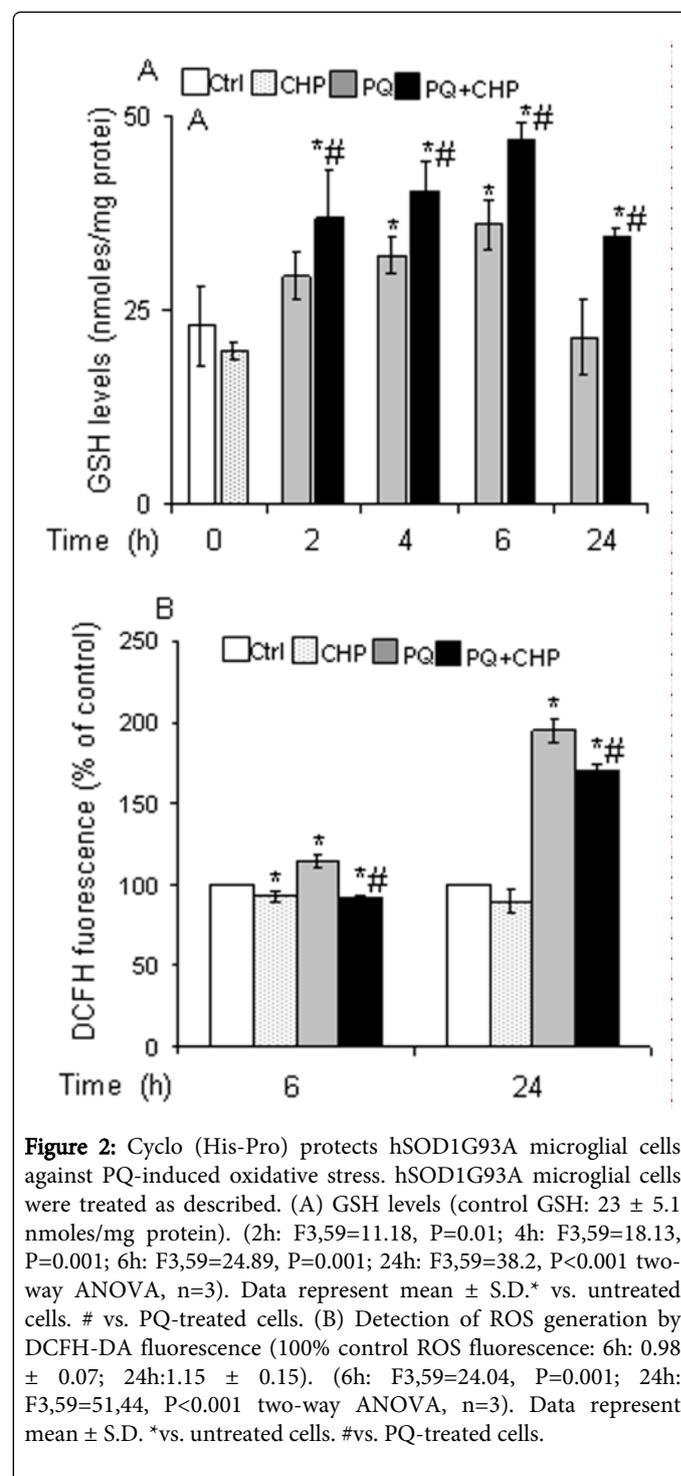
Results

Cyclo (His-Pro) alleviates paraquat toxic effects in hSOD1G93A microglial cells.

To determine whether the cyclic dipeptide (His-Pro) is capable of counteracting the deleterious effects of PQ, we first evaluated PQ toxicity in hSOD1G93A microglial cells (Figure 1A).

Figure 1: PQ induced cell-toxicity and protective effects of cyclo (His-Pro) in hSOD1G93A microglial cells. (A) hSOD1G93A microglial cells were treated with increasing PQ concentration and cell redox activity determined by MTT assay (6h: $F_{3,0}=36.68$, P<0.001; 24h: $F_{3,0}=328.16$, P<0.001 and 48h: $F_{3,0}=262.65$, P<0.001, one-way ANOVA, n=3). hSOD1G93A microglial cells, pre-treated with 50μM cyclo (His-Pro) for 24h, were exposed to 25 μM PQ and used for: (B) cell redox activity assessed by MTT assay at the indicated time points. Absorbance of untreated cells (6h: 0.73 ± 0.008; 24h: 0.98 ± 0.02; 48h: 1.34 ± 0.15) was assumed as 100% (6h: $F_{3,59}=6.77$, P=0.031; 24h: $F_{3,59}=17.79$, P=0.003 two-way ANOVA, n=3). Data represent mean ± S.D. * vs. untreated cells, # vs. PQ-treated cells; (C) viability after a 24h PQ exposure (viable cells in control sample: 2.5×10^4/ml assumed as 100%) ($F_{3,59}=6,9$, P=0.03 two-way ANOVA, n=3). Data represent mean ± S.D. # vs. PQ-treated cells; (D) morphological assessment after a 24h PQ exposure. Magnification 20X ; and (E) Apoptotic index after a 24h PQ exposure.

We found that decreases in cell redox activity were concentration-dependent up to a 24h exposure, whereas, a 48h exposure resulted in a marked decrease in cell redox activity even at very low PQ concentration. In our study we used 25 μM PQ, since a 24h exposure caused the loss of 40% cell redox activity. A 24h pre-treatment with 50 μM cyclo (His-Pro), confirmed as the minimum effective concentration [23], significantly protected the hSOD1G93A microglial cells from PQ-induced toxicity by rescuing cell redox activity (Figure 1B).

Figure 2: Cyclo (His-Pro) protects hSOD1G93A microglial cells against PQ-induced oxidative stress. hSOD1G93A microglial cells were treated as described. (A) GSH levels (control GSH: 23 ± 5.1 nmoles/mg protein). (2h: $F_{3,59}=11.18$, P=0.01; 4h: $F_{3,59}=18.13$, P=0.001; 6h: $F_{3,59}=24.89$, P=0.001; 24h: $F_{3,59}=38.2$, P<0.001 two-way ANOVA, n=3). Data represent mean ± S.D.* vs. untreated cells. # vs. PQ-treated cells. (B) Detection of ROS generation by DCFH-DA fluorescence (100% control ROS fluorescence: 6h: 0.98 ± 0.07; 24h:1.15 ± 0.15). (6h: $F_{3,59}=24.04$, P=0.001; 24h: $F_{3,59}=51,44$, P<0.001 two-way ANOVA, n=3). Data represent mean ± S.D. *vs. untreated cells. #vs. PQ-treated cells.
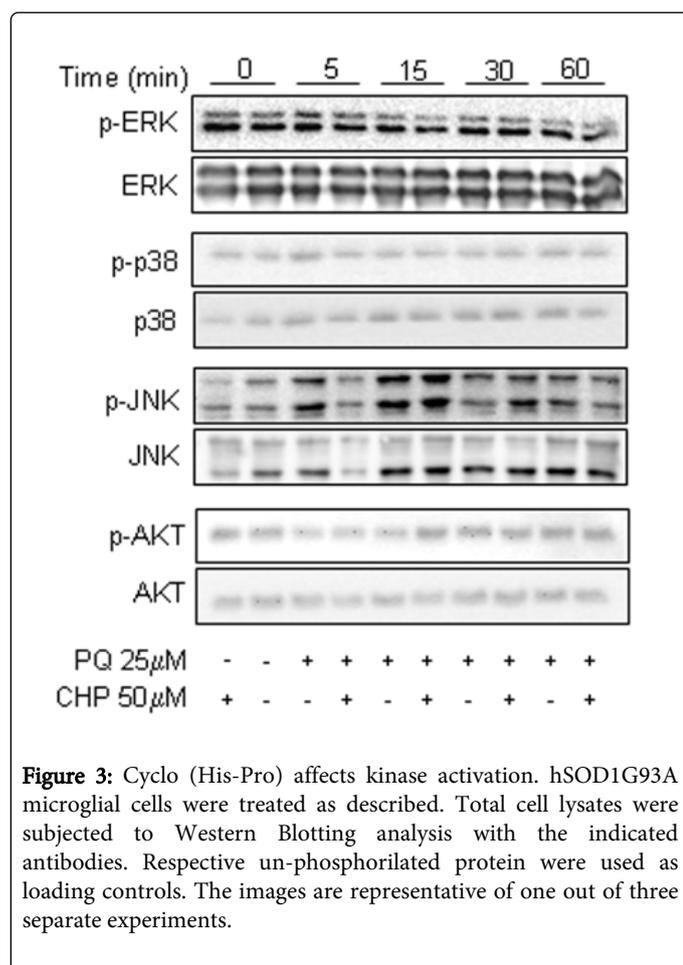
While PQ exposure did not decrease cell viability, cyclo (His-Pro) significantly increased cell viability vs. PQ (Figure 1C). Phalloidin-stained images showed a striking change of microglia shape following

a 24h PQ-exposure (Figure 1D) but cellular morphology nearly reverted to control in mutant microglial cells pre-treated with cyclo (His-Pro), indicating an attenuation of PQ toxicity. PQ did not cause increases in apoptosis (Figure 1E) nor NO production (data not shown). We also observed that cyclo (His-Pro) caused an early and sustained increase in GSH cellular levels (Figure 2A) and significantly decreased PQ-induced ROS generation (Figure 2B).

Cyclo (His-Pro) acts via MAP kinases in hSOD1G93A microglial cells.

We have previously shown that the effects of cyclo (His-Pro) are mediated by the concomitant activation and/or disactivation of various MAPK pathways [23]. To investigate the mechanism of cyclo (His-Pro) at the signalling level, we examined the activation of extracellular-signal-regulated kinases (ERK 1/2), p38 kinase, C-Jun N-terminal Kinase (JNK), and protein kinase B (Akt), known to be involved in the responses to various stressors and in cell survival (Figure 3).

Figure 3: Cyclo (His-Pro) affects kinase activation. hSOD1G93A microglial cells were treated as described. Total cell lysates were subjected to Western Blotting analysis with the indicated antibodies. Respective un-phosphorilated protein were used as loading controls. The images are representative of one out of three separate experiments.

Cyclo (His-Pro) inhibited PQ-induced ERK 1/2 phosphorylation at 15 min without affecting phospho-p38 levels, also unmodified by PQ. On the other hand, the cyclic dipeptide delayed the PQ-induced AKT inactivation and slightly decreased the JNK phosphorylation at 60 min.

Cyclo (His-Pro) activates Nrf2-antioxidant response in hSOD1G93A microglial cells.

We have already provided evidence consistent with the notion that cyclo (His-Pro) exerts cytoprotection by interfering with the Nrf2–NF-κB systems, the former controlling the antioxidant and the latter the pro-inflammatory cellular response [12,14-16]. To verify whether cyclo (His-Pro) uses the same mechanism to relieve the PQ-induced cytotoxic effects in the hSOD1-mutated microglial cells, we analysed the activation of Nrf2 and NF-κB. We found that cyclo (His-Pro) induces a remarkable nuclear translocation of Nrf2 that lasts up to 3h, while not modifying the slight PQ-induced activation of NF-κB (Figure 4A and 4B). Nuclear translocation can be regarded as a marker of activation [24,25].

Indeed, we found that cyclo (His-Pro) induced a robust up-regulation of the mRNA levels of Nrf2-driven genes, such as Gclc, Gclm, xCT, and Nqo1, while no effect was observed on the transcription of the NF-κB–driven genes, such as Tnfα, Prdx1, and gp91phox (Figure 4C and 4D). It is noteworthy that the up-regulation of glutathione synthesising enzymes and cystine-glutamate transporter are in accordance with the increased GSH levels found in cyclo (His-Pro)-treated hSOD1G93A microglia cells.

To establish a causal link between the up-regulation of genes such as Nqo1 by cyclo (His-Pro) and decreased ROS production, we treated hSOD1G93A microglial cells with dicumarol, a NQO1 inhibitor, and with apocynin, an inhibitor of NADPH oxidase activity which generates ROS independent of NQO1. We then determined the level of ROS generation in the presence of PQ and cyclo (His-Pro) (Figure 4E). The inhibition of NQO1 abrogated the cyclo (His-Pro)-induced decreases in ROS production whereas the inhibition of NADPH oxidase did not counteract the cyclo (His-Pro) effects in decreasing ROS generation.

Cyclo (His-Pro) induces brain-derived neurotrophic factor expression in hSOD1G93A microglial cells.

Microglial cells modulate the pathological and /or regenerative state of the brain by producing a variety of physiologically-active substances, such as neurotrophic molecules [26]. To further investigate the beneficial effects of cyclo (His-Pro) on the PQ-stressed hSOD1G93A microglial cells, we determined the transcriptional effects of the cyclic dipeptide on the expression of various neurotrophins (Figure 5A). Although PQ treatment did not alter Bdnf gene expression, the cyclic dipeptide caused a robust up-regulation of the mRNA levels either alone or in combination with PQ. As a functional read-out of the Bdnf up-regulation, we used neuroblastic PC12 cells as a model of neurite outgrowth induced by neurotrophic factors [27,28].

We analysed PC12 neuronal differentiation in the presence of conditioned media obtained by cyclo (His-Pro)-treated hSOD1G93A microglial cells (Figure 5B). Conditioned media obtained from hSOD1G93A microglial cells treated with cyclo (His-Pro) alone caused a drive towards neuronal differentiation in PC12 cells. In addition, conditioned media from hSOD1G93A microglial cells treated with cyclo (His-Pro) before PQ exposure were still able to drive, although at a minor extent, PC12 towards neuronal differentiation.
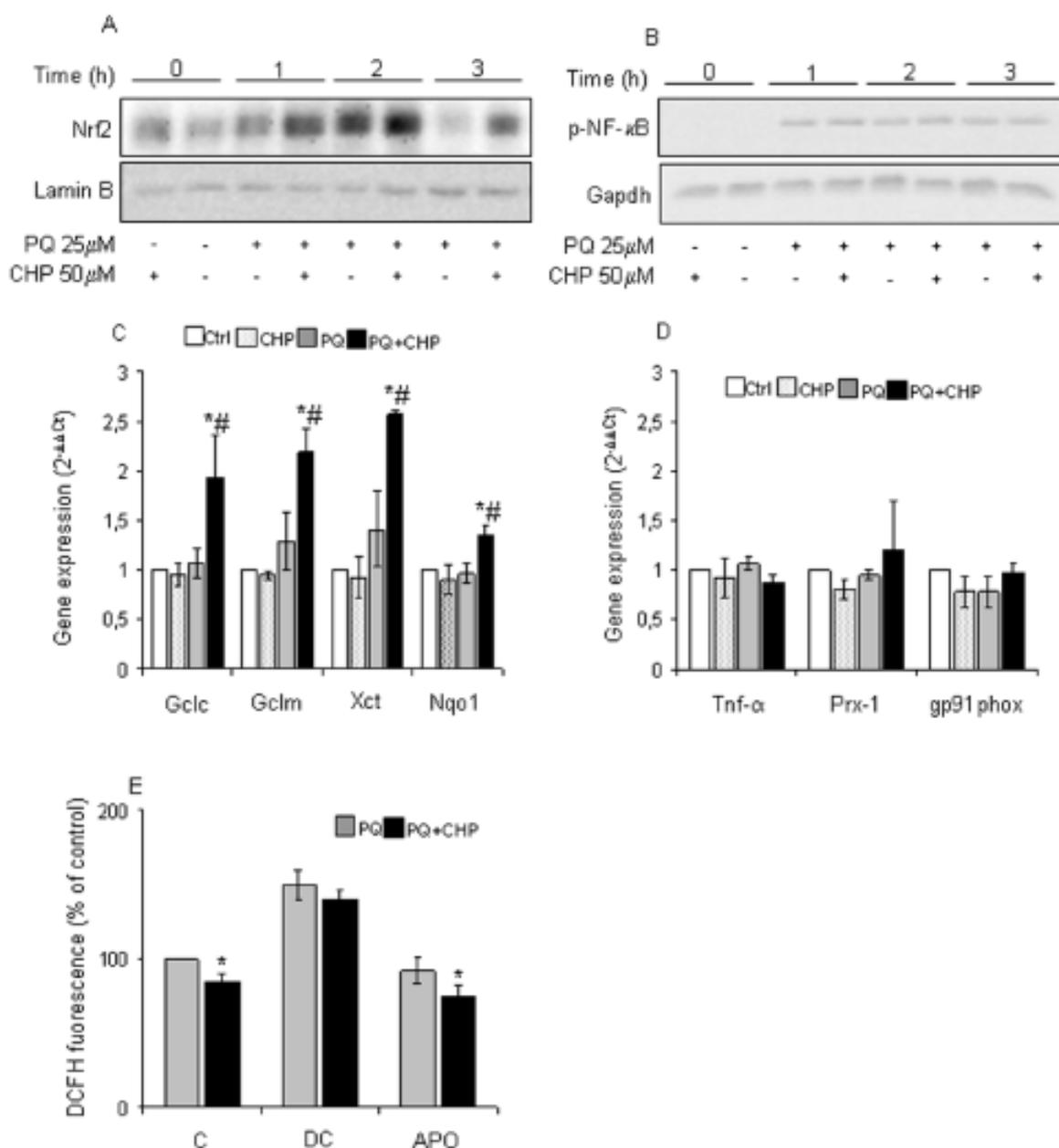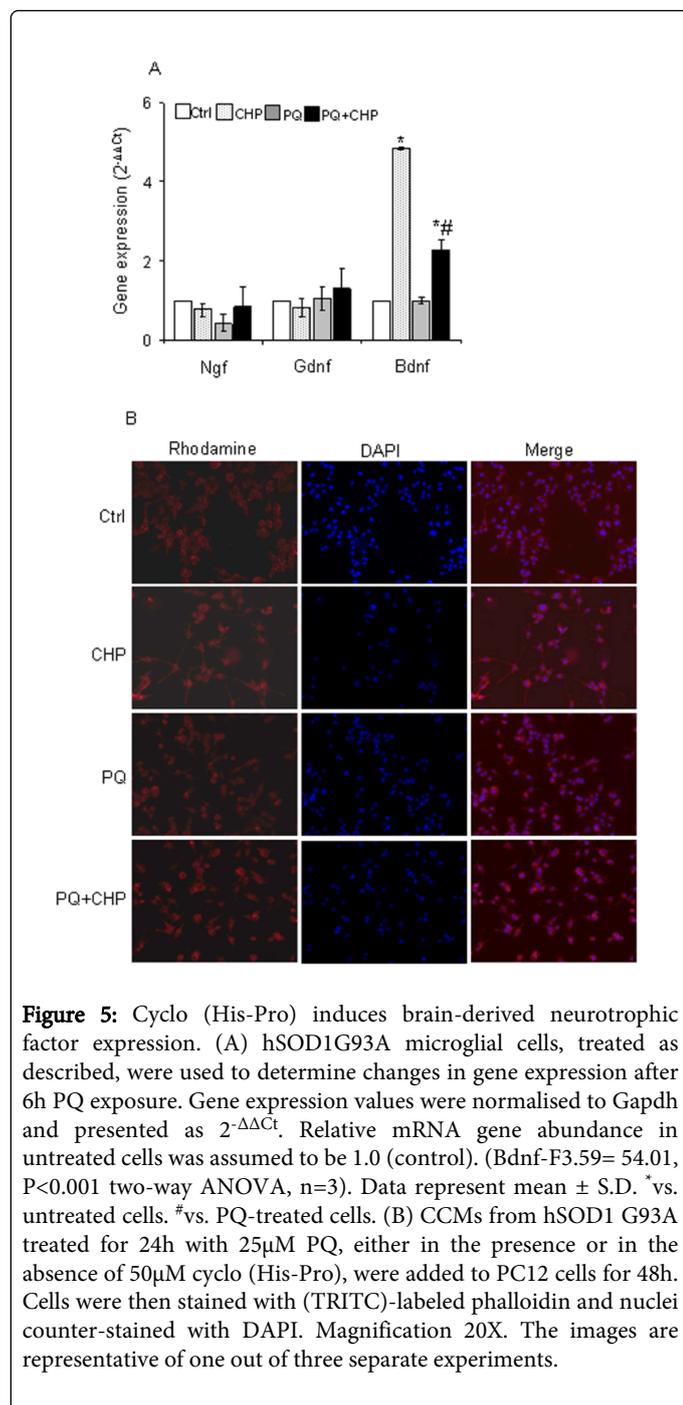
Figure 4: Cyclo (His-Pro) affects transcription factor activation leading to changes in gene expression. (A) and (B) Cells were treated as described and, at each indicated time, cells were collected and nuclear/total extracts were subjected to Western Blotting with indicated antibodies. Anti-Lamin B and anti-GAPDH antibodies were used as marker for nuclear and total extracts, respectively; (C) and (D) Cells, treated as described, were used to determine changes in gene expression after a 6h PQ exposure. Gene expression values were normalised to Gapdh and presented as $2^{-\Delta\Delta Ct}$. Relative mRNA gene abundance in untreated cells was assumed to be 1.0 (control). (Gclc-F3.59=22.12, P=0.002; Gclm-F3.59=23.22, P=0.001; xCt-F3.59= 13.55, P=0.006; Nqo1-F3.59= 5.86, P=0.008; two-way ANOVA, n=3). Data represent mean ± S.D. *vs. untreated cells, # vs. PQ-treated cells. (E) 50 µM dicumarol (DC) and 500 µM apocynin (APO), were used as described in material and methods. ROS generation was detected by DCFH-DA fluorescence after 24h PQ exposure. Fluorescence of PQ-treated cells (1.70 ± 0.08) was assumed as 100%. (C-F6.61=27.21, P=0.007; Apo- F6.61= 7,98, P=0.04; one-way ANOVA, n=3). Data represent mean ± S.D. *vs. respective PQ-treated cells.

Figure 5: Cyclo (His-Pro) induces brain-derived neurotrophic factor expression. (A) hSOD1G93A microglial cells, treated as described, were used to determine changes in gene expression after 6h PQ exposure. Gene expression values were normalised to Gapdh and presented as $2^{-\Delta\Delta Ct}$. Relative mRNA gene abundance in untreated cells was assumed to be 1.0 (control). (Bdnf-F3.59= 54.01, P<0.001 two-way ANOVA, n=3). Data represent mean ± S.D. *vs. untreated cells. #vs. PQ-treated cells. (B) CCMs from hSOD1 G93A treated for 24h with 25μM PQ, either in the presence or in the absence of 50μM cyclo (His-Pro), were added to PC12 cells for 48h. Cells were then stained with (TRITC)-labeled phalloidin and nuclei counter-stained with DAPI. Magnification 20X. The images are representative of one out of three separate experiments.

Discussion

Two major observations are reported in this study. First we showed that, even in the presence of the hSOD1 mutation, cyclo (His-Pro) is still able to act as an antioxidant agent. Second, the striking increase in Bdnf expression induced by cyclo (His-Pro) might lead to the proposal of cyclo (His-Pro) as a neurotrophic agent. A very complex combination of environmental and genetic factors underlies the pathophysiological origins of neurodegenerative disorders. However, in many of these disorders, processes such as inflammation and oxidative stress activate common final pathways leading to toxicity and cellular death. High levels of oxidative damage within the brain and the activation of neuroinflammatory factors are a prominent feature in patients with Amyotrophic Lateral Sclerosis (ALS) [29,30]. Although the brain, isolated from the systemic circulation by the protective blood-brain barrier has been long considered an immune privileged organ [31], the relationship between neuroinflammation and neurodegeneration is now well accepted [32-34]. Moreover, the importance of glial cells in the progression of neurodegeneration has also been recognised, since they migrate to the damaged cells where they clear the dead cell debris. However, in the process, microglia release Reactive Oxygen Species (ROS), pro-inflammatory cytokines, complement factors, and neurotoxic molecules, exacerbating inflammation and leading to further neuronal dysfunction and death [35-42]. Many data from autoptic spinal cord and blood examinations of ALS patients, as well as animal and cellular models, support a role for the immune system in ALS pathogenesis [43]. Paraquat, a widely used herbicide, has strong neurotoxic effects and is one of the major environmental risk factors for Parkinson's disease occurrence [44]. As a redox cycling agent, PQ, by interfering with the electron transfer, produces destructive ROS and oxidative stress with associated toxicity [45-48]. Cyclo (His-Pro), originally discovered in the brain, is an endogenous cyclic dipeptide structurally related to the hypothalamic thyrotropin-releasing hormone [17]. The most intriguing function of this cyclic dipeptide is related to its neuroprotective role, first reported in traumatic injuries of the spinal cord [11], and then confirmed in an in vivo model of LPS-induced reactive gliosis [12]. Nuclear factor-like 2 (Nrf2) is a transcription factor that regulates the constitutive and inducible expression of antioxidant and phase 2 detoxification enzymes via a cis-acting DNA element called electrophile responsive element / antioxidant responsive element (EpRE/ARE) [49]. The Nrf2–ARE pathway represents a physiological adaptation to oxidative stress and its activation is the major mechanism in terminating the NF-κB-driven immune response [13,24,25]. Here we found that the treatment of hSOD1G93A microglial cells with cyclo (His-Pro) markedly reduced PQ–induced ROS production. However, it is worth noting that the production of ROS by hSOD1G93A microglial cells appeared to be a slow process and, at early time points such as a 6h PQ-exposure, only slight increases in ROS levels were observed. We initially assumed that the slow ROS generation could be explained by the fact that the SOD1 enzyme normally functions as a free radical scavenger and many SOD1 ALS mutants have superoxide-scavenging activity comparable with that of wild type SOD1 [50,51]. Moreover, we speculated that the PQ concentration used in the study, although incapable of causing apoptosis, was enough to trigger the antioxidant response by activating the Nrf2 signalling. This process resulted in significantly increased cellular GSH levels at each time-point thus leading to a robust cellular ROS detoxification. In addition, the observed up-regulation of the Nqo1 gene and the fact that cyclo (His-Pro) acts mainly via augmented NQO1 activity to reduce the ROS load might also contribute to the slow increase in ROS production. Data from the literature show that ERK 1/2, JNK and AKT are central to PQ-toxic effects while p-38 is minimally involved in cytotoxicity [46,52-55]. Consistent with these data, we found that, at the signalling level, the protective effects of cyclo (His-Pro) were achieved by inhibiting ERK 1/2 activation and delaying the inactivation of the AKT pathway. On the other hand, the PQ-mediated JNK activation was only marginally altered by the cyclic dipeptide. The discrepancy of our findings with data from other groups might reside in the fact that we used a PQ–concentration which did not cause apoptosis since the robust activation of JNK, which follows exposure to higher concentrations of PQ, drives caspase-3 activation and apoptosis. Thus,

the mutated glial cells used in the study might be responsible for the lack of apoptosis even in the presence of marked early and transient JNK activation. Finally, we showed that cyclo (His-Pro) markedly up-regulated Bdnf expression in hSOD1G93A microglial cells and, in the presence of PQ, the cyclic dipeptide could robustly up-regulate Bdnf gene expression. The fact that cyclo (His-Pro) alone can induce such a strong up-regulation of the Bdnf gene by hSOD1G93A microglia cells might underpin a potentially beneficial use of the cyclic dipeptide in the course of the ALS disease.

In conclusion, we showed: i) an exogenous oxidative stress on hSOD1G93A microglial cells can worsen the known deleterious effects of the mutated microglia cells; ii) the use of cyclo (His-Pro), by diminishing the oxidative burden and triggering the defence response, can, at least partially, attenuate PQ toxicity.

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