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Tyrosol, a simple phenol from EVOO, targets multiple pathogenic mechanisms of neurodegeneration in a *C. elegans* model of Parkinson's disease

Calahorra J. García-Moreno [a], Montserrat Porta de la Riva [b], Esther Martínez-Lara [a], Eva Siles [a], Ana Cañuelo [a,*]

[a] Biochemistry and Molecular Biology Section, Department of Experimental Biology, University of Jaen, Jaen, Spain
[b] Cancer and Human Molecular Genetics, C. elegans Core Facility, Bellvitge Biomedical Research Institute-IDIBELL, L'Hospitalet de Llobregat, Barcelona, Spain

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ABSTRACT

Parkinson's disease (PD) is a common neurodegenerative disorder involving α-synuclein (α-syn) aggregation, oxidative stress, dysregulation of redox metal homeostasis, and neurotoxicity. Different phenolic compounds with known antioxidant or antichelating properties have been shown to also interfere with aggregation of amyloid proteins and modulate intracellular signaling pathways. The present study aims to investigate for the first time the effect of tyrosol (TYR), a simple phenol present in extra-virgin olive oil, on α-syn aggregation in a *Caenorhabditis elegans* model of PD and evaluate its potential to prevent α-syn toxicity, neurodegeneration, and oxidative stress in this model organism. Our results show that TYR is effective in reducing α-syn inclusions, resulting in a lower toxicity and extended life span of treated nematodes. Moreover, TYR delayed α-syn-dependent degeneration of dopaminergic neurons *in vivo*. TYR treatment also reduced reactive oxygen species level and promoted the expression of specific chaperones and antioxidant enzymes. Overall, our study puts into perspective TYR potential to be considered as nutraceutical that targets pivotal causal factors in PD.

1. Introduction

Aggregation of the presynaptic protein α-synuclein (α-syn) in dopaminergic (DA) neurons of the *substantia nigra pars compacta* is one of the main toxic mechanisms in the pathogenesis of Parkinson's disease (PD). Under physiological conditions, α-syn is found in its free disordered cytosolic native form, but under cellular stress, α-syn can also appear as an α-helix-rich form with a high propensity to aggregate (Burré et al., 2013; Fauvet et al., 2012). Progressive accumulation of this protein in the form of oligomers and subsequent fibrils results in the formation of cytoplasmic inclusions known as Lewy bodies, also including proteins such as ubiquitin and chaperone Hsp70, among others (Sharma and Priya, 2017; Wakabayashi et al., 2007). Recently, studies have pointed to α-syn oligomers and protofibrils as the true toxic species that trigger neurodegeneration and suggest that the formation of Lewy bodies could constitute a cytoprotective mechanism in PD (Ingelsson, 2016; Winner et al., 2011).

Reactive oxygen species (ROS) in DA neurons have been strongly associated to the development of PD (Dias et al., 2013). In contrast to other brain regions, the *substantia nigra* is especially susceptible to attack by ROS, considering its additional oxidative stress burden due to dopamine metabolism, a lower antioxidant level, a high composition in fatty acids prone to peroxidation and its high iron concentration which enhances ROS generation through Fenton reaction (Reeve et al., 2014; Reynolds et al., 2007; Zhao et al., 2017). In addition, elevated ROS levels in neurons is known to affect α-syn aggregation into toxic oligomers, leading to pathological events such as synaptic dysfunction, mitochondrial inhibition, and interruption of correct chaperone function (Dias et al., 2013). Altogether, these factors finally converge in neurodegeneration and cell death (Schildknecht et al., 2013). The use of antioxidant therapies has been shown to promote either protection or slowdown of cell death progression in PD (Filograna et al., 2016; Liu et al., 2007; Wang et al., 2006).

The invertebrate model *C. elegans* provides several appealing advantages to investigate the connection between oxidative stress,

* Corresponding author at: Biochemistry and Molecular Biology Section, Department of Experimental Biology, University of Jaén, Campus Las Lagunillas, 23071 Jaén, Spain. Tel.: +34953212767; fax: +34953211875.
E-mail address: acanuelo@ujaen.es (A. Cañuelo).

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α-syn aggregation, and toxicity. In this sense, transgenic strains that reproduce distinct aspects of PD on human α-syn expression in muscle cells or in DA neurons have been developed in the last decade (Harrington et al., 2012; Van Ham et al., 2008). Moreover, its transparent body enables in vivo visualization of dopaminergic neurodegeneration and α-syn inclusions in body wall cells, making it a simple but useful system to investigate both cell loss and α-syn aggregation in PD (Chakraborty et al., 2013).

In the last decades, several phenolic compounds present in olive leaf and fruit have shown important beneficial effects against different pathologies including neurodegenerative disorders, although the mechanisms involved in the latter are not clearly defined yet (Coccia et al., 2016; Grossi et al., 2013; Luccarini et al., 2014; Rosillo et al., 2014; Santangelo et al., 2016; Vauzour et al., 2010). In previous studies, our group has reported a prolongevity effect of tyrosol (TYR), one of the main polyphenols in olive leaf and fruit, also present in extra-virgin olive oil, using *wild-type* strains of *C. elegans* (Cañuelo et al., 2012, 2015a; Cañuelo and Peragón, 2013). Although we have described interesting biological effects of this phenol in delaying aging and protecting from thermal and oxidative stress, its therapeutic potential in a neurodegeneration context has not been examined before in this model organism.

In this study, we have analyzed the effect of TYR on α-syn aggregation using a transgenic strain of *C. elegans* which constitutively overexpresses this human protein in body wall cells thus exhibiting a progressive paralysis phenotype. We have also evaluated the effect of TYR on neurodegeneration in vivo using a *C. elegans* strain that expresses α-syn exclusively in DA neurons, which show accelerated degeneration over time. Finally, we have measured TYR effect on both life span and ROS level and determined the expression of specific chaperones and antioxidant enzymes in this experimental model to assess its potential protective role in PD pathogenesis.

2. Materials and methods

2.1. Strains and growth conditions

The *C. elegans* strains N2, NL5901 (pkIs2386; unc-54p: α-syn:YFP + unc-119 (+)) and UA44 (bal11; Pdat-1:α-syn, Pdat-1:gfp) used in this study were obtained from the CGC (Caenorhabditis Genetics Center). The strain NL5901 overexpresses α-syn:YFP within worm body wall muscle cells and also exhibits age-dependent mobility defects associated with α-syn:YFP aggregation, which can be easily monitored. The strain UA44 expresses α-syn:GFP under the control of the dopamine transporter *dat-1* promoter, causing an age-dependent neurodegeneration of DA neurons. Worms were propagated at 21 °C either on solid nematode growth media (NGM) seeded with the *Escherichia coli* strain OP50 (Brenner, 1974) or in liquid media (S-complete medium) as described by Solis & Petrascheck (Solis and Petrascheck, 2011).

2.2. Treatment with TYR

For all the analysis, TYR (2-(4-hydroxyphenyl) ethylalcohol); (Extrasynthese, France) was dissolved in DMSO and added to its final concentrations to NGM previously autoclaved and cooled to 50 °C. The media was immediately dispensed into Petri dishes that were kept protected from light and stored at 4 °C until use. For liquid media experiments, TYR dissolved in DMSO was added directly to S-complete medium. A final DMSO concentration of 0.1% (v/v) was maintained in all experimental groups. All the experiments involving TYR were always carried out in parallel with a control group that contained only DMSO.

2.3. Life span assays

The screening procedure was carried out as previously described (Solis and Petrascheck, 2011). Briefly, L1-synchronized adult worms were grown in S-complete medium containing 5 mg/mL *E. coli* OP50 and a mixture of penicillin-streptomycin 1% (v/v). An aliquot of the worm suspension was transferred to each well of a 96-well plate, mixed for 2 minutes and incubated for 45 hours at 20 °C until the animals reached the L4 stage. The animals were sterilized by adding 30 μL of a stock solution of 0.6 mM fluorodeoxyuridine (FUDR) to each well. After 24 hours, on day 1 of adulthood, TYR was added at 4 different concentrations ranging from 50 μM to 1 mM, the plates were sealed and incubated at 20 °C. Every 7 days, 5 μL of the *E. coli* OP50 (100 mg/mL) were added to each well. The number of surviving animals was monitored daily until death. Nematodes were considered to be dead when they did not respond to a mechanical stimulus with a platinum wire or no pharyngeal pumping was observed. An average of 120 nematodes were used per experimental condition.

2.4. Quantification of aggregates

TYR was added to the NGM of synchronized L4 worms and maintained during the whole assay. At the 6th day of adulthood, worms (n = 60/treatment) were mounted in a 5 μL drop of 10 mM levamisole (Sigma) on a 3% agarose pad, covered with a 24 × 24 mm coverslip and observed under confocal microscopy to visualize fluorescent α-syn inclusions in the head region. Confocal microphotographs were obtained with Leica TCS SP5 II and equal adjustment of brightness and contrast on control and matched experimental images was done using confocal software LAS-AF. For a more accurate quantification of individual inclusions, z-stack overlay images were analyzed using Image J software. An average of 20 worms per experimental condition were analyzed.

2.5. Paralysis assay in liquid culture

Synchronized populations were obtained using hypochlorite extraction. Worms were grown on solid media up to day 1 of adulthood. FUDR 0.12 mM was added when worms reached the L4 stage. At adult day 1, N2 or NL5901 worms per well were transferred to S-medium with OP50 *E. coli* (optical density 0.5) in a flat-bottom 96-well plate at 20 °C. TYR 1 mM was added and locomotion under control and treatment conditions was assessed for 90 minutes after 3, 7, 9, and 11 days using WMicrotracker (Phylumtech, Santa Fe, Argentina). A total of 180 worms were used per experimental condition.

2.6. Preparation of worm protein extracts and western blotting

For protein extraction, 20 worms per condition were manually collected at the 6th day of adulthood and pooled in 15 μL of Laemmli buffer. After freezing in liquid nitrogen, the mix was heated at 95 °C for 10 minutes. Proteins were separated by SDS-PAGE in a 4%–20% gradient polyacrylamide gel (BioRad) and transferred to Immobilon P PVDF membranes (Millipore). For α-syn:YFP detection, blots were probed with anti α-syn monoclonal antibody (Invitrogen, Camarillo, CA) at a 1:1000 dilution. An anti-rabbit secondary antibody at a 1:5000 dilution was used (Sigma-Aldrich). Actin was used as loading control. ECL Prime kit (GE Healthcare Life Sciences) was used for signal detection, following manufacturer's instructions.

2.7. DA neurons degeneration assay

For neurodegeneration assay, the *C. elegans* transgenic strain UA44 (bal11; Pdat-1:α-syn, Pdat-1:gfp) was used. TYR was added to the NGM media of synchronized L4 worms and maintained during the whole assay. Worms were transferred to fresh treatment plates with OP50 every 2–3 days. On the 14th day of adulthood, nematodes were placed on glass slides with 10 μL of levamisole and GFP was visualized with a CX 31 (Olympus) fluorescence microscope fitted with a camera (C-7070 Wide Zoom, Olympus). The integrity of the six anterior DA neurons, four cephalic (CEP) and two anterior deirid (ADE), was evaluated for neurodegeneration according to previously described criteria (Berkowitz et al., 2008). Briefly, animals with complete GFP fluorescence in all 4 CEP and 2 ADE neurons were scored as *wild-type*, whereas individual animals exhibiting loss of DA neurons or signs of degeneration such as axonal blebbing were scored as non–*wild-type* or *degenerating*. An average of 40 worms per experimental condition were analyzed.

2.8. Measurement of ROS

The 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) assay have been adapted from a previously described protocol (Wang and Joseph, 1999). Briefly, L1 synchronized worms were grown in S-complete medium (10–15 worms per 20 μL) containing 5 mg/mL *E. coli* OP50. In L4 stage, worms were sterilized by adding 0.6 mM FUDR and after 24 hours, on day 1 of adulthood, TYR was added. On adult day 6, ten worms per well were transferred to a 96-well plate with 75 μL of PBS per well. Subsequently 25 μL of DCF-DA 150 μM dissolved in PBS buffer was added and immediately fluorescence was measured for 125 minutes at 10 minutes intervals, using excitation and emission λ at 485 nm and 535 nm, respectively (Synergy HT, BioTek). An average of 60 worms per experimental condition were analyzed.

2.9. RNA isolation

Synchronized nematodes were collected at the 6th day of adulthood, washed 3 times in M9 buffer and pelleted by centrifugation for RNA isolation. After centrifugation, worms were resuspended in 350 μL of TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), flash frozen in liquid nitrogen and thawed at 37 °C three times for disruption and total RNA was purified using the RNeasy Mini kit (Qiagen, Venlo, the Netherlands) following the manufacturer recommended protocol. Final volume of isolated RNA was 50 μL per biological sample.

2.10. Real-time qPCR

Briefly, cDNA was synthesized from 1 μg of total RNA using the Maxima First Strand cDNA Synthesis Kit for real-time qPCR (Thermo Scientific, Waltham, MA, USA). qPCR was performed in a CFX384 Touch Real-Time PCR Detection System (Bio-Rad) using Kapa SYBR FAST qPCR kit (Kapa Biosystems, Wilmington, MA, USA) with 1 μL cDNA in a 10 μL reaction volume using the following gene-specific oligonucleotide primers: *hsp-70* (NM_060084.2), CGTTTCGAAGAGCTCTGTGCTGATCTTTTCCGC (F) and TTAATCAACTTCCTCTACAGTAGGTCCTTGTGG (R); *hsp-4* (NM_063135), GCAACCAAGATGCCTCTACTG (F) and CCTCCCGCCGAGTAAAGTTT (R); *hsp-12.6* (NM_069267.1), ATGATGAGCGTTCCAGTGATGGCTGACG (F) and TTAATGCATTTTTCTTGCTTCAATGTGAAGAATTCC (R); *hsp-16.1* (NM_072953.3), GTCACTTTACCACTATTTCCGTCCAGCTCAACGTTC (F) and CAACGGGCGCTTGCTGAATTGGAATAGATCTTCC (R); *gst-4* (NM_069447.5), TTTTCTATGGAAGTGACGCTGA (F) and TTTTCTATGGAAGTGACGCTGA (R); *sod-3* (NM_078363.5) CAAACCAGGATCCTTTGGAA (F) and TGGCAAATCTCTCGCTGATA (R); *sod-1* (NM_001026785.2), TGGATCACACAGAAGTCCGA (F) and GGAATCCATGAAGACCGGGA (R); *gpx-1* (NM_060197.1), CAAAGAATTGCTCGATGTGTACA (F) and TGCATCGAACATGAATCCACC (R); *gpx-2* (NM_001306618.1), GGAGCTCCTCGATGTGTACA (F) and ACCACCGAATTGATTGCACG (R); *ctl-1* (NM_064578.4), TCCATTTCAAGCCTGCTCAAG (F) and AATGGCATTGAACAGGTCGC (R); *ctl-2* (NM_001027302.5), GCTGAGGTTGAACAATCCGC (F) and AAGGCGGTGGAAATGAGTGT (R); *ama-1* (NM_068122.6), AAGCTATAGCCCTTCGTCGC (F) and CGAGGATGGAGTGTACGTCG (R). All samples were run in triplicate. Cycle thresholds of amplification, expression levels of the target genes normalized to the housekeeping gene *ama-1* and relative FC for transcripts were calculated using the CFX Manager (Bio-Rad).

2.11. Statistical analysis

Data are expressed as mean values ± SEM of at least three independent experiments. Statistical comparisons between the different experimental groups and their corresponding controls were made with Student's *t*-test or log-rank test, accepting $p < 0.05$ as the level of significance, using GraphPad Prism 6 software (GraphPad Software Inc).

3. Results

3.1. TYR extends life span in a C. elegans model of PD

In a previous study by our group, we described that TYR was able to induce a significant life span extension in a *wild-type C. elegans* strain (Cañuelo et al., 2012). In the present study, we have used the transgenic strain NL5901 as a *C. elegans* model of PD. Overexpression of α-syn in body-wall cells of this strain has been reported to induce significant toxicity and reduced life span, as well as impaired motility and pharyngeal pumping as compared with the *wild-type* N2 strain (Bodhicharla et al., 2012). Thus, we decided to start our study by analyzing if TYR was also capable of stimulating longevity in this transgenic strain. With this aim, we performed life span assays adding four different TYR doses to the NGM. As shown in Fig. 1, TYR at 1 mM concentration exerted a significant life span increase in worms compared with control group (mean survival days: control 18.67 ± 0.33 vs. TYR 1 mM 21.33 ± 0.66; *t*-test $^{*}p = 0.0232$), supporting our previously reported TYR effects on *C. elegans* longevity. However, lower TYR doses did not induce significant increases in survival.

3.2. TYR decreases the amount of α-syn inclusions without affecting its expression

The transgenic *C. elegans* strain NL5901 used in this study, constitutively expresses YFP-fused human α-syn protein in body wall cells, allowing in vivo quantification of fluorescent inclusions of this protein. Thus, we also chose this strain to study the effect of TYR treatment on α-syn aggregation. As shown in Fig. 2A–B, by adult day 6, a pronounced decrease of α-syn inclusions was observed in the 1 mM TYR group as compared with nontreated control nematodes (n° aggregates/animal: control 58.72 ± 3.47 vs. TYR 1 mM 22.63 ± 5.17; *t*-test $^{**}p = 0.0044$). Nevertheless, no changes in total α-syn:YFP levels were observed (Fig. 2C). These results suggest that TYR supplementation protects against the aggregation of α-syn, a critical process in PD development in humans.

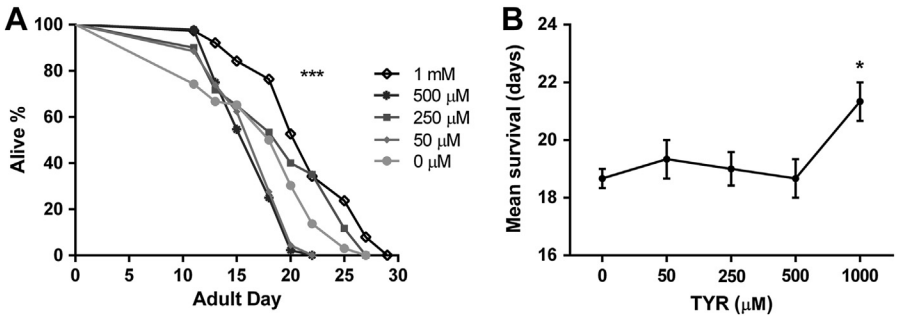

Fig. 1. (A) Representative graph of a life span assay at different TYR doses on NL5901 nematodes cultured in 96-well plates at 22 °C. Nematodes were treated with TYR from adult day 1 until death and monitored for survival. The median survival for 0 μM, 50 μM, 250 μM, 500 μM, and 1 mM tyrosol were (days): 19, 18, 20, 20, and 22, respectively. 1 mM TYR dose exerted a significant increase in life span as compared with vehicle-treated nematodes (median survival days: control 19 vs. TYR 1 mM 22; $^{***}p < 0.0005$; log-rank test). (B) Mean survival values from three independent experiments showing that 1 mM TYR significantly extends the life span of NL5901 strain (control 18.67 ± 0.33 vs. TYR 1 mM 21.33 ± 0.66; *t*-test $^{*}p = 0.0232$). Abbreviations: TYR, tyrosol.

3.3. *Motility impairment in NL5901 strain is ameliorated in response to TYR*

α-syn toxicity in NL5901 strain is associated with its aggregation in body wall cells causing age-dependent mobility defects which can be monitored (van Ham et al., 2010). As shown in Fig. 3, NL5901 strain exhibits a premature and progressive decline in body movement compared with the wild-type strain N2 (Fig. 3A). This decline becomes more evident by adult day 7 in both, control, and TYR treated NL5901 nematodes. However, by adult day 9, 1 mM TYR treated nematodes exhibited a significantly faster body movement as compared with untreated controls (n° of activity counts per 30 minutes: control 7.7 ± 1.81 vs. TYR 1 mM 20.9 ± 5.62; *t*-test $^{*}p = 0.0364$) (Fig. 3B). By adult day 11, both groups showed a similar level of body movements, close to 100% paralysis. These results may be related to the lower amount of α-syn inclusions shown earlier in

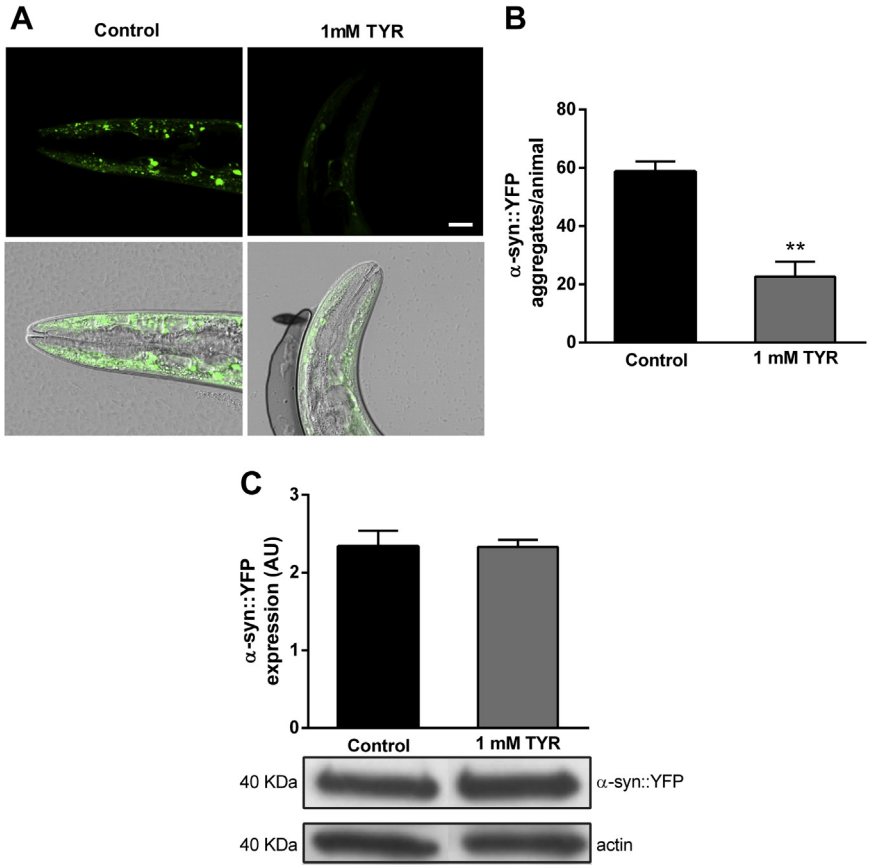

Fig. 2. (A) Confocal microscopy images showing α-syn:YFP aggregates in muscle cells of the head region of 6-day adult NL5901 worms grown in the presence of 0 μM or 1 mM TYR (scale bar 20 μm). (B) Quantification of α-syn:YFP aggregates (size above 6 μm^2) per worm in each experimental condition (control 58.72 ± 3.47 vs. TYR 1 mM 22.63 ± 5.17; *t*-test $^{**}p$ value = 0.0044). (C) Western blot densitometry showing α-syn:YFP levels in TYR-treated worms at the same experimental conditions as in (A–B). All lanes were loaded with equal protein extract from synchronized 6-day adult NL5901 worms. Actin was used as loading control. No significant differences in the total amount of α-syn:YFP were found. Abbreviations: TYR, tyrosol.

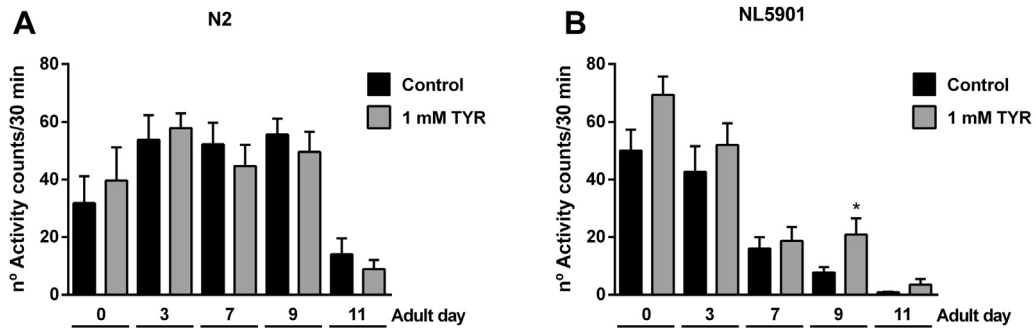

Fig. 3. Effect of TYR treatment on worm locomotion quantified as number of activity counts in 30 minutes. 1 mM TYR was added on day 1 of adulthood and locomotion under control and treatment conditions was assessed after 3, 7, 9, and 11 days. (A) In N2 (*wild-type*) strain no significant differences were observed in locomotion after TYR treatment and paralysis started between days 9 and 11 of the assay. (B) In NL5901 strain, paralysis started earlier than in the *wild-type* strain (between days 3 and 7). TYR treatment delayed worm paralysis compared with nontreated worms. This difference was more evident by day 9 (control 7.7 ± 1.81 vs. TYR 1 mM 20.9 ± 5.62; *t*-test $^{*}p = 0.0364$). Abbreviations: TYR, tyrosol.

treated worms, supporting the notion that TYR treatment could prevent α-syn toxic effects by interfering with its cytoplasmic aggregation process in this model organism.

3.4. TYR delays dopaminergic neurodegeneration in a C. elegans model of PD

DA neurons are the main neuronal type affected in PD (Spillantini et al., 1998). To investigate if the observed effect of TYR on α-syn aggregation could be also extrapolated to a *C. elegans* model of DA neurodegeneration induced by α-syn, we used the transgenic strain UA44 (bal11; Pdat-1:α-syn, Pdat-1:GFP). The expression of human α-syn under the control of the dopamine transporter *dat-1* promoter in this strain causes an age-dependent neurodegeneration of DA neurons, allowing the easy identification of these neurons by the simultaneous expression of GFP (Ray et al., 2015). Under our culture conditions, we established that degenerative changes were more evident in nontreated worms at adult days 12–14, when approximately 40% of nematodes have their 6 anterior DA neurons intact. Treatment with 1 mM TYR induced a significant increase in the percentage of nematodes with intact neurons at 14th day of adulthood as compared with controls (% wild-type animals: control 45.33 ± 3.52 vs. TYR 1 mM 80 ± 2.30; *t*-test $^{**}p = 0.0012$) (Fig. 4). Thus, our data show that TYR is also protective against α-syn-dependent neurodegeneration in vivo.

3.5. TYR decreases ROS level in a C. elegans model of PD

Oxidative stress is thought to be an underlying mechanism for the pathology of PD in both sporadic and familial forms. In line with this, increases in the oxidized lipids, proteins, and DNA were observed in the brain of patients with PD (Hwang, 2013). In a previous study, we showed that TYR treatment was able to promote oxidative stress resistance induced by paraquat in *wild-type C. elegans* strains (Cañuelo et al., 2012). Herbicides and pesticides such as rotenone, paraquat, and maneb are commonly used as PD models that result in increased ROS production and altered mitochondrial energetics (Blesa et al., 2012). In a recent study, Dewapriya et al.

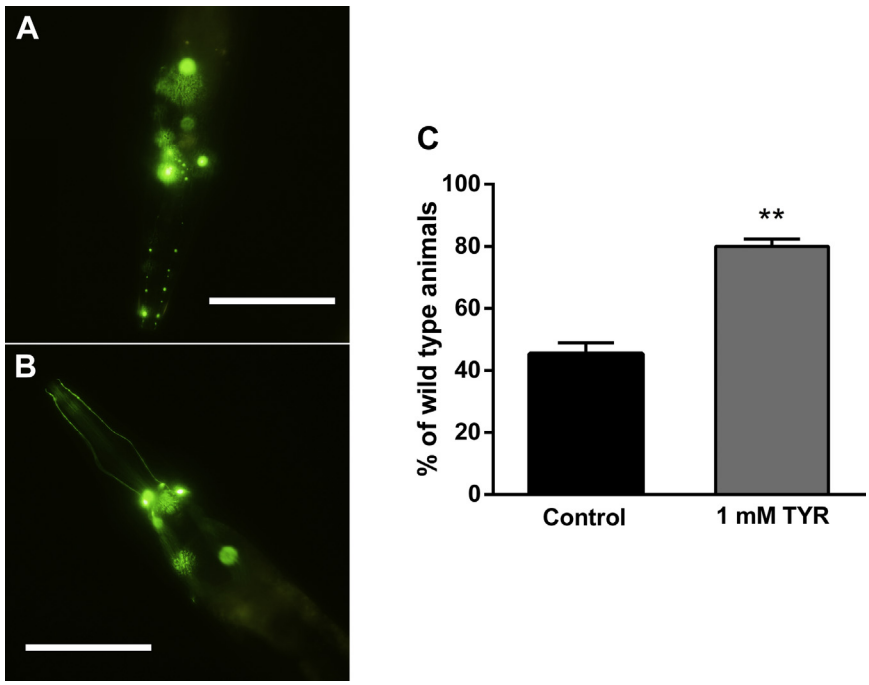

Fig. 4. (A–B): GFP expression pattern in DA neurons of transgenic *C. elegans* (UA44) at adult day 14. A: Untreated control; B: TYR treated nematode (1 mM). Scale bar, 50 μm. (C) Percentage of UA44 worms that had the six anterior DA neurons (CEP and ADE) intact, expressed as *wild-type* animals, at day 14. The graph represents the average of three independent assays (Control 45.33 ± 3.52 vs. TYR 1 mM 80 ± 2.30; *t*-test $^{**}p$ value = 0.0012). Abbreviations: TYR, tyrosol; DA, dopaminergic; CEP, cephalic; ADE, anterior deirid.

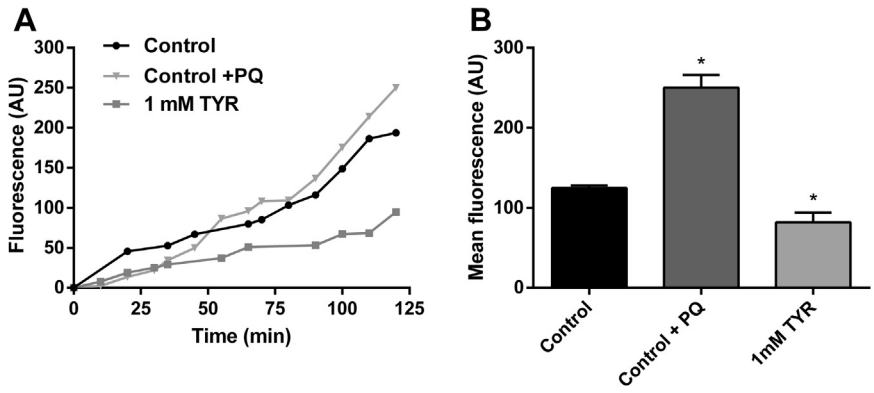

Fig. 5. (A) Effect of 1 mM TYR on ROS level in NL5901 *C. elegans* strain measured by DCF fluorescence. Paraquat was used as a control of oxidative stress. TYR treatment induces a lower accumulation of intraworm ROS levels as revealed by DCFH-DA fluorescence over time. (B) Mean fluorescence at the end point of three DCF assays showing a significant decrease in ROS level in TYR-treated nematodes compared to controls (control 124.5 ± 3.5 vs. TYR 1 mM 82 ± 12.06; *t*-test $^{*}p = 0.0347$). Abbreviations: TYR, tyrosol; ROS, reactive oxygen species.

reported a protective effect of TYR treatment against MPP+-induced cytotoxicity in mouse brain–derived catecholaminergic neuron cells by suppressing the oxidative and nitrosative stress in these cells (Dewapriya et al., 2013). Thus, we decided to assess whether TYR could lower the intracellular ROS level in NL5901 nematodes by using CM-H2DCFDA, a fluorescent probe that reacts with ROS. As expected, paraquat treatment used as positive control, induced a marked increase in intraworm ROS levels compared with control group (fluorescence AU: control 124.5 ± 3.5 vs. control + PQ 250 ± 16.17; *t*-test $^{*}p = 0.0132$) (Fig. 5A and B). However, pretreatment with TYR significantly reduced ROS accumulation compared with control (control 124.5 ± 3.5 vs. TYR 1 mM 82 ± 12.06; *t*-test $^{*}p = 0.0347$) and with paraquat-treated worms, confirming that this phenol has an effective antioxidant effect in this *C. elegans* strain.

3.6. The reduction of α-syn accumulation by TYR is accompanied by the upregulation of specific chaperone and antioxidant enzymes

In *C. elegans*, coordinated induction of genes involved in antioxidant/heat-shock response and detoxification results in a longer life span and an improved proteostasis (Honda and Honda, 1999; Johnson et al., 2002). To investigate the molecular mechanism for TYR protective effect in an α-syn-overexpression scenario, we performed qPCR analysis of several known oxidative/heat stress response genes in NL5901 transgenic nematodes.

Worms were cultured with 0 or 1 mM TYR from L1 stage. On day 5 of adulthood, the mRNA level of target genes was analyzed using qPCR. We detected a significant increase in the chaperone genes *hsp-70* (relative mRNA levels: control 1 ± 0.30 vs. TYR 1 mM 4.2 ± 0.85; *t*-test $^{**}p = 0.0037$), *hsp-12.6* (control 1 ± 0.05 vs. TYR 1 mM 2.038 ± 0.36; *t*-test $^{*}p = 0.047$) and *hsp-16.2* (control 1 ± 0.15 vs. TYR 1 mM 1.934 ± 0.32; *t*-test $^{*}p = 0.011$) in response to TYR as compared with controls (Fig. 6). In addition, the antioxidant gene *gst-4* (control 1 ± 0.056 vs. TYR 1 mM 1.64 ± 0.15; *t*-test $^{*}p = 0.017$) was also significantly upregulated in response to TYR treatment. However, TYR did not affect the expression of other genes analyzed (*hsp-4*, *sod-3*, *sod-1*, *ctl-1*, *ctl-2*, *gpx-1*, and *gpx-2*).

4. Discussion

In previous studies, we had shown that TYR supplementation was able to induce life span increases and promote stress resistance in the model organism *C. elegans* (Cañuelo et al., 2012). Thus, we next sought to assess the effect of this simple phenol in transgenic *C. elegans* strains that express human α-syn and recapitulate key pathogenic processes in PD.

To determine the most effective TYR concentration in the *C. elegans* strain NL5901, we first performed life span assays in parallel adding TYR at different doses ranging from 50 μM to 1 mM. We observed a moderate increase in life span at the higher dose of TYR (1 mM). Recently, different phytochemicals have been also shown to promote longevity in this strain (Govindan et al., 2018; Jadiya et al., 2011; Ji et al., 2016) indicating a clear connection between life span and α-syn expression in these nematodes. Although significant, the observed life span increase promoted by TYR in this strain was less prominent compared with the one previously described in *wild-type* nematodes and this effect was achieved at a higher TYR dose (1 mM instead of 250 μM) (Cañuelo et al., 2012); these differences suggest that TYR may act through additional cellular mechanisms in the context of α-syn expression that could influence its effect on longevity in this specific strain.

One of the main findings in this study is that TYR treatment is able to substantially reduce the amount or formation of α-syn inclusions in a well-studied *C. elegans* model of PD. This effect was accompanied by a delay in the onset of motility dysfunction induced by α-syn in this model. These results, together with the increased survival observed in this PD strain, support a positive effect of TYR supplementation in reducing α-syn-associated toxicity in vivo. In recent studies, different plant extracts have been shown to also reduce α-syn aggregation in the *C. elegans* strain NL5901 (Chalorak et al., 2018; Jadiya et al., 2011; Ji et al., 2016). Although the precise mechanisms underlying their effect remain to be clarified, it has been suggested that it may involve binding to α-syn monomers and oligomers via hydrophobic interaction with β-structures, which leads to delay of the nucleation process and further assembly of toxic oligomers (Ji et al., 2016). During the last decade, much investigation has focused on small natural molecules, rich in aromatic groups (such as polyphenols) as amyloid inhibitors with very variable results. In this sense, different natural polyphenols such as apigenin, curcumin, epigallocatechin-3-gallate among others have been proven effective inhibiting α-syn misfolding and aggregation in vitro (Dhouafli et al., 2018). Nevertheless, their capacity to prevent DA neurons degeneration in vivo has not been evaluated in depth. Regarding TYR, although there have been a few studies suggesting protective effects of this phenol in PD (Dewapriya et al., 2013; Vauzour et al., 2010), none of them have assessed the direct effect of this phenol in DA neurons expressing α-syn in vivo. In this sense, our study using the *C. elegans* strain UA44 provides another relevant result, showing for the first time that TYR treatment is able

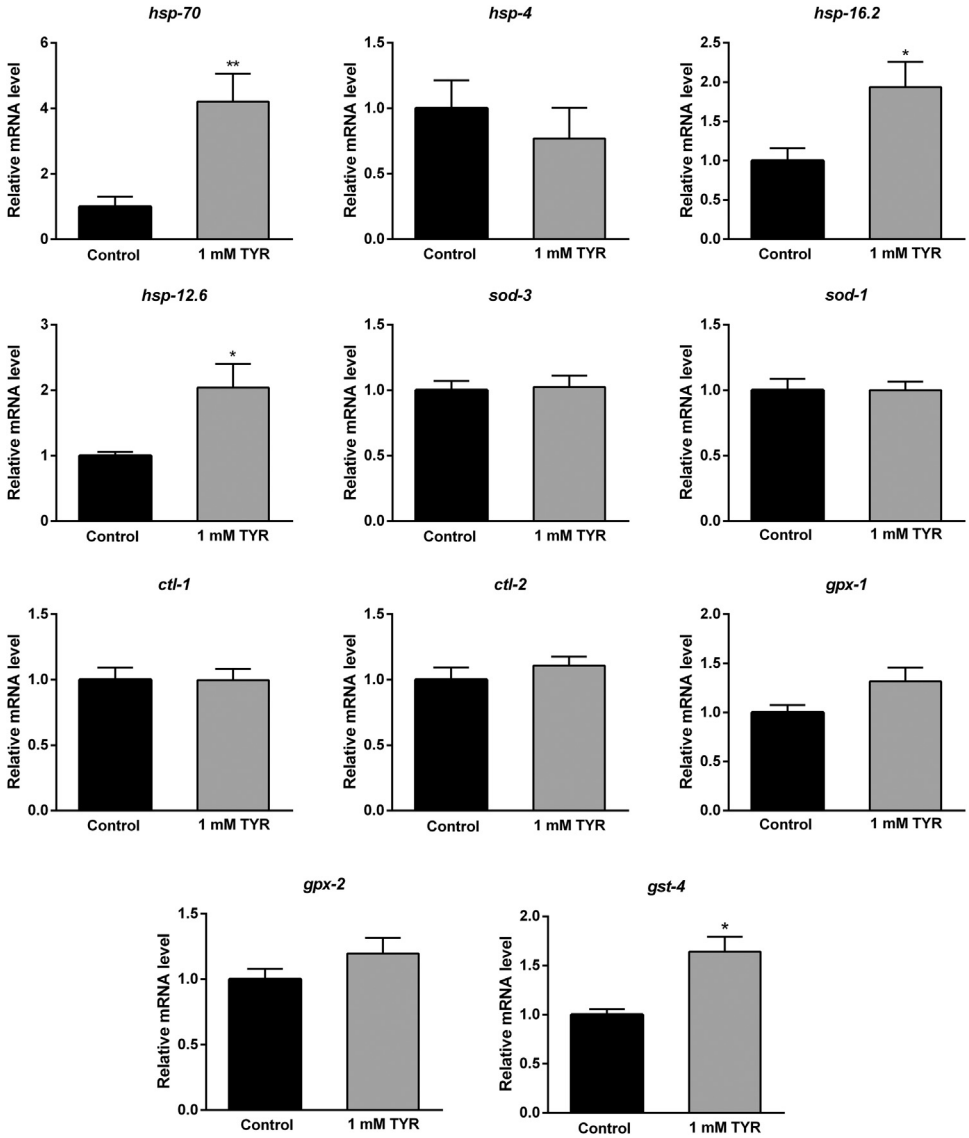

Fig. 6. Effect of TYR on the mRNA expression of *hsp-70*, *hsp-4*, *hsp-16.6*, *hsp-12.2*, *sod-3*, *sod-1*, *ctl-1*, *ctl-2*, *gpx-1*, *gpx-2*, and *gst-4*. Results are expressed as normalized mRNA expression relative to control worms using *ama-1* as housekeeping gene. *hsp-70* (control 1 ± 0.30 vs. TYR 1 mM 4.2 ± 0.85; *t*-test $^{**}p = 0.0037$), *hsp-12.6* (control 1 ± 0.05 vs. TYR 1 mM 2.038 ± 0.36; *t*-test $^{*}p = 0.047$), *hsp-16.2* (control 1 ± 0.15 vs. TYR 1 mM 1.934 ± 0.32; *t*-test $^{*}p = 0.011$), *gst-4* (control 1 ± 0.056 vs. TYR 1 mM 1.64 ± 0.15; *t*-test $^{*}p = 0.017$). Abbreviations: TYR, tyrosol.

to induce a significant delay in dopaminergic neurodegeneration associated with α-syn expression in a model organism. This effect on neurodegeneration is probably related to the observed decrease in α-syn aggregation also induced by TYR in *C. elegans*, suggesting the potential of this simple phenol to reduce the toxicity of α-syn in a dopaminergic environment.

Fibrils of α-syn in Lewy bodies and the formation of aggregates have been associated with an increased oxidative stress (Dias et al., 2013). In this sense, it has been suggested that oxidative conjugation of dopamine with α-syn protein inhibits its transition from protofibrils to mature fibrils, leading to the potential accumulation of cytotoxic soluble protofibrils in DA neurons. Moreover, the addition of antioxidants has the ability to reverse the formation of these adducts, suggesting that catechol oxidation can contribute to the accumulation of α-syn protofibrils (Chinta and Andersen, 2008; Conway et al., 2001). Experiments both in vivo and using cultured cells suggest that ROS are able to induce α-syn aggregation, which in turn increases ROS, creating a vicious cycle leading to neurodegeneration (Nieto et al., 2006; Tabrizi et al., 2000). In this sense, our results showing an effective decrease of intraworm ROS accumulation after TYR treatment suggest that the reported reduction of α-syn inclusions may also be related to the ability of TYR to act as an ROS scavenger. Thus, TYR could ameliorate the cellular oxidative environment that contributes to the formation of toxic α-syn species.

It has been reported that, when overexpressed *in vivo* and *in vitro*, the molecular chaperone *hsp-70* is able to reduce the amount of misfolded, aggregated α-syn protein, also protecting from its toxicity (Klucken et al., 2004). Hence, the reduction in α-syn inclusions observed in TYR-treated nematodes could be related to the detected increase in this chaperone expression. On the other hand, *hsp-16.2* and *hsp-12.6* are members of the 16 kDa and 12 kDa families of small heat shock proteins (smHSPs) known to function as molecular chaperones in *C. elegans*, preventing the aggregation of denatured proteins and guiding misfolded proteins to refold to their native state (Horwitz, 1992; Leroux et al., 1997). In particular,

hsp-16.2 is likely to function as a passive ligand temporarily preventing unfolded proteins from aggregating and it has been shown to interact with intracellular human beta amyloid peptide, a primary component of the extracellular plaques found in Alzheimer's disease (Fonte et al., 2008). The expression of smHSPs is activated by transcription factors HSF-1 and DAF-16 in response to stress, in turn promoting *C. elegans* longevity (Hsu et al., 2003). Interestingly, we have previously reported that TYR supplementation is able to induce specific smHSP expression both, at the mRNA and protein level in *C. elegans*, and this effect was associated to an increased life span and stress resistance in *wild-type* strains (Cañuelo et al., 2012, 2015b; Cañuelo and Peragón, 2013). Moreover, we described that this effect was partially dependent on HSF-1. GST-4 (glutathione transferase-4) is involved in the phase II oxidative stress response and several studies have reported that specific GSTs are able to modulate the response to oxidative stress in *C. elegans* as mediated by SKN-1 (Park et al., 2009). Thus, overexpression of this gene increased resistance to oxidative stress in this animal model (Ayyadevara et al., 2005; Leiers et al., 2003). The fact that TYR induces the expression of this antioxidant gene in α-syn expressing nematodes, may explain the lower ROS level observed as well in TYR-treated animals.

In summary, although further analyses should be conducted to narrow down the molecular mechanisms involved, our study reveals important and novel biological activities of TYR, a simple phenol naturally present in the typical Mediterranean diet, that would act in a synergic manner to prevent or delay the main pathogenic causal factors in PD. Moreover, our results provide an interesting source for molecular docking analyses using TYR structure as a model molecule in drug design aimed to discover new PD neuroprotective therapies.

5. Conclusions

The present study reports for the first time that dietary supplementation with TYR is able to significantly reduce the amount of α-syn aggregates in a *C. elegans* model of PD and, more importantly, delay the onset of dopaminergic neurodegeneration *in vivo*. In parallel, TYR ameliorated the toxic effects of α-syn aggregation on nematode mobility, also promoting a significant life span increase. Moreover, our results show that TYR treatment induces an effective decrease of cellular ROS level in the *C. elegans* strain used, supporting a protective effect of this phenol against oxidative stress in vivo. These results were concomitant to the upregulation of the antioxidant gene *gst-4* as well as chaperones *hsp-70*, *hsp-12.6,* and *hsp-16.2*. In conclusion, although we cannot rule out the involvement of other mechanisms in the observed TYR effects in this PD model, it seems quite feasible that TYR neuroprotective effects may result from combining direct inhibition of α-syn aggregation, chaperone modulation, and ROS scavenging, making it a suitable candidate as nutraceutical compound in PD.

Disclosure

On behalf of all the authors, the corresponding author states that there is no conflict of interests.

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