Hydroxytyrosol improves mitochondrial energetics of a cellular model of Alzheimer's disease

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Abstract

Mitochondrial energetic deficit is one of the hallmarks of neurodegenerative disorders, e.g. Alzheimer´s disease (AD). Adherence to a Mediterranean diet is associated with lower incidence of cognitive decline and AD and extra virgin olive oil's (poly)phenols such as oleuropein and hydroxytyrosol (HT) are being actively studied in this respect. In this study, we assessed the effects of HT on mitochondrial energetic dysfunction in the 7PA2 cells cellular model, i.e. one of the best cellular models of Aβ toxicity with a well-characterized mitochondrial dysfunction typically observed in AD. We report an increase of new mitochondria at 8 hours post HT-treatment, which was followed by higher mitochondrial fusion. Further, ATP concentrations were significantly increased after 24 hours of treatment with HT as compared with controls.

Our data suggest that HT may revert the energetic deficit of a cellular model of AD by potentiating mitochondrial activity. Because HT is being proposed as dietary supplement or component of functional foods, future studies in appropriate animal models and - eventually - humans are warranted to further investigate its potential neuroprotective actions in AD.

Keywords: hydroxytyrosol, mitochondria, Alzheimer´s disease

1. INTRODUCTION

Mitochondrial energetic deficit is an intracellular dysfunction shared by many neurodegenerative disorders, e.g. Alzheimer´s disease (AD). AD is one the main aging-related diseases whose prevalence increases from 65 years of age: approximately 46.8 million people worldwide are affected by AD and this figure will triple by 2050 [1]. Therefore, it is indispensable to elucidate the physiopathology of AD to design new strategies to prevent, mitigate, and/or delay AD development. AD is characterized by an irreversible memory loss, partly triggered by the formation of extracellular senile plaques, accumulations of amyloid β peptide (Aβ), and intracellular neurofibrillary tangles of hyperphophorylated tau protein, which negatively affect synaptogenesis [2]. This dynamic neurobiological process is dependent on high levels of energy, provided by mitochondria. Specifically, the most amount of cellular adenosine triphosphate (ATP) derives from the mitochondrial electron transport chain. The electron flow from complex I to complex IV simultaneously generates an electrochemical proton gradient in the inner mitochondrial membrane, which is used by complex V (ATP synthase) to produce ATP. A supply of reduced nicotinamide adenine dinucleotide (NADH) to mitochondria as well as oxygen are necessary to trigger this transport. Aralar, the mitochondrial carrier of aspartate/glutamate, is a component of the malate/aspartate shuttle that is essential to provide NADH and to ensure the oxidative phosphorylation performance in the mitochondria [3]. In summary, mitochondria are crucial to a proper neuronal activity. In AD, both Aβ peptide as well as hyperphosphorylated tau protein accumulations actively exacerbate mitochondrial dysfunction by inducing many mitotoxic effects [4, 5]. Numerous studies have suggested that Aβ inhibits mitochondrial respiration

leading to a reduced ATP production [6, 7]. Further, Aβ is able to enter into the mitochondria and interact with the ATP synthase subunit α, leading to lower ATP production [8, 9]. A decreased respiratory complexes V, III, and II is commonplace in mitochondria from frontal cortices of AD patients [10]. In transgenic AD mice, there is a deregulation of complex I tau-dependent, at both protein and activity levels [11]. To date, all studies suggest that the reduced levels of mitochondrial ATP actively contribute to neuronal cell death and to AD progression.

Mitochondrial biogenesis (mitochondriogenesis) is the process by which cells increase the amount of their mitochondria; it is also impaired in AD brain, where an unbalanced mitochondriogenesis contributes to mitochondrial energetic dysfunction [12]. Members of the peroxisome proliferator-activated receptor γ coactivator1 α (PGC-1α) family are key regulators of mitochondrial biogenesis and function in highly metabolic tissues, such as liver, kidneys, and brain. In particular, PGC-1α acts as a transcriptional coactivador of nuclear genes involved in the mitochondriogenesis induced by energy needs. Further, PGC-1β participates on the maintenance of basal mitochondrial function [13]. Moreover, various studies have showed that the expression of PGC-1α, as well as of other nuclear transcriptional factors are significantly decreased in hippocampus of AD, indicating altered mitochondriogenesis [14, 15]. In addition, fusion and fission mitochondrial processes are also tightly related to mitochondrial integrity and function, as mitochondria are dynamic organelles which constantly divide and fuse with each other in response to different physiological state. Various studies have found altered levels of fusion and fission proteins in cell and animal models

of AD, indicating that the presence of abnormal mitochondrial dynamics could represent an early marker of AD [16, 17].

Adherence to a Mediterranean diet is associated with lower incidence of cognitive decline and AD [18-21]. Extra virgin olive oil's (poly)phenols such as oleuropein [22-25] and hydroxytyrosol (HT) [18, 26-32] are being actively studied in this respect and are being attributed neuroprotective properties. As an example, HT restores the impaired insulin signalling pathway in a cell model of AD [32] and prevents cognitive decline in a mouse model of Aβ-deposition [33]. In terms of cerebral mitochondrial dysfunction, some studies have suggested that HT could improve it in animals exposed to high oxidative stress conditions, i.e. in db/db mice and in arsenic-treated rats [34, 35]. Of note, all these works focused on the purported antioxidant actions of HT [18]. Yet, the effect of HT on the reduced mitochondrial ATP production associated to AD has been poorly investigated.

We wanted to ascertain the effects of HT on mitochondrial energetic dysfunction in a cellular model of AD, using 7PA2 cells because it is one of the best cellular models of Aβ toxicity with a well-characterized mitochondrial dysfunction typically observed in AD [36, 37].

2. MATERIALS AND METHODS

2.1 Cell line and culture conditions

The 7PA2 cell line was a kind gift of Prof. Dennis J. Selkoe, the Neurologic Diseases Institute at Harvard Medical School (Boston. USA). These cells were originally obtained from the Chinese hamster ovary (CHO) cell line stably

transfected with cDNA encoding human amyloid precursor protein APP751, a protein that bears the Val717Phe familial AD mutation related to the development of early AD [38]. Cells were routinely cultured in 100 mm culture dishes in Dulbecco´s modified Eagle´s medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 0.01% glutamine, 100 U/ml penicillin-streptomycin, and 200 µg/ml G-418 (to maintain the phenotype of transfected cells). All these reagents were purchased form Lonza (Basel, Switzerland). Cells were grown at 37 ºC in an atmosphere containing 5% CO_2.

2.2 Treatment of the cells

For all experiments, cells were plated in 60 mm culture dishes (100.000 cells/cm^2) with supplemented DMEM. Once cells reached 80% confluence, they were maintained for 24h with DMEM without FBS to inhibit cellular proliferation. Subsequently, cells were treated with 5 µM of HT for 4, 8, or 24 hours. HT was kindly donated by Seprox Biotech (Madrid, Spain) and was dissolved in absolute ethanol to the desired concentrations. The controls received equivalent volume of the vehicle and all cells were recollected at 4, 8, or 24 hours. Three different experiments were performed in duplicate for each assay.

2.3 Measurement of ATP levels

Intracellular ATP levels were quantified by a fluorometric assay (Sigma-Aldrich, St Louis MO, USA). Cells were homogenized and deproteinized using a 10kDa molecular weight cut-off spin filter (Millipore Corp., Bedford, Mass.). The assay was performed according to the manufacturer´s protocol and fluorescence (λ_{ex} = 535/ λ_{em} = 587) was measured in a microplate reader (Biochrom Asys UVM 340, Cambridge, UK).

2.4 Protein extraction and quantification

7PA2 were exposed to the treatments previously described. The media were removed and cells were collected on ice in 200 µL of lysis buffer pH 7.6 containing 50 mmol/L HEPES, 10mM EDTA, 50 mmol/L sodium pyrophosphate, 100 mmol/L sodium fluoride, 10 mmol/L sodium orthovanadate, 1% Triton X-100, 2 mmol/L phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, and 10 µg/ml aprotinin. Samples were homogenized, incubated overnight at – 80 ºC, and then centrifuged at 14,000 rpm for 30 min at 4 ºC to remove cellular debris. Clear supernatants were transferred to new tubes to determine the total protein concentration by BCA (Pierce, Thermo Fisher Scientific).

2.5 Western Blot analysis

Total proteins (30 µg) were resolved on a 10% SDS-PAGE gel and then transferred to polyvinyl difluoride (PVDF) membranes. Membranes were blocked with Tris-buffered saline containing 0.1% Tween 20 (TTBS) and 5% (w/v) milk powder during 2 h at 25 ºC. Blots were incubated with primary antibodies diluted in TTBS at 4 ºC overnight. Antibodies included anti-citrate synthase; anti- PGC-1α (1:1000 dilution) from Santa Cruz Biotechnology (Santa Cruz, CA); anti-ATP synthase (1:1000 dilution); anti-Mitofusin 2 (1:5000 dilution) from Abcam PLC (Cambridge, UK); and anti-Aralar which was a gift from Professor A del Arco [39]. The membranes were subsequently washed and incubated with the corresponding secondary antibody conjugated with horseradish peroxidase diluted (1:2000) in TTBS during 90 min at 25 ºC. Proteins were detected by chemiluminescence with a West Pico substrate (Thermofisher). All blots were reblotted with their corresponding total forms or with anti-glyceraldehyde-3-

158 phosphate dehydrogenase (GADPH) to normalize each sample for gel-loading
159 variability. Bands were quantified by densitometry using Adobe Photoshop´s
160 (Adobe systems, Inc., Mountain View, CA).

161 *2.6 RNA extraction and quantification*

162 Total RNA from cells was obtained using the Tripure Isolation reagent
163 (Sigma-Aldrich, San Luis, Misuri, USA), following the manufacturer's instructions.
164 RNAs were quantified using the NanoDrop 2000 (Take3, BioTek).
165 Complementary DNA (cDNA) was synthesized from 1 µg of DNase-treated RNA
166 [40].

167 *2.7 Real time qPCR analysis*

168 Real time quantitative PCR (q-PCR) was performed by using ABI PRISM
169 7500 Fast Sequence Detection System instrument and software (Applied
170 Biosystem, Foster City, CA). Relative quantification of target cDNA in each
171 sample was performed from 10 ng of cDNA in TaqMan One-Step real time PCR
172 Master Mix and using Pre-Developed TaqMan Assay Reagents (PE Applied
173 Biosystem) for PGC1α (Rn00580241_m1) and 18S rRNA (Hs99999901_s1) with
174 VIC as real time reporter was used as control to normalize gene expression. The
175 $^{\Delta\Delta}$CT method was used to calculate the relative differences between experimental
176 conditions and control groups as fold change in gene expression [41].

177 *2.8 Mitochondrial DNA (mtDNA) quantification*

178 Total genomic DNA was isolated from cells using Tripure Isolation reagent
179 (Sigma-Aldrich, San Luis, Misuri, USA) following the manufacturer's instructions.
180 The mtDNA copy number quantification ($2^{-\Delta\Delta CT)}$) was evaluated by quantitative
181 PCR as previously reported [42, 43] using 16S rRNA as a mtDNA marker (sense

primer 5´-AGAGTTTGATCCTGGCTCAG-3´; antisense primer 5´-CTACGGCTACCTTGTTACGA-3´) and β-actin as a nuclear DNA (nucDNA) marker (sense primer 5´-GGTATGGAATCCTGTGGCATCCATGAAA-3´; antisense primer 5´-GTGTAAAACGCAGCTCAGTAACAGTCC-3´)

2.9 Mitochondrial permeability transition (MPT) assay

7PA2 cells were plated in 24-well plates at the density of approximately 104 cells/well and treated as described above. After washing with PBS, cells were stained with Mitotracker® Red CMX Ros (Invitrogen) diluted in PBS (100 nM). After being incubated at 37 ºC for 15 min, the samples were washed with PBS and with paraphormaldehyde 4% for 15 min. Nuclei were labelled with bis-benzimide (Hoechst 33258; 1 mg/mL for 10 min at RT). Coverslips were mounted on glass slides with fluorescent mounting medium. Finally, samples were photographed by a laser scanning confocal microscope (Leica, TCS SP5, Germany). The images were analyzed using ImageJ NIH software that allowed quantification of the signal strength corresponding to mitotracker.

3. Statistical analyses

Data are expressed as means ± SEM. Statistical analysis was performed using one-way ANOVA (GraphPad Prism 5.03 software, GraphPad Software, Inc., San Diego, CA). When the main effect was significant, the Bonferroni post-hoc test was applied to determine individual differences between means. Statistical significance was set at $p< 0.05$. Pearson´s correlation coefficient r was used to measure the degree of association between different variables measured in each group. Two-tailed p values < 0.05 were considered significant.

206 ## 4. RESULTS

207 *4.1 HT improves the energetic status of 7PA2 cells*

208 Krako and collaborators [36] reported that 7PA2 transfected cells display
209 decreased ATP production from their respiratory chain, as well as a reduced
210 mitochondrial membrane potential in comparison with untransfected CHO cells.
211 Furthermore, they suggested that the high production of toxic Aβ could be
212 triggering the severe bioenergetic impairment in 7PA2 cells. We assessed the
213 effect of HT treatment on ATP production in 7PA2 cells by a fluorometric assay.
214 After 24 hours of treatment with HT, ATP concentrations were significantly
215 increased as compared with controls. However, HT did not modify ATP levels at
216 4 and 8 hours (Table 1.). To shed some light on this HT-induced effect, we wanted
217 to find out whether it could be due to an increased mitochondrial content or/and
218 activity in 7PA2 cells.

219 *4.2 HT alters the mitochondrial content of 7PA2 cells*

220 In order to determine whether HT increased ATP levels via increased
221 mitochondriogenesis, we measured some markers of mitochondrial mass. First,
222 we assessed the concentrations of citrate synthase (CS), a mitochondrial matrix
223 protein implicated in oxidative metabolism and commonly used as a marker of
224 mitochondrial content [44, 45]. We found an increased CS expression in 7PA2
225 cells treated with HT for 8 hours. Conversely, we observed no significant effect 4
226 and 24 hours after HT-supplementation (Fig. 1A).

227 Based on this result, we hypothesized that the HT-induced increased
228 mitochondrial mass could be related to the formation of new mitochondria.
229 Therefore, we measured messenger RNA (mRNA) levels and protein expression

of the transcriptional factor PGC-1α as well as the mtDNA copy number in HT-treated 7PA2 cells. PGC-1α levels were significantly increased at 8 hours post HT-treatment. Nevertheless, HT treatment for 4 and 24 hours did not alter the expression of this mitochondrial regulator (Fig. 1B and C).

Several reports have suggested that the evaluation of mtDNA copy number can be a good indicator of the amount of mitochondrial mass, because it remains almost constant in a normal physiological state [46-48]. Interestingly, we found a HT-induced mtDNA copy number increase in treated 7PA2 cells, at 8 hours (Fig. 1D). Moreover, mtDNA copy number positively correlated with the PGC1α and CS concentrations in the 7PA2 cells that were treated for 8 hours (correlation coefficient (r) = 0.8377; $p < 0.001$*** and $r = 0.7098$, $p < 0.01$**; respectively), suggesting that HT induces the formation of new mitochondria in 7PA2 cells after eight hours.

Together, these findings show that the increased ATP production observed in 7PA2 cells 24 hours after HT-treatment is unlikely to be due to an increased mitochondrial mass.

4.3 HT boosts mitochondrial activity

Considering the aforementioned results, we wanted to determine whether the high ATP levels observed 24 hours after HT-treatment could be produced by an increase in mitochondrial activity. Therefore, we quantified - by mitotracker probes - the number of active mitochondria in HT-treated 7PA2 cells. As shown in Figure 2A, HT increased the number of active mitochondria 24 hours after treatment in comparison with controls. Moreover, we found an increased level of aralar in HT-treated 7PA2 cells after 8 and 24 hours, corroborating the finding

that HT facilitates the provision of NADH to the respiratory chain (Fig. 2B). Likewise, the levels of the mitochondrial ATP synthase were significantly increased in HT-treated 7PA2 cells (after 24 hours) whereas the 8-hour treatment induced a small and non-significant increase (Fig. 2C.).

In summary, all these findings indicate that HT increases mitochondrial activity in 7PA2 cells after 24 hours of treatment.

4.4 HT favors mitochondrial fusion

We wanted to ascertain whether the HT-induced augmented mitochondrial activity could be related to changes in the mitochondrial machinery. Therefore, we studied the levels of a membrane protein involved in the fusion of mitochondria. Mitofusin is a GTPases embedded in the outer membrane of the mitochondria, which maintains the balance of the mitochondrial network [49]. Interestingly, increased mitofusin levels were found in HT- treated 7PA2 cells after 24 hours (Fig. 3), indicating that HT could be inducing mitochondrial fusion in 7PA2 cells in that period. However, we did not detect changes in mitofusin levels at 4 and 8 hours after treatment with HT.

5. DISCUSSION

This study provides evidence that HT is able to improve the cellular energetic state of an “in vitro” model of AD. Various investigators suggested that HT acts on mitochondrial dysfunction in different pathologies such as obesity, type 2 diabetes [35, 50] and inflammatory angiogenesis as associated with cancer and vascular disease [51]. As regards AD, Peng and collaborators [52] showed that HT ameliorates mitochondrial dysfunction and cognitive behaviour

in the cerebral cortex of APP/PS1 mice, an animal model of AD. However, the Authors only focused on evaluating mitochondrial markers of oxidative stress and the inflammatory response induced by Aβ accumulation.

In our study, we elucidated how HT is able to restore the mitochondrial energetic deficit of 7PA2 cells, a cellular model of AD with an Aβ-induced mitochondrial impairment. We assessed both the mitochondrial content and activity of such cells to clarify the intracellular process responsible for the HT-induced bioenergetic improvement.

The 7PA2 cell line exhibits impaired mitochondrial machinery, leading to lower ATP production similar to that found in AD patients' brains [36, 53, 54]. We observed that HT significantly increased ATP concentrations after 24 hours of supplementation. This agrees with Reutzel et al, who fed aged mice a mixture of (poly)phenols typical of olive oil [55] and with Schaffer et al. who used HT-rich olive mill waste water [28, 29]. Also, HT was able to augment the intracellular ATP concentration in degenerated mouse muscle cells [56]. Our data show that the altered mitochondrial function of 7PA2 cells could be restored by HT.

To shed some light on the mechanisms by which HT increases ATP production by AD cells, we ascertained whether this phenomenon was due to a change in the quantity of mitochondria or in their activity. To measure the mitochondrial mass, we quantified the levels of CS, a mitochondrial protein, PGC1α, a transcriptional regulator of mitochondriogenesis, and the mtDNA copy number after HT treatment. We showed that all these parameters were increased in the 7PA2 cells treated for 8 hours with HT and we computed significant correlations among them, strongly suggesting that HT increases the

mitochondrial content of 7PA2 cells 8 hours after supplementation. Similar results were found in HT-treated adipocytes, although the concentration of HT and the treatment period were different than the ones used in our study [57]. Here we demonstrate that HT increases the amount of mitochondria in a cellular model of AD, suggesting that the HT-induced cognitive improvement found in previous studies and the association between olive oil consumption and lower incidence of neurodegeneration could be partly explained by such action [52]. The HT-induced mitochondriogenesis could be, indeed, responsible for the increased ATP production observed in 7PA2 cells 24 hours post HT-treatment. Nevertheless, an active respiratory chain is necessary to increase ATP levels. Therefore, we evaluated mitochondrial activity by measuring some of its markers. Mitochondrial label assays carried out with mitotracker probes showed an increased number of active mitochondria in 7PA2 cells treated with HT for 24 hours. The same experimental group also exhibited a higher expression of two molecules essential for a proper activity of the respiratory chain, i.e. the transporter Aralar and ATP synthase, the mitochondrial complex V. These data indicate that HT augments the NADH flux mediated by Aralar and also favours ATP synthesis, increasing the levels of the key enzyme involved in this process. An in vitro study performed in serum-starved fibroblasts demonstrated that HT regulates the biogenesis of OXPHOS complexes, activating Protein kinase A and CREB pathways [58]. Another study reported that HT supplementation increases mitochondrial complex I activity (NADH-dependent) in muscle from rats subjected to excessive physical activity [59]. Soni and collaborators [34] demonstrated that HT restores the enzymatic activities of mitochondrial complexes I, II and IV in brains of rats with arsenic-induced mitochondrial dysfunction, which fits with our

finding because the NADH necessary to activate the mitochondrial complex I could be provided by Aralar, whose expression is potentiated by HT. Moreover, we have found high levels of ATP synthase at 24 hours after HT-treatment, which could partially contribute to the increased ATP production found in 7PA2 cells at the same treatment time. An increase in mitochondrial function should be associated with higher mitochondriogenesis. Conversely, we showed an increase of new mitochondria at 8 hours post HT-treatment rather than at 24 hours. Given that mitochondria are dynamic organelles, we hypothesize that the lack of increased mitochondrial content at 24 hours after treatment could be caused by the fusion of the new mitochondria generated at 8 hours, which become fully activated at 24 hours. To corroborate this hypothesis, we measured mitofusin levels as a mitochondrial fusion indicator. Indeed, immunoblots revealed high mitofusin levels at 24 hours, which suggests that, between 8 and 24 hours after supplementation, HT could be inducing mitochondrial fusion in 7PA2 cells. This is noteworthy because, according to Wang et al [16], mitochondrial fusion is decreased - via APP overexpression - in AD. Consistent with our data, another study proposed that HT induces mitochondrial fusion in muscles of rats subjected to high intensity exercise [59]. In summary, accrued evidence supports the hypothesis that the putative protective role of HT in AD is partly due to the its inducting mitochondrial fusion.

In conclusion, our data suggest that HT may revert the energetic deficit of a cellular model of AD by potentiating mitochondrial activity. Because HT is being proposed as dietary supplement or component of functional foods, future studies in appropriate animal models and - eventually - humans are warranted to further investigate its potential neuroprotective actions in AD.

Conflict of Interest

The authors declare no conflict of interest associated with this work.

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Table 1. Effect of HT (5 µM) on the ATP levels (pmol/µl) of 7PA2 cells, at 4, 8, and 24 hours.

	ATP pmol/µl	
	CONTROL	**HYDROXYTYROSOL**
4 hours	639.90 ± 93.97	553.30 ± 35.96
8 hours	530.30 ± 47.56	619.70 ± 36.69
24 hours	586.60 ± 17.99	963.50 ± 82.33**

Values are means ± SEM of three independent experiments that were performed in duplicate. ***p< 0.001 after ANOVA as compared with control group.

Fig 1.

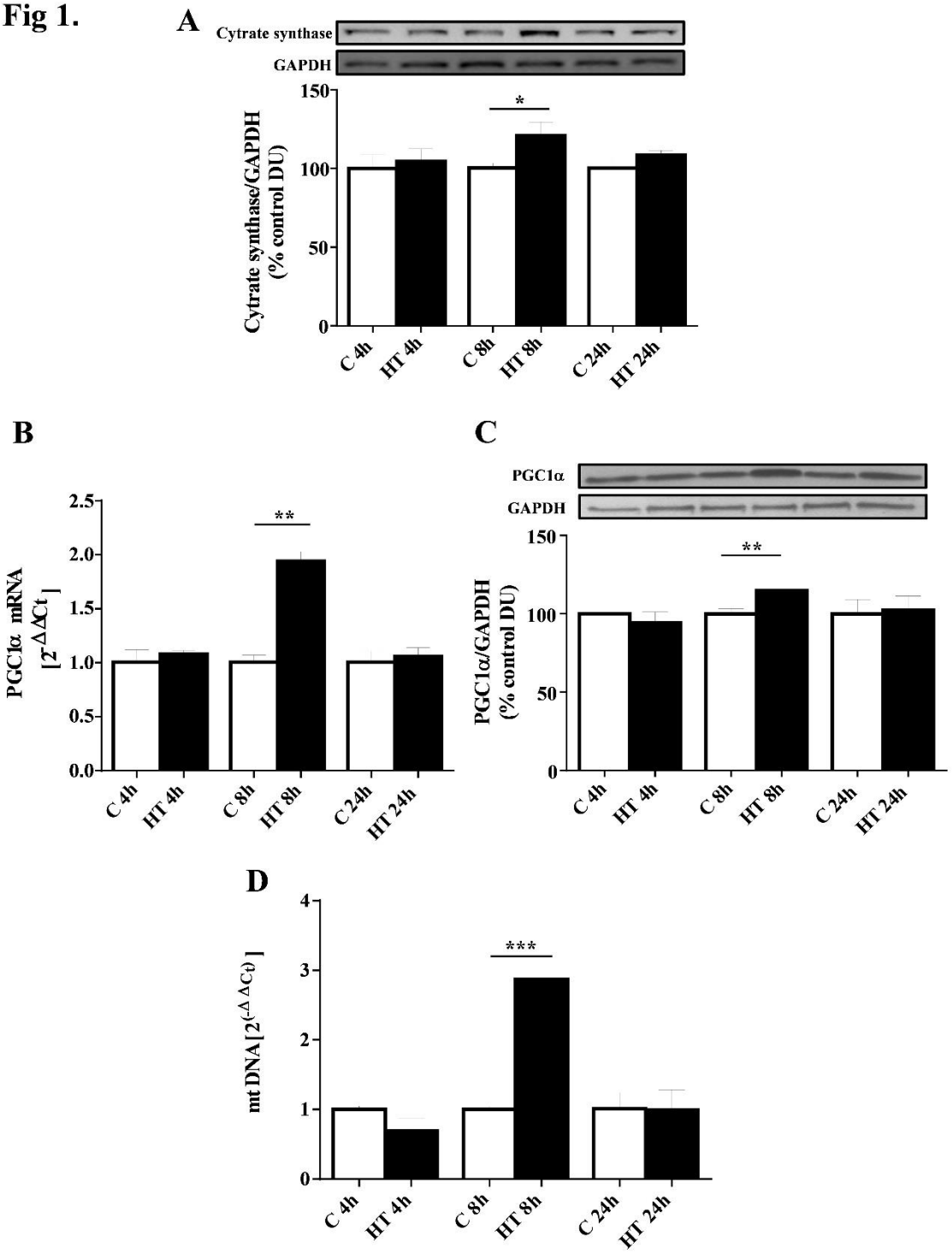

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Fig 2.

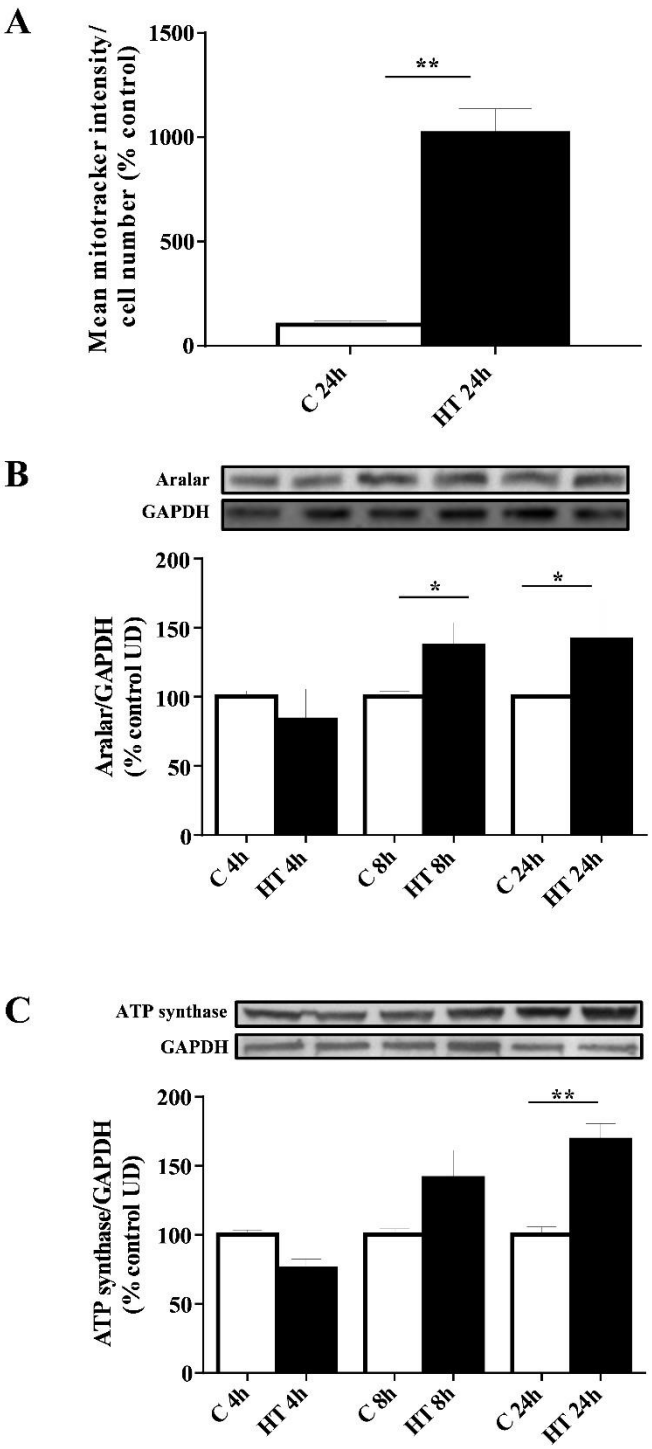

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Fig 3.

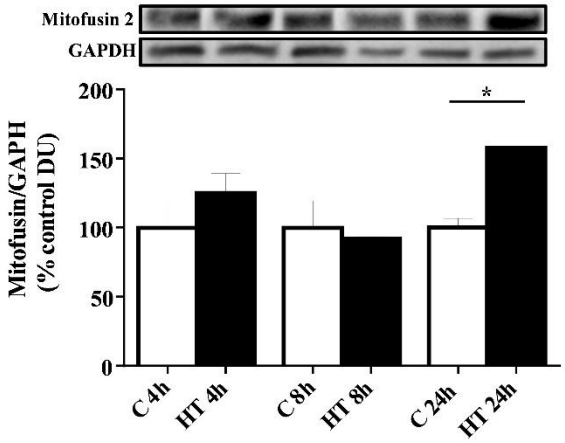

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Figure Legends

Figure 1. Effect of hydroxytyrosol (5 µM) on markers of mitochondrial mass in 7PA2 cells treated for 4, 8, and 24 hours. Control groups (C) are shown as open bars and treated groups (HT) as solid bars. (A) Relative protein levels of citrate synthase (CS) and (C) peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α). (B) Relative levels of mRNA of PGC-1α and (D) mitochondrial DNA. Data are means ± SEM of three different experiments carried out in duplicate. DU, densitometry units. **P*< 0.05; ***P*< 0.01; ****P*< 0.001 after ANOVA.

Figure 2. Effect of hydroxytyrosol (5 µM) on markers of mitochondrial activity in 7PA2 cells treated for 4, 8, and 24 hours. Control groups (C) are shown as open bars and treated groups (HT) as solid bars. (A) Mean intensity of active mitochondria labelled with mitotracker probes in 7PA2 cells treated for 24 h with HT. (B and C) Western blot and densitometry from immunoblots derived from the protein expression levels of Aralar and ATP synthase in control and treated 7PA2 cell groups. Data are means ± SEM of three different experiment carried out in duplicate. DU, densitometry units. *P< 0.05; **P< 0.01 after ANOVA.

Figure 3. Densitometry from immunoblots derived from the Western blot analysis of the relative mitofusin 2 protein levels in 7PA2 cells treated with hydroxytyrosol for 4, 8, and 24 hours. Control groups (C) are shown as open bars and treated groups (HT) as solid bars. The data are percentages of the respective control means ± SEM of three different experiments performed in duplicate. DU, densitometry units. *P< 0.05 after ANOVA.