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AGING AND OXIDATIVE STRESS DECREASE PINEAL ELONGATION FACTOR 2: IN VIVO PROTECTIVE EFFECT OF MELATONIN IN YOUNG RATS TREATED WITH CUMENE HYDROPEROXIDE†

Mario F. Muñoz[1+], Sandro Argüelles[2+], Mercedes Cano[3], Francesco Marotta[4] and Antonio Ayala[1]*

+ These authors contributed equally to this work.

[1]Department of Biochemistry and Molecular Biology Faculty of Pharmacy. University of Sevilla. Spain
[2]China-US (Henan) Hormel Cancer Institute
Zhengzhou, China.
[3]Department of Physiology and Zoology. Faculty of Pharmacy. University of Sevilla. Spain.
[4]ReGenera Research Group for Aging Intervention & Milano Medical Center. Milano. Italy.

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***Correspondence:**
Departamento de Bioquímica y Biología Molecular.
Facultad de Farmacia.
Universidad de Sevilla.
C/. Tramontana s/n. 41012-Sevilla. Spain.
E-mail: aayala@us.es

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Abstract

We studied the alterations of Elongation Factor 2 (eEF2) in the pineal gland of aged rats as well as the possible protective role of exogenous melatonin on these changes in young rats treated with cumene hydroperoxide (CH), a compound that promotes lipid peroxidation and inhibits protein synthesis. The study was performed using male Wistar rats of 3 (control), 12 and 24 months and 3 month-old rats treated with CH, melatonin and CH plus melatonin. We found that pineal eEF-2 is affected by aging and CH, these changes being prevented by exogenous melatonin in the case of CH-treated rats. The proteomic studies show that many other proteins are affected by aging and oxidative stress in the pineal gland. The results suggest that one of the possible mechanisms underlying pineal gland dysfunction during aging is the effect of lipid peroxidation on eEF-2, which is a key component of protein synthesis machinery. This article is protected by copyright. All rights reserved

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Introduction

The translation is a complex process requiring a lot of energy, many participant molecules and is affected by various circumstances, such as aging. When organisms age, a considerable reduction of protein synthesis takes place (Rattan and Clark, 1996;Ryazanov and Nefsky, 2002;Thornton et al., 2003). The causes of this decrease are still not clear, since there are many participating components involved in the three translation steps. The hepatic and renal elongation steps have been shown to be especially affected by aging (Ayala et al., 1996;Parrado et al., 2003;Piwien-Pilipuk et al., 2002). This delay is caused by alterations of Elongation Factor 2 (eEF2), the protein that catalyses movement of the ribosome along the mRNA. During aging, eEF2 molecules lose activity, display greater oxidations levels and are fragmented, specifically by the action of lipid peroxidant agents (Arguelles et al., 2006). Also, eEF2 have been reported to form adducts with low molecular weight aldehydes such as malondialdehyde (MDA) and 4-hydroxynonenal (HNE), which are generated *in vivo* as a consequence of the lipid peroxidation processes (Arguelles et al., 2006;Arguelles et al., 2009;Arguelles et al., 2011;Ayala et al., 2014). According to these results, one possible mechanism responsible for the decline of protein synthesis during aging could be the alteration of eEF2 levels, secondary to lipid peroxidation and adduct formation with these aldehydes.

Old age is associated with functional decline in all body tissues. Many common physiological changes encountered over time can be related to neuroendocrine phenomena, where the endocrine components have been suggested to have an important central role in the aging process. With aging, there are multiple changes in the production, efficacy and the clearance of many different hormones (Morley, 2003). Among these changes is the decreased ability of the pineal gland to produce melatonin (Mel) and to work as a central structure in the circadian system under control of the suprachiasmatic nucleus (SCN). Through Mel production, the pineal gland influences the rest of the body. With reduced activity of the pineal gland in aged organisms, any physiological effects resulting from Mel may be assumed to diminish (Hardeland, 2012;Stokkan et al., 1991). In fact,

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there is a great deal of evidence indicating that aging is characterized by a progressive deterioration of circadian timekeeping and disrupted Mel production (Hardeland, 2013). With respect to the possible mechanisms underlying pineal gland dysfunction, it has been suggested the age related alterations of neural and humoral systems that regulate its activity as well as the molecular changes of the gland itself (Moller and Baeres, 2002;Simonneaux and Ribelayga, 2003). The levels of enzymes, metabolites, and receptors involved in melatonin synthesis also decrease during aging, resulting in low levels of melatonin in the elderly (Sarlak et al., 2013). Because of its role in the circadian system, many rhythmically expressed genes have been detected in the pineal gland (Maronde and Stehle, 2007;Wunderer et al., 2013) with 100-fold variations between day and night. This means that pineal gland protein synthesis is a particularly important process that must always work properly, not only in the long run but also in a 24-h interval. Any deviation from this situation of fine regulation would lead to serious consequences in the role of this master gland.

Considering protein synthesis inhibition and pineal changes reported during aging, we hypothesized that the translation process can be affected in the gland of old organisms. Consequently, the main goal of the current study was to determine the alterations of eEF2 in the pineal gland of aged rats as well as the possible role of exogenous Mel on these changes in young rats treated with cumene hydroperoxide (CH), a compound that promotes lipid peroxidation and inhibits protein synthesis (Ayala et al., 2014). Besides eEF2, the proteome of the pineal gland was studied to see whether or not other proteins are affected by CH and aging.

Materials and Methods

Animals

Forty male Wistar rats of 3-month-old (control), five 12- and five 24-month-old were used in this study. The rats were maintained under an automatically controlled temperature (22–23º C) in a 12-h

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light–dark cycle (lights on, 7 a.m. -7 p.m.). The care and use of animals was performed according to the Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes (http://ec.europa.eu/environment/chemicals/lab_animals/home_en.htm).

Treatments

Cumene hydroperoxide –CH- (Sigma–Aldrich, Inc.St. Louis, MO, USA) was dissolved in saline and the solution sonicated for 5 s at 50 W. CH-treated rats (3-month-old rats) received an intraperitoneal injection of 40 mg/kg/day CH at 10 a.m. for 14 (n=5 rats) and 28 (n=5 rats) days. On the other hand, to test the possible protective effect elicited by Mel (Sigma–Aldrich, Inc.St. Louis, MO, USA), 3- month- old rats were randomly divided into four groups of five rats. The first group, designated as the control group, received saline solution (equal volume of CH solution, i.p.) at 10 a.m. daily for 28 days. Seven days after starting the treatment, they were administered with water daily at 6 p.m. (equal volume of Mel solution, orally with a feeding tube). The second group was treated with 15 mg/kg/day of CH for 28 days (i.p.) at 10 a.m. and 7 days after starting the treatment water was administered daily at 6 p.m. (equal volume of Mel solution) until the end of the treatment. The third group received a daily saline solution (i.p.) at 10 a.m. for 28 days, and 7 days after starting the treatment, Mel (10 mg/kg/day) was administered orally at 6 p.m. with a feeding tube until the end of the treatment. The fourth group received 15 mg/kg/day of CH for 28 days (i.p.) at 10 a.m. and 7 days after starting the treatment, Mel (10 mg/kg/day) was administered orally at 6 p.m. until the end of the treatment. After the treatments, the rats were deeply anesthetized and the whole pineal glands were removed during nighttime (12 a.m.) in the dark, the procedures were performed under a dim red light. The whole pineal gland and serum were stored at -80ºC until biochemical determinations were performed.

Immunoblot analysis

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For Western blot analysis, the whole pineal glands were homogenized in lysis buffer: 150 mM NaCl, 1% Triton X-100, 1.2 mM deoxycholic acid, 0.1% SDS, 10 mM EDTA, 0.02% NaN_3, 1mM phenylmethyl sulfonyl fluoride, and sonicated for 20 s. The homogenate was then vortexed (10 s) and centrifuged at 15,000 x g (4ºC, 20 min). 20 µg of protein was separated by sodium dodecyl sulfate 10% polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to a nitrocellulose membrane (Hybond-C Extra, GE Healthcare Bio-Science, Uppsala, Sweden). The transfer buffer consisted of 25 mM bicine, 25 mM bis-Tris, and 1 mM EDTA, pH 7.2. The power conditions were 120V, 1 h. After transfer, membranes were stained with Ponceau red to indicate that the same amount of protein is present across different lanes. The membranes were blocked with blocking buffer (2% dry milk in 20 mM Tris–HCl, pH 7.5, 500 mM NaCl, 0.05% Tween 20) for 1 h at room temperature. The membranes were then incubated with the polyclonal anti-eEF2 (produced in our laboratory) antibody 1:1000 overnight at 4ºC. This antibody was obtained against an amino terminal sequence that has neither a phosphorylation site nor the diphthamide residue. Therefore, what we detect is the total eEF-2. After incubation, the membranes were washed in 20 mM Tris–HCl, pH 7.5, 500 mM NaCl, 0.05% Tween 20, and incubated with peroxidase-conjugated anti-Ig secondary antibodies (DAKO, Denmark). The proteins were visualized using the chemiluminescence kit (Santa Cruz Biotechnology, CA, USA). The bands were analyzed by densitometry using the Quantity One1 1-D Analysis Software (Bio-Rad, Hercules, CA, USA). The Western blots were normalized by the total proteins in the Ponceau red stained membranes, which is a validated method to assess protein loading and transfer(Rivero-Gutiérrez et al., 2014; Gilda & Gomes, 2013; Romero-Calvo et al., 2010). The optical densities of eEF2 bands were divided by the optical densities of proteins (using a thin strip through the center of the lane running from top to bottom) in the Ponceau red stained membranes. We used a thin strip because allows that majority of proteins are included (Fig. 1A).

Two-dimensional electrophoresis (2-DE)

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A 2-DE gel was made from the whole pineal of each individual animal (n = 5 in each groups). Samples of pineal glands protein in 340 µl of urea rehydration solution (8 M urea, 2% CHAPS, 0.01% bromophenol blue, 20 mM DTT and 1% IPG Buffer – GE Healthcare Bio-Science, Uppsala, Sweden) were loaded onto 18-cm precast immobilized pH gradient strips, pH 3-10 (GE Healthcare) for the first dimension. The strips were rehydrated for 12 h at 30 V and 20º C using the Ettan IPGphor 3 (GE Healthcare). The voltage during isoelectrofocusing at 20º C was programmed as follows: 1 h at 500 V, 1 h at 1000 V, 1 h at 2000 V (gradient), 5 h at 8000 V (gradient) and 5 h at 8000 V. Immediately following isoelectrofocusing separation, the strips were equilibrated for 15 min in equilibration buffer [50 mM Tris/HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, trace of bromophenol blue]. In the first equilibration step, 1 % DTT was added to the solution. In the second equilibration step, 4% iodoacetamide was added. For the second dimension, the 18-cm strips were loaded onto a 10% SDS-PAGE (25 cm x 20 cm x 0.15 cm) and run 2 h at 100 V and 200 V until complete. Gels were stained with DodecaTM Silver Stain Kit (Bio-Rad, USA) compatible with mass spectrometry.

Protein visualization and image analysis

The gels (n = 5 in each groups) were scanned in Gel DocTM XR, and the protein spot patterns were analyzed using Progenesis SameSpots software (Nonlinear Dynamics, Newcastle, UK). This software performs an alignment that makes it possible to accurately compare different images by removing the positional variation introduced during the electrophoresis process. Once the alignment has been performed, each spot on each image will be in the same location as the matching spot on the other gels. Before quantification, background subtraction is carried out to correct for the intensity level of the scanner bed and staining variations across the gel. After that, the software compares the different groups and the fold change value of each spot presented in a table along with their ANOVA p-value.

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Mass spectrometry

Each protein spot was excised from the gel with a sterile blade; it was placed in an eppendorf with 500 µl of distilled water and sent to the Proteomic Service of Universidad de Cordoba, Spain. Proteins were digested with trypsin and peptide masses were determined using a mass spectrometer (MALDI-TOF/TOF). After spectrum calibration and removal of trypsin auto-digestion and keratin peaks, the peptide mass fingerprint data was submitted to the NCBI non-redundant (NCBInr) database using the MASCOT software (www.matrixscience.com). The following parameters were used for the database search: Max missed cleavage 1; significance threshold $p<0.05$; fixed modification carbamidomethyl on cystein; variable modification oxidation of methionine; mass values monoisotopic; fragment mass tolerance 0.2 Da; and peptide mass tolerance 100 ppm but the observed mass accuracy was usually better than 50 ppm for identified peptides.

Determination of lipid hydroperoxide by oxidation of Fe^{2+} in the presence of xylenol orange.

The protocol for lipid peroxidation measurements (Jiang et al., 1991) was adapted for a microplate reader. Forty µg of proteins were incubated with 90 µl of H_2SO_4 for 30 min. Following addition of 100 µl FOX reagent (0.5 mM ferrous ammonium sulfate, 0.2 mM xylenol orange and 200 mM sorbitol in 25 mM H_2SO_4) the mixture was incubated at room temperature for 45 min, protected from light. The formation of ferric ions was detected measuring the resulting colored complex with xylenol orange at 540 nm.

Melatonin analysis

Serum melatonin was measured by enzyme-linked immunosorbent assay (ELISA) following manufacturer´s instructions (GenWay Biotech Inc., San Diego, CA, USA).

Protein determination

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Protein content of the samples of pineal glands was determined using DC™ Protein Assay (Bio-Rad, Hercules, CA, USA) and the protein concentration in serum was determined by the Lowry assay (Lowry et al., 1951).

Statistical analysis

Data are shown as means ± SEM. Graph plotting and statistical analysis used GraphPad Prism Version 5.03 (Graph Pad Software). Statistical evaluation was performed by one-way ANOVA, followed by Tukey's test. A *p*-value of ≤ 0.05 was considered significant.

Results

Aging decreases eEF2 levels and increases lipid peroxidation in the pineal gland and serum

The levels of eEF2 were determined in the pineal glands of 3-, 12- and 24- month old rats. Figure 1A shows a typical membrane containing a single band corresponding to eEF2 (92 kDa) from these tissues. Aging produced a significant decrease of 53.1% ($P<0.001$) and 27.8% ($P<0.01$) in total levels of eEF2 in 24- and 12- month-old rats, respectively (Fig 1A, B). To determine oxidative damage in the pineal gland and serum, lipid peroxides were measured in samples from the experimental groups. Aging significantly increased the production of lipid peroxides by 74.7% ($P<0.001$) and 37.6% ($P<0.01$) in the pineal gland of 24- and 12-month-old rats, respectively (Fig. 1C). Serum levels of lipid peroxides were increased by 152.7% ($P<0.001$) and 92.7% ($P<0.001$), respectively, in these groups (Fig. 1D).

Oxidative stress induced by exposure to CH decreases eEF2 levels and increases lipid peroxidation in pineal gland and serum

Next, levels of eEF2 were determined in the pineal glands of rats treated with CH. In order to compare the results with those obtained using aging models, we decided to use high CH concentration to produce a strong and measurable effect. More precisely, rats treated with 40

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mg/kg/day of CH for 14 and 28 days. Several controls were performed using several doses of CH. CH affected the levels of eEF2 in a dose dependent manner (results not shown). Figure 2A shows a typical membrane containing eEF2 bands from these tissues. The amount of eEF2 significantly decreased by 64.9% (P<0.001) and 44.8% (P<0.001) with respect to control in rats treated with CH for 28 and 14 days, respectively (Fig. 2B). CH treatment increased the production of lipid peroxides as a function of the length of the treatment (Fig. 2C) in the pineal glands. Levels of lipid peroxides were significantly elevated by 87.9% (P<0.001) and 62.6% (P<0.001) in rats treated with CH for 28 and 14 days, respectively. Serum levels of lipid peroxides were also measured. As seen in Fig. 2D, lipid peroxides significantly increased up to 186.2% (24 months) and 141.1 % (12 months) with respect to control values.

Oxidative stress induced by exposure to CH and aging affects other proteins than eEF2 in the pineal gland

To study whether CH treatment and aging affect proteins other than eEF2 in the pineal gland, we studied the proteome of this tissue in rats treated with 40 mg/kg/day of CH for 14 and 28 days and in 12- and 24-month-old rats. After analyzing the proteome, an average of 850 spots was detected on silver stained 2-DE gels (pH 3–10). Eighty spots were affected by either CH treatment or aging (Fig. 3). Twenty-five spots were affected by both CH and aging. After MS analyses of these 25 spots, ten of them were identified. After MS analyses of these 25 spots, ten of them were identified (Fig. 4 and Table 1). Predicted and actual pI and size of all proteins identified were nearly identical for all spots. All the proteins listed in Table 1 are the first protein candidate in the search result lists and their scores are normally significantly higher than the scores for the second candidates. In most cases, these proteins are also the only candidate having significant score, leading to their unambiguous identifications.

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Melatonin prevents eEF2 decrease and lipid peroxidation increase in the pineal gland and serum associated with oxidative stress induced in vivo by CH

To test the possible protective effect elicited by Mel, young rats were treated with mild CH concentration alone and CH plus Mel as described in Materials and Methods, specifically 15 mg/kg/day of CH 28 days and 10 mg/kg/day of Mel. Several controls were performed using several doses of CH. CH affected the levels of eEF2 in a dose dependent manner (results not shown). The levels of eEF2 and lipid peroxides were determined in the pineal glands and serum. The treatment with CH significantly affected eEF2 levels so that it was 28.2% ($P<0.001$) lower with respect to controls (Fig. 5A, B). While treatment with Mel alone did not significantly affected the eEF2, this hormone prevented the loss of total eEF2 under lipid peroxidation conditions when co-administered with CH. CH treatment significantly increased lipid peroxides in pineal glands *in vivo* up to a value that was 50.7% ($P<0.001$) higher than controls (Fig. 5C). This increase was completely prevented by co-treatment with Mel. Serum levels of lipid peroxides were also measured in the experimental groups. As seen in Fig. 5D, CH treatment leads significant increase of 85.4% ($P<0.001$) in the levels of lipid peroxides. Treatment with exogenous Mel alone did not affect the serum levels of lipid peroxides. However, when Mel was co-administered with CH, it prevented the increase of lipid peroxidation. Concerning Mel serum levels, the amount significantly decreased 2.4-fold ($P<0.001$) after the treatment with CH, and, obviously, significantly increased 1.7-fold ($P<0.001$) after treatment with exogenous Mel. The co-treatment of Mel partially prevented the effect of CH (Table 2).

Discussion

In rats the pineal gland represents a complex with two parts-a superficial pineal at the brain surface, and deep pineal close to the third ventricle. The superficial and deep parts are connected by a thin pineal stalk (Moller and Baeres, 2002). The parenchyma of the pineal gland consists of the predominant Mel-producing pinealocytes (about 95%), astrocyte-like interstitial cells (about 4%)

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and macrophages (< 1%) (Moller and Baeres, 2002). The pineal gland plays an important role in the modulation of many aspects of mammalian physiology. This gland exerts its effects through a variety of functions: as an endocrine gland, as a transducer, as a regulator of hormones and as a circadian oscillator (Macchi and Bruce, 2004). The ability of the pineal gland to produce Mel in rhythmic fashion declines significantly with age. Reduced Mel concentrations during aging, especially nocturnal levels have been reported (Wu and Swaab, 2005;Wu and Swaab, 2007). The pineal gland itself shows clear age-related changes (Skene and Swaab, 2003).

eEF2 has a key role as a cytoplasmic component of the protein synthesis machinery, where it is an essential regulatory protein of the translational elongation step (Browne and Proud, 2002) that catalyzes the movement of the ribosome along the mRNA. When organisms age, a considerable reduction of protein synthesis takes place (Rattan, 2010). According to previous work one possible mechanism responsible for the decline of protein synthesis during aging could be the alteration of eEF2 level (Parrado et al., 1999). Considering protein synthesis inhibition and pineal gland changes reported during aging, we have studied the alterations of eEF2 in this gland of aged rats.

Our results show that eEF2 levels decrease with aging in the pineal gland (Fig. 1), this loss being bigger in 24-month-old rats. Due to the fact that eEF2 is a key element in the translation process (Browne and Proud, 2002;Ryazanov et al., 1988), protein synthesis should be affected in the pineal gland. This circumstance could result not only in the deregulation of the day-night variations in protein levels, but also in the alteration of circadian Mel production. The loss in eEF2 in the pineal gland during aging is accompanied by a concomitant increase of the LP levels (Fig. 1). Among the oxidative stress processes, lipid peroxidation seems to affect eEF2 especially (Arguelles et al., 2006).

In order to study the potential relationship between lipid peroxidation and the decrease of eEF2, young rats were treated for 14 and 28 days with CH, a compound that promotes lipid peroxidation

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and inhibits protein synthesis (Poot et al., 1988). In the pineal gland, CH decreased the levels of eEF2 as a function of the length of the treatment (Fig. 2). At the same time, LPs were increased. Again, this result suggests that some of the possible factors causing the alteration of this molecule with aging in the pineal gland can be the lipid peroxidation process. There is abundant evidence showing that lipid peroxidation products are capable of modifying proteins (Ayala et al., 2014;Sayre et al., 2006) including eEF2 (Arguelles et al., 2006;Arguelles et al., 2009) and, in some cases, breaking the protein backbone directly, resulting in protein fragmentation (Giron-Calle and Schmid, 1996;Grune et al., 2005).

Because its participation in determining many circadian processes in the body, many genes are rhythmically expressed in pineal gland (Bustos et al., 2011;Maronde and Stehle, 2007). Consequently, pineal gland must have proteins with various turnover rates depending on the time of day. This means that in order to work properly, the pineal gland must have optimal translation machinery by which proteins are synthesized at the right moment according to changes in its internal metabolism. Therefore, all elements of the translational machinery should be present at certain levels in this tissue. In a previous study using 2-DE gel and MS analysis Moller and colleagues (Moller and Baeres, 2002) reported in the superficial pineal gland of the Wistar rat an up-regulation of 25 and 35 proteins during the night and daytime respectively. This up-regulation of proteins, both at night and daytime, reflects changes in internal metabolism, protein translation and protein synthesis.

We have analyzed the proteome of the pineal gland in rats killed during nighttime, when the enzymatic activity related to Mel synthesis and secretion are higher (Macchi and Bruce, 2004;Skene and Swaab, 2003). With respect to the CH, several proteins were affected (Fig. 3 and 4). Among these proteins, ten of them were identified as indicated in Table 1, but 15 proteins spots were not identified probably because it is difficult to identify proteins of certain types on 2-DE gels, such as

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membrane proteins, basic proteins and proteins of low concentrations. Most of spots not identified were obvious proteins of low expression levels on the 2-DE gels. Some of the proteins identified are protective and antioxidants, others are involved in metabolism, signalling, and protein processing. A lower expression level was observed in all cases by the action of CH. When comparing the effect of CH and aging, only the expression of aspartate aminotransferase, haptoglobin and glutathione S-transferase changed in the same direction in all situations. In 24-month-old rats, only the changes in the expression levels of calcium binding protein-1, triosephosphate isomerase-1 and peptidylprolyl isomerase-A were opposite of that which occurs during CH treatment. Although the changes observed in various specific proteins may be of value, more research is needed to confirm these changes by additional procedures, as well as to identify and determine the relevance to pineal physiology/biochemistry of these proteins unidentified in this study.

The fact that in the current experimental study the Mel prevented the decrease of eEF2 levels secondary to oxidative stress induced by exposure to CH in the pineal gland, strongly suggests that Mel might have a potential role of avoiding alteration of expression levels of many proteins in this gland as a consequence of oxidative stress and aging, which surely affects the tissue homeostasis. These changes should be especially important in tissues such as the pineal gland where there is a circadian gene expression since the resulting proteins should be finely regulated at specific times of day so that accurate information is sent to all circadian rhythms of body functions. Under our experimental conditions, there is correlation between lipid peroxides levels in serum and in pineal gland of old rats and young rats treated with CH. The present results confirm our previous published findings (Arguelles et al., 2004), showing that the serum levels of oxidative stress biomarker (lipid peroxides) is indicative of oxidative stress in tissues of an individual.

As a potential protective substance to prevent these changes in eEF2, the focus was on Mel because Mel and its metabolites are effective antioxidants able of deactivating a wide variety of reactive

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oxygen species, including hydroperoxyl radical (Galano et al., 2013;Garcia et al., 2014). Previously, we have reported the protective effect of Mel on the molecular changes of eEF2 caused by oxidative stress induced by exposure to CH in the hypothalamus, hypophysis and liver (Arguelles et al., 2011). Our present results show that Mel prevented partial- or totally the effects of oxidative stress on pineal eEF2 induced by CH *in vivo* (Fig. 5). The effect of Mel on lipid oxidative stress agrees with previous reports (Salvi et al., 2001). The effect of melatonin may not only be the consequences of its antioxidant effects but also due to its anti-inflammatory properties (Reiter et al., 2000;Reiter et al., 2013).

As eEF-2 is regulated by several mechanisms such as phosphorylation, ribosylation, and subcellular location, each one having a physiological effect (Arguelles et al., 2013;Arguelles et al., 2014), whether or not Mel affects all these mechanisms under oxidative stress conditions remain to be determined.

Theoretically, if the translation machinery is challenged in general, one of the biological consequences of eEF2 in pineal gland alterations would be a lower production of Mel. To test whether the oxidative stress could be involved in the decline of hormone levels, we measured the levels of Mel in CH-treated rats. Our results show that the levels of this hormone declines with CH treatment (Table 2). A decline in the levels of other hormones as a function of the age has been described previously (Frutos et al., 2007;Matsumoto, 2002;Smith et al., 2005). According to this, once Mel levels go down, the Mel protection on pineal eEF2 would be lost, entering in a vicious cycle which could only be broken by exogenous administration of Mel. Obviously, when Mel is co-administered with CH, serum levels of the hormone are kept above the control values. We cannot affirm that these increased levels are the consequence of Mel protecting the protein synthesis in the pineal gland. In any case, the fact that its serum levels have increased with respect to the CH-treated group allowing Mel to perform its chronobiotic, antioxidant and multifaceted cell protective functions under conditions of elevated oxidative stress (Reiter et al., 2010). Together, these results indicate that pineal eEF2 is affected by aging and oxidative stress induced by exposure to CH, and

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Mel supplementation is able to prevent the effects of oxidative stress on eEF2 in pineal glands induced by CH *in vivo*. It remains to be determined whether Mel administration also prevents changes of eEF-2 due to aging.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Figure 1. Evidence that aging decreases eEF2 levels and increases lipid peroxidation in the pineal gland and serum. Rats of 3 (control) 12 and 24 months were used in this study. **A**: Typical nitrocellulose membrane immunostained with anti-eEF2 antibody and the same lanes stained with Ponceau red. **B**: Optical densities of eEF2 bands were divided by the total proteins in the Ponceau red stained membranes, **C**: Effect of aging on lipid peroxidation in the pineal gland. **D**: Effect of aging on lipid peroxidation in serum. Results are expressed as percentage with respect to the amount found in control and are the mean ± SEM of 5 animals. ANOVA followed by Tukey's test. ** P<0.01, ***P<0.001 vs. control.

Figure 2. Evidence that oxidative stress induced by exposure to CH decreases eEF2 levels and increase lipid peroxidation in pineal gland and serum. 3-month-old rats were treated with CH for 14 and 28 days. **A**: Typical nitrocellulose membrane immunostained with anti-eEF2 antibody and the same lanes stained with Ponceau red. **B**: Optical densities of eEF2 bands were divided by the total proteins in the Ponceau red stained membranes, **C**: Effect of CH on lipid peroxidation in the pineal gland. **D**: Effect of CH on lipid peroxidation in serum. Results are expressed as percentage with respect to the amount found in control and are the mean ± SEM of 5 animals. ANOVA followed by Tukey's test. ***P<0.001 vs. control.

Figure 3. Image of a 2-D silver stained gel of rat pineal gland. Protein solution (80 µg of total protein) was loaded onto pH 3-10 NL strip gels. The second dimension was run onto 10% SDS-gels. An average of 350 spots was detected. Eighty spots were affected by either CH treatment or aging. Twenty-five spots were affected by both CH and aging and 10 of them (green circles) were identified. See Table 1 for identification and significant changes of spots.

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Figure 4. In vivo effect of CH and aging on proteins expression other than eEF-2 in pineal glands. Silver stained two dimensional gels of pineal glands. Protein solution (80 µg of total protein) was loaded onto pH 3-10 NL strip gels. The second dimension was run onto 10% SDS-gels. The spots were analyzed for peptide mass fingerprint and confirmed by tandem mass spectrometry. See Table 1 for identification and significant changes of spots.

Figure 5. Evidence that Melatonin prevents eEF2 decreases and lipid peroxidation increases in the pineal gland and serum associated with oxidative stress induced in vivo by CH. 3-month-old rats were treated with vehicle, CH (15 mg/kg/day, i.p.), Mel (10 mg/kg/day, orally) and CH (15 mg/kg/day, i.p.) + Mel (10 mg/kg/day, orally). **A**: Typical nitrocellulose membrane immunostained with anti-eEF2 antibody and the same lanes stained with Ponceau red. **B**: Optical densities of eEF2 bands were divided by the total proteins in the Ponceau red stained membranes, **C**: Effect of Mel on lipid peroxidation in the pineal gland. **D**: Effect of Mel on lipid peroxidation in serum. Results are expressed as percentage with respect to the amount found in control and are the mean ± SEM of 5 animals. ANOVA followed by Tukey's test. *$P<0.05$, ***$P<0.001$ vs. control, †††$P<0.001$ vs CH- treated.

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Table 1. Oxidative stress and aging affects other proteins than eEF2 in the rat pineal gland.

Spot ID	Protein score	PS C.I%	Protein name	Mr	pI	Accession No.	%Secuence coverage	% Variation CH		% Variation Aging	
								14 days	28 days	12 mo	24 mo
82	342	100	Calcium binding protein-1	47589.9	4.95	gi|488838	32	25.8 ↓	19.1 ↓	29.8 ↑	32.7 ↑
179	110	100	Aspartate aminotransferase	46628.0	6.73	gi|122065118	29	35.8 ↓	14.5 ↓	53.4 ↓	30.9 ↓
213	98	100	Haptoglobin	39052.0	6.10	gi|60097941	25	61.2 ↓	68.4 ↓	12.1 ↓	48.3 ↓
308	305	100	Carbonic anhydrase I	28339.0	6.86	gi|157817869	39	71.1 ↓	46.8 ↓	10.3 ↑	42.5 ↓
317	394	100	Triosephosphate isomerase 1	27344.9	6.89	gi|117935064	57	22.9 ↓	85.4 ↓	15.2 ↑	28.2 ↑
322	229	100	Phosphoserine phosphatase	25179.8	5.49	gi|57527332	35	37.6 ↓	54.6 ↓	30.3 ↑	22.7 ↓
343	142	100	Glutatione-S-transferase	23652.2	6.89	gi|25453420	35	14.7 ↓	20.7 ↓	17.9 ↓	10.1 ↓
355	361	100	Phosphatidylethanolamine binding protein	20902.0	5.48	gi|8393918	36	27.3 ↓	13.3 ↓	20.7 ↑	24.1 ↓
363	206	100	Peroxiredoxin 2	21941.0	5.34	gi|8394432	27	31.6 ↓	15.6 ↓	17.6 ↑	19.5 ↓
387	318	100	Peptidylprolyl isomerase A	18091.0	8.34	gi|8394009	51	14.4 ↓	24.0 ↓	19.7 ↑	28.7 ↑

Analyses of the 2DE gels were performed using the software Progenesis SameSpots, which allows automated image alignment, background subtraction and spot quantification and comparison. Results are expressed as percentage with respect to the amount found in control, and are the mean ± SEM of five experiments. ANOVA followed by Tukey's test. $P<0.05$ vs. Control. PS C.I%, protein scores confidence interval; Mr, nominal mass; pI, calculated isoelectric point value.

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Table 2. Levels of Melatonin in serum

	Melatonin
Control	148.8 ± 4.4
CH	61.8 ± 5.4***
Mel	247.6 ± 7.1***
Mel + CH	170.8 ± 8.1

Rats (3 months old rats) were treated with vehicle, CH (15 mg/kg/day, i.p.), Mel (10 mg/kg/day, orally) and CH (15 mg/kg/ day, i.p.) – Mel (10 mg/kg/day, orally). Mel was determined as described in Materials and Methods. The results (pg/ml) are means ± SEM of five experiments. ***Significantly different from rats control. ANOVA followed by Tukey's test (P<0.001).

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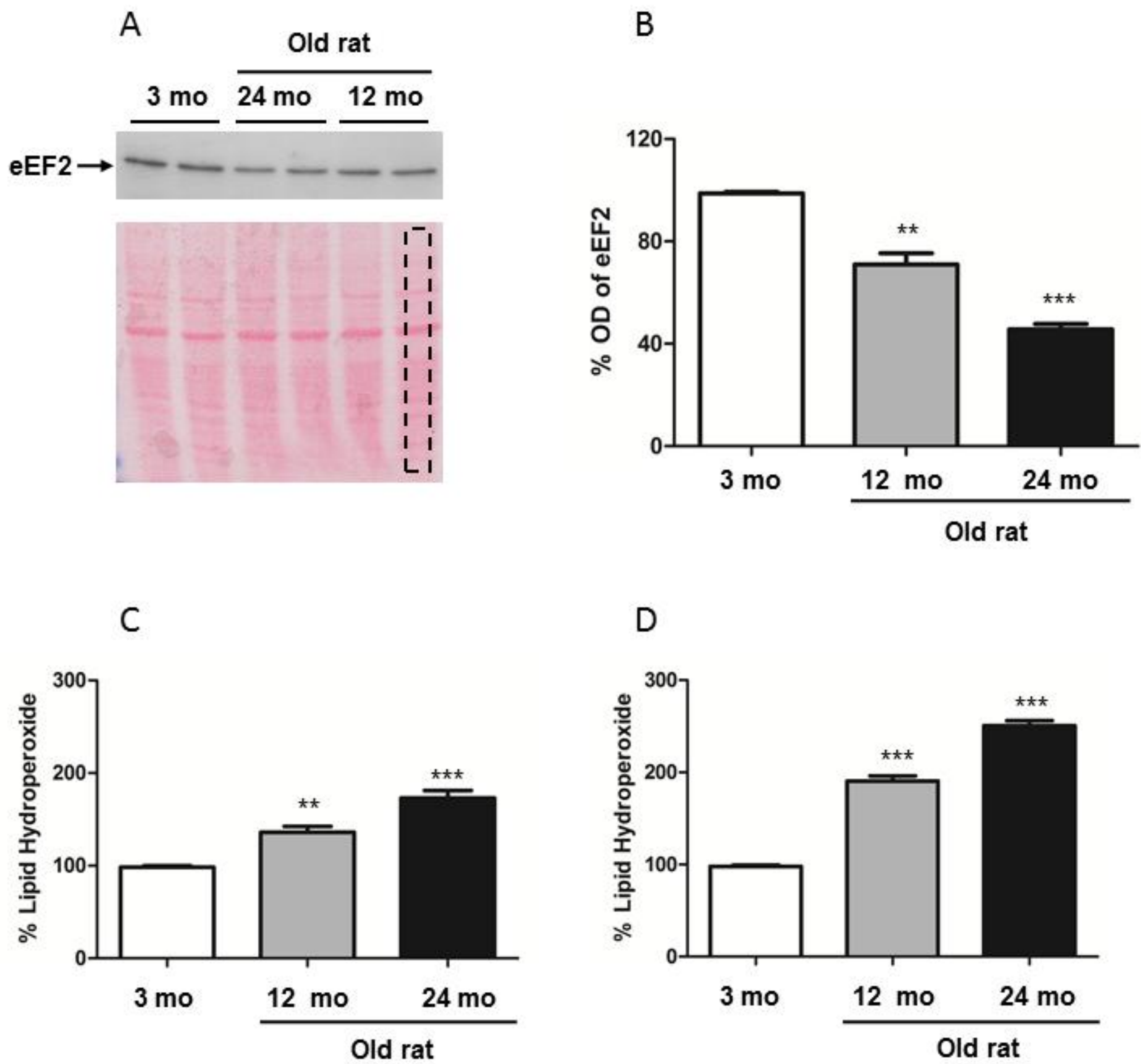

Figure 1

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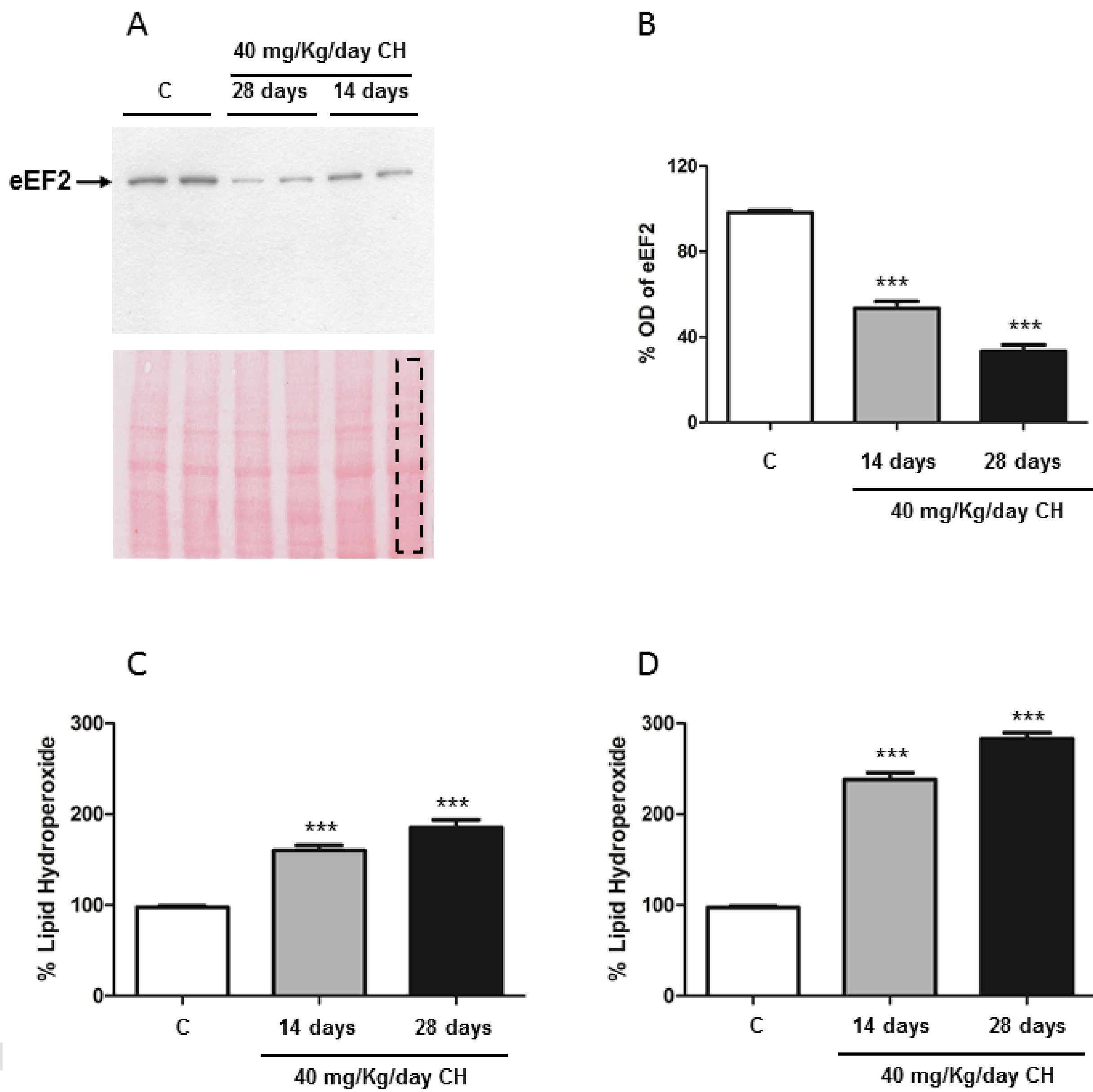

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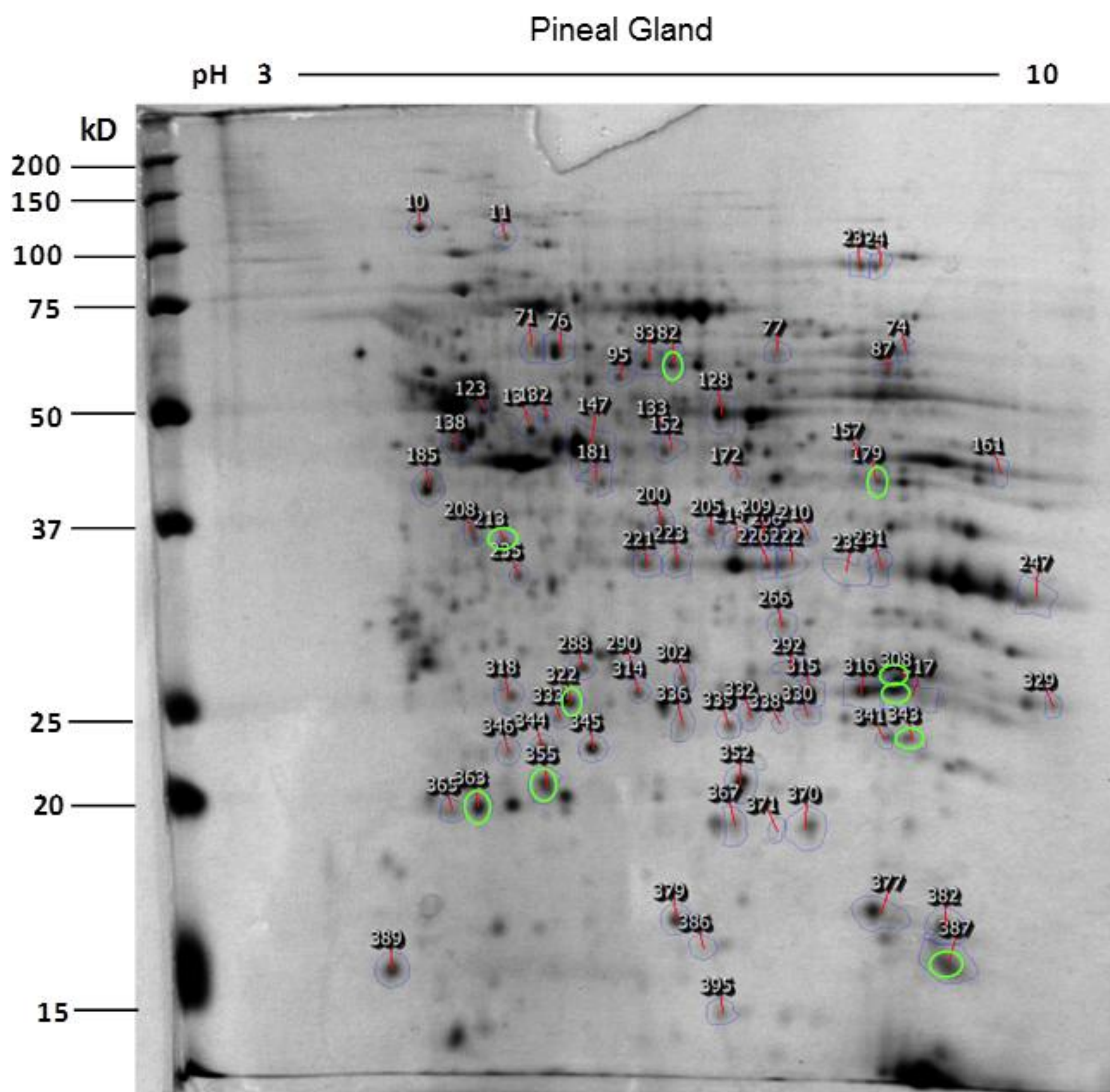

Figure 3

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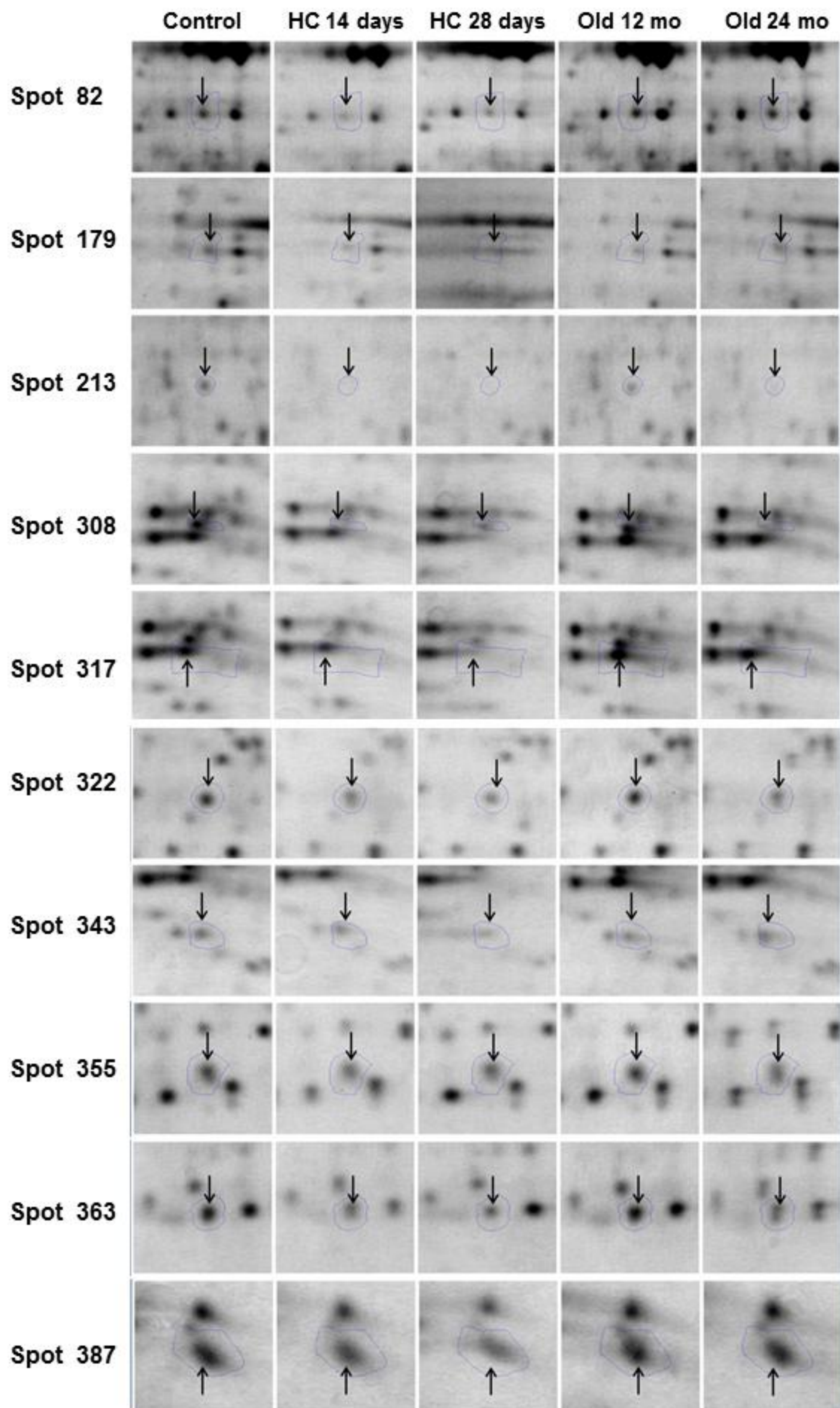

Figure 4

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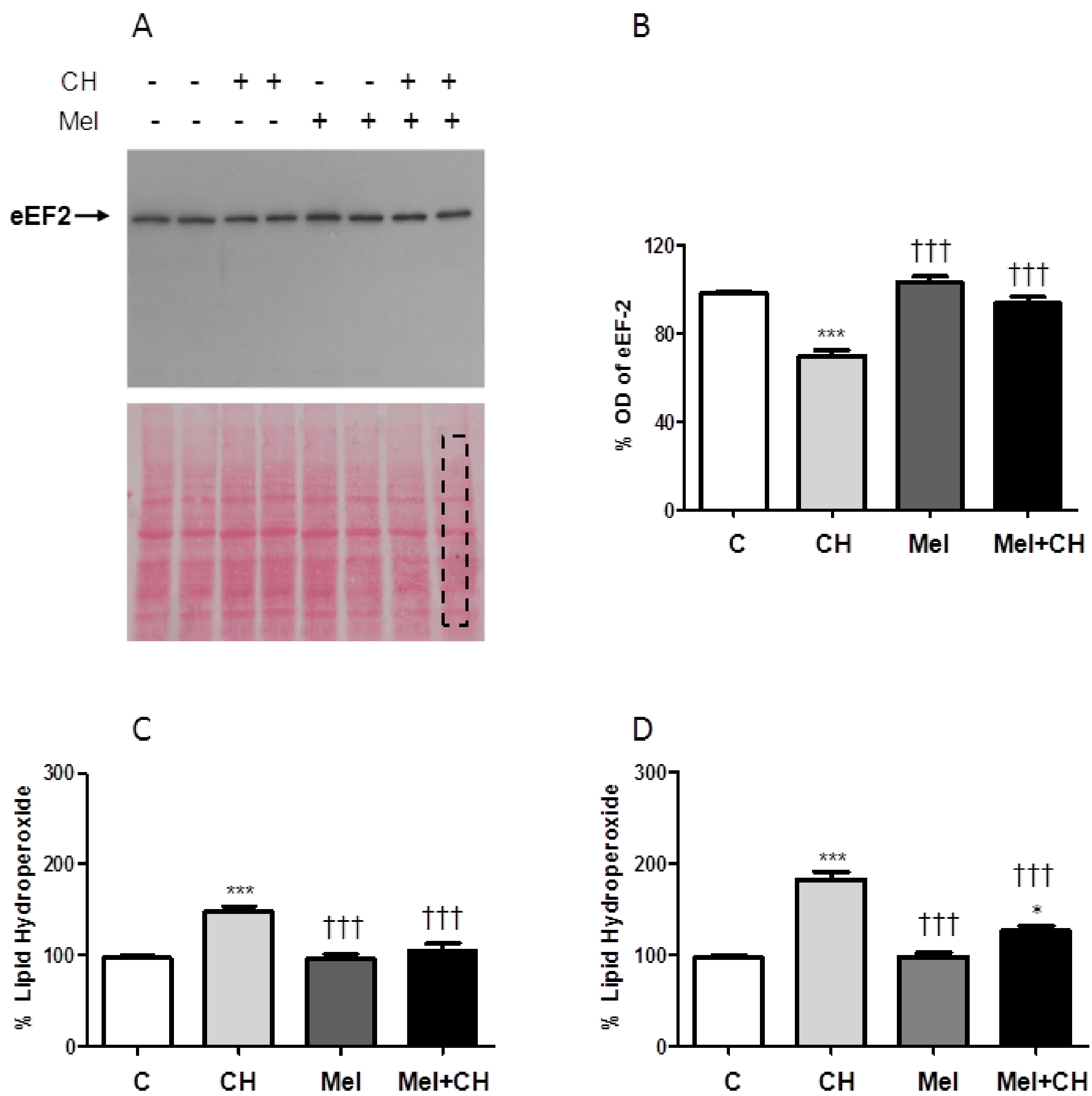

Figure 5

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