IN VITRO AND IN VIVO PROTECTION BY MELATONIN AGAINST THE DECLINE OF ELONGATION FACTOR-2 CAUSED BY LIPID PEROXIDATION: PRESERVATION OF PROTEIN SYNTHESIS.

Sandro Argüelles[1], Mario F. Muñoz[1], Mercedes Cano[2], Alberto Machado[1] and Antonio Ayala[1]

[1]Departamento de Bioquímica y Biología Molecular, Facultad de Farmacia, Universidad de Sevilla, Sevilla, Spain

[2]Departamento Fisiología y Zoología, Facultad de Farmacia, Universidad de Sevilla, Sevilla, Spain

Abstract: As organisms age, a considerable decrease in protein synthesis takes place in all tissues. Among the possible causes of the decline of translation in old animals are the modifications of elongation factor-2 (eEF-2). eEF-2 occupies an essential role in protein synthesis where it catalyzes the ribosomal translocation reaction. eEF-2 is particularly sensitive to increased oxidative stress. However, all oxidants do not affect eEF-2, only compounds that increase lipid peroxidation. As peroxides are unstable compounds, they decompose and generate a series of highly reactive compounds, including aldehydes malondialdehyde (MDA) and 4-hydroxynoenal (HNE). We have previously reported that hepatic eEF-2 forms adducts with low-molecular weight aldehydes, MDA and HNE. Therefore, the protection of eEF-2 must be specifically carried out by a compound with lipoperoxyl radical-scavenging features such as melatonin. In this article, we show the ability of melatonin to protect against the changes that occur in the eEF-2 under conditions of lipid peroxidation induced by cumene hydroperoxide (CH), a compound used experimentally to induce lipid breakdown. As experimental models, we used cultured cells and rats treated with this oxidant compound. eEF-2 levels, adduct formation of this protein with MDA and HNE, and lipid peroxides were determined. In the cultured cells, protein synthesis rate was also measured. Our results show that melatonin prevented the molecular changes in eEF-2 and the decline in protein synthesis rate secondary to lipid peroxidation. The results also show that serum levels of several hormones were affected by CH-induced oxidative stress, which was partially or totally prevented by melatonin.

Key words: 4-hydroxynoenal, adduct, cumene hydroperoxide, elongation factor 2, ghrelin, growth hormone, lipid peroxides, malondialdehyde, melatonin, testosterone, thyroxine

INTRODUCTION

One of the biochemical pathways that is universally affected by aging in all organisms is protein synthesis [1–3], which is essential for the maintenance of cellular homeostasis. In old animals, protein synthesis is decreased around 60–80% [4]. Previous reports from our laboratory have shown that the elongation step is especially affected by aging [5]. This effect seems to be related to post-translational alterations of elongation factor-2 (eEF-2). eEF-2 occupies an essential role in protein synthesis where it catalyzes the ribosomal translocation reaction, resulting in the movement of ribosomes along the mRNA [6]. This protein is regulated by several mechanisms, which suggest that it may be involved in other biochemical reactions besides translation [7]. eEF-2 is particularly sensitive to increased oxidative stress that occurs during aging; however, all species of oxidants do not affect eEF-2. Only compounds that increase lipid peroxidation such as cumene hydroperoxide (CH) and t-butyl- hydroperoxide affected eEF-2 [8].

Lipid peroxidation is a phenomenon that occurs as a consequence of free radical attacks on lipids. Its toxicity goes beyond the site of lipid peroxides (LP) origin and it is translocated to subcellular sites [9, 10]. Lipid peroxidation has been linked to a variety of disorders, including atherogenesis, ischemia–reperfusion injury, and UV-induced carcinogenesis [11, 12]. Generated peroxides trigger a sequential cascade of new free radicals and more peroxides. As peroxides are unstable compounds, they decompose and generate through uncontrolled nonenzymatic reactions, a series of highly reactive compounds including aldehydes malondialdehyde (MDA) and 4-hydroxynoenal (HNE), which contribute to the deleterious effects of oxidative stress.

In a previous report, we have shown that the formation of adducts between MDA and HNE with hepatic eEF-2 in 12- and 24-month-old rats [13]. Concomitant to the adduct formation, a decrease in the levels of eEF-2 was found and an increase in the amount of lipid peroxidation. These results suggest that one possible mechanism responsible for the decline of protein synthesis during aging could be the alteration of eEF-2 levels, secondary to lipid peroxidation and adduct formation with these aldehydes.

One consequence of these findings is that protection of eEF-2 must be specifically carried out by a compound with lipoperoxyl radical-scavenging features. In this article, the focus is on the possible role of melatonin in preventing eEF-2 oxidative changes. Melatonin participates in several important, physiological functions and also is an effective antioxidant [14–17]. Many of its proposed therapeutic or preventive uses are based on this antioxidant activity [18–21]. Melatonin has consistently been found to be highly efficient in limiting the peroxidation of lipids [22–24]. This may be a result of its ability to scavenge the initiating agents, for example, $OH^{\cdot}$, ONOO–, etc., rather than being because of its apparent limited capability as a direct $LOO^{\cdot}$ scavenger [24]. Because melatonin can diffuse through biological membranes, it exerts actions in almost every cell and cellular organelle in the body [25, 26]. We have previously reported on a daily circadian rhythm of oxidative stress where levels of this stress increased throughout the day [27], when circulating melatonin levels are at their lowest [28, 29]. We hypothesized the release of melatonin by the pineal gland during darkness may be a key step in determining this oxidative stress cycle.

To test the role of melatonin in preventing the oxidative damage of eEF-2 caused by lipid peroxidation, cell culture and young rats were treated with CH, a compound that has been often used in experimental models to induce lipid peroxidation. For the in vivo experiments, several tissues of the rats (i.e. liver, hypothalamus, and hypophysis) were used in our study. Hypothalamus and hypophysis were chosen because they secrete peptide hormones whose synthesis requires the integrity of the translation machinery. Liver was studied because of its

high activity in synthesizing proteins. In these tissues, eEF-2 levels, adduct formation of this protein with MDA and HNE, and LP were determined and the results were compared with rats cotreated with melatonin. At the same time, serum levels of several hormones were determined. In cultured cells, the protein synthesis rate was also measured.

MATERIALS AND METHODS

Animals

Three-month-old male Wistar rats weighing 250–300 g were used in this study. The rats were maintained under an automatically controlled temperature (22–23°C) and in a 12-h light–dark cycle. Animal care complied with the Guide for the Care and Use of Laboratory Animals (National Academy Press, Washington, DC, USA, 1996).

Treatment of cell culture

The human cervical cancer (HeLa) cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Life Technologies Invitrogen, Inc., Paisley, UK), supplemented with 10% fetal bovine serum, 50 U/mL penicillin G sodium, 50 µg/mL streptomycin, and 2 mM L-Glutamine (Life Technologies Invitrogen, Inc.). The cells were cultured in a humidified atmosphere at 37°C and 5% CO2. The cell cultures were treated for 3 hr with melatonin (50 and 10 µM) diluted in DMEM. Cells were washed three times with PBS and treated with 5 mM CH in DMEM 10 min at 37°C. The cell cultures were again washed three times with PBS.

Treatment of animals

Rats were randomly divided into four groups. The first group was designated as the control group, which received saline solution (i.p.) and water daily (equal volume of melatonin solution, orally with a feeding tube) for 28 days. The second group was treated with CH (15 mg/kg/day, i.p.) for 28 days and 7 days after starting the treatment, water was administered daily (equal volume of melatonin solution) until the end of the treatment. The third group received a daily saline solution (i.p.) for 28 days and 7 days after starting the treatment, melatonin was administered (10 mg/kg/day, orally with a feeding tube) until the end of the treatment. The fourth group received CH (15 mg/kg/day, i.p.) for 28 days and 7 days after starting the treatment, melatonin was administered (10 mg/kg/day, orally with a feeding tube) until the end of the treatment. After the treatment, the rats were decapitated and tissue samples were stored at −80°C until biochemical determinations were performed.

Western blotting

Fifty microgram of total proteins was loaded on 10% SDS-PAGE and transferred to a nitrocellulose membrane (Hybond-c extra, Amersham Life Science). The transfer buffer was 25 mM Bicine, 25 mM Bis–Tris, 1 mM EDTA, pH 7.2. The power conditions were 60 V, 2 hr. After transfer, membranes were stained with Ponceau red to indicate that the same amount of protein is present across the 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) 1 hr at room temperature. The membranes were then incubated with the polyclonal anti-eEF-2 antibody at a dilution 1:1000 for 3 hr at room temperature. After incubation with the primary antibodies, the membranes were washed in 20 mM Tris–HCl, pH 7.5, 500 mM NaCl, and 0.05% Tween-20 and incubated with peroxidase-conjugated anti-Rabbit immunoglobulins secondary antibodies (Dako, Produktionsvej, Denmark). The proteins were visualized using the

chemiluminescence's kit of Pierce (Rockford, IL, USA). All experiments were repeated in triplicate. The bands were analyzed by densitometry using AnalySIS software (Soft Imaging System GmbH, Münster, Germany). The amount of protein layered was controlled by quantification of three easily distinguishable bands in the Ponceau red stained membranes.

Determination of lipid hydroperoxide by oxidation of Fe2+ in the presence of xylenol orange (FOX reagent)

Lipid hydroperoxides were determined as previously described [30]. FOX reagent was prepared with 100 µM xylenol orange, 4 mM butylated-hydroxyl-toluene, 25 mM sulphuric acid, and 250 µM ammonium ferrous sulphate. The samples were mixed with 900 µL of FOX reagent and 50 µL of methanol. After mixing, the samples were incubated at room temperature for 30 min. The vials were centrifuged at 2400 g for 10 min. Absorbance of the supernatant was measured at 560 nm ($\varepsilon = 4.3 \times 104/M/cm$).

Analysis of adduct formation with eEF-2

Tissue homogenates (350 µg) were incubated in RIPA buffer (10 mM Tris, 1% Triton X-100, 1.2 mM deoxycholic acid, 0.1% SDS, 10 mM EDTA, 0.02% NaN3, 50 mM NaCl, 1 mM PMSF) with either 10 µL of polyclonal goat anti-MDA or 10 µL of polyclonal goat anti-HNE on a rotator for 2 hr at 4°C. Then, 30 µL protein A (10%) beads (Sigma-Aldrich, St Louis, MO, USA) was added and incubated on a rotator overnight at 4°C. After incubation, the immune complexes were collected by centrifugation, washed five times with 10 mM HEPES buffer, resuspended and separated by 10% SDS–PAGE. The eEF-2 levels were analyzed by immune staining on nitrocellulose membrane.

Measurement of [3H]-Leucine incorporation

HeLa cells, grown in 12-well culture dishes were washed three times with PBS and then incubated for 0, 15, 30, 45, and 60 min in DMEM in the presence of [3H]-Leucine (2 µCi/mL; MoravekBiochemical's, Inc., Brea, CA, USA) at 37°C. Labeling was stopped by the addition of 1.5 mL ice-cold 1 M trichloroacetic acid. Cells were scraped and suspensions were centrifuged for 5 min at 4000 g. The precipitate was washed three times with 250 µL of 0.6 M trichloroacetic acid. The final pellet was washed with 70% ethanol and solubilized with 0.5 M NaOH at 37°C for the determination of proteins and radioactivity (Beckman scintillation counter LS 6500 Multi-purpose, Beckman Coulter, Inc., Brea, CA, USA). As a control, protein synthesis inhibition was performed using 5 µM cycloheximide (Riedel-de Haën, Basel, Switzerland).

Hormone analysis

Serum hormones were measured by enzyme-linked immunosorbent assay (ELISA) following manufacturer's instructions: Testosterone (Alpha Diagnostic International, San Antonio, TX, USA), growth hormone, ghrelin (Millipore, St Charles, MO, USA), melatonin, and thyroxine (GenWay Biotech Inc., San Diego, CA, USA).

Protein determination

Protein content of the samples was measured by Lowry's method using BSA as the standard [31].

Statistical analysis

One-way analysis of variance (ANOVA) followed by Tukey's test were used to compare the results.

RESULTS

The protective effect of melatonin was measured in cultured cells. The levels of eEF-2 were determined by quantifying optical densities of the eEF-2 band in nitrocellulose membranes immune-stained with anti-eEF-2 antibody (Fig. 1A). As shown in Fig. 1B, CH leads to a 72% decrease in the levels of eEF-2. To control that the differences in signal corresponding to the eEF-2 are not because of a different loading of proteins, the optical density of eEF-2 bands were divided by the average of optical density of three distinguishable bands in the Ponceau red stained membranes (Fig. 1A). This result is similar to that already found by our group in liver homogenates treated directly with CH [5]. Pretreatment of the cell cultures with the two doses of melatonin (50 and 10 μM) partially prevented this decrease in eEF-2 levels caused by CH so that its levels were 40% and 44% lower than controls (Fig. 1B). This protective effect was not proportional to the dose of melatonin used. Melatonin alone had no effect on the levels of eEF-2.

We concomitantly measured changes in eEF-2 and global protein synthesis rates treatment of CH. CH produced a marked decrease in protein synthesis rate, which was 85% less than control (Fig. 1C). This result agrees with those obtained previously by other authors [32]. Pretreatment of these cells with melatonin partially prevented this inhibition of protein synthesis with no significant differences between the two doses. It is noteworthy that melatonin alone did not affect the protein synthesis rate. Controls of the effect of ethanol, in which melatonin was dissolved, were made to take away its effect on the results.Melatonin also exerted a downward effect of LP. LP was induced by CH (440% of the control). This increase was partially prevented by the two doses of melatonin and the LP remained elevated with respect to controls. Thus, LP was 274% and 211% of the control value when cells were cotreated with 50 and 10 μM melatonin, respectively (Fig. 1D). No effect was observed in cells treated with melatonin alone.

To test whether the protective effect of melatonin observed in vitro also occurred in vivo, the rats were treated with CH and CH plus melatonin as described in Materials and methods. The levels of eEF-2, adduct formation and LP were determined in hypophysis, hypothalamus, and liver.

Like cultured cells, eEF-2 levels in the hypophysis were quantified by measuring the optical density of the bands present in nitrocellulose membranes (Fig. 2A). Again, the amount of protein layered was controlled by quantification of three easily distinguishable bands in the Ponceau red stained membranes. In this tissue, the treatment with CH affected eEF-2 levels so that they were 55% with respect to controls (Fig. 2B). While treatment with melatonin alone did not significantly affect the eEF-2, this indoleamine prevented the loss of this protein under lipid peroxidation conditions (Fig. 2B) when coadministered with CH. Also, it is noteworthy that melatonin completely prevented the formation of adducts between HNE and MDA with the eEF-2 (Fig. 2C,D). These adducts are formed with many proteins and relevant molecules in a reaction that usually is irreversible and leads to their inactivation. The levels of adducts between eEF-2 and the MDA and HNE increased 38% and 40%, respectively, after treatment with CH in the pituitary (Fig. 2C,D). While basal levels of adducts were not diminished by melatonin alone, this hormone prevented the increase generated by CH. In regards to LP, the same protective effect was observed. CH treatment increased LP in vivo up to a value that was 66% higher than controls (Fig. 2E). This increase was completely prevented by cotreatment with Mel, the effect being more effective in vivo than in vitro.

In hypothalamus, only a single band was obtained when nitrocellulose membranes were immunostained with anti-eEF-2 (Fig. 3A). The amount of protein layered was controlled by quantification of three easily distinguishable bands in the Ponceau red stained membranes. In this tissue, the decrease in eEF-2 caused by CH was 40% (Fig. 3B) where melatonin was able to prevent this decline. Similarly, melatonin also prevented the formation of adduct between eEF-2 and MDA and HNE (Fig. 3C,D). These adducts were increased up to values that were 157% of the control values in the case of MDA (Fig. 3C) and 166% in the case of HNE (Fig. 3D). The cotreatment with melatonin prevented any molecular changes on the eEF-2 molecule. The LP increased with CH dose in this tissue, reaching levels 98% higher than controls (Fig. 3E). This increase was completely prevented by cotreatment with melatonin.

In liver, the levels of eEF-2 band were quantified from the nitrocellulose membranes (Fig. 4A). CH affected levels of this protein so that the levels were 35% of control values (Fig. 4B). Cotreatment with melatonin partially prevented this decline but eEF-2 levels remain significantly lower than control (50%). Regarding the formation of adducts, surprisingly, in liver, CH induced the adduct formation to a greater extent than on the hypophysis and hypothalamus: 626% with respect to controls in the case of MDA and 794% in the case of HNE (Fig. 4C,D). At the same time, the effect of the cotreatment with melatonin was lower and that level of these adducts remained high compared with controls: 374% in the case of MDA and 427% in the case of HNE. However, the increase in LP caused by CH was similar to that found in the hypothalamus: 200% with respect to control (Fig. 4E), this increase being prevented by cotreatment with melatonin.

Serum levels of several hormones were measured in the experimental groups. As seen in Fig. 5A–E, CH treatment leads to a decline in the levels of testosterone (82%), growth hormone (88%), ghrelin (54%), melatonin (60%), and thyroxin (47%), respectively. In general, the treatment with exogenous melatonin alone did not affect the serum levels of these hormones except in the case of ghrelin, whose levels were significantly reduced by melatonin (23% decrease). Obviously, the amount of serum melatonin was increased by 59% after the treatment with exogenous melatonin. The cotreatment of melatonin partially prevented the effect of CH on testosterone, growth hormone, and ghrelin. The preventive action of melatonin cotreatment was total in the case of thyroxine.

DISCUSSION

Herein, we show the ability of melatonin to ameliorate the changes that occur in the eEF-2 under conditions of lipid peroxidation induced by CH. As experimental models, we used cultured cell and rats treated with this oxidant compound. For the in vivo study, the hypothalamic/pituitary axis was selected, which is a major part of the neuroendocrine system [33]. The output of this unit regulates many body functions [34, 35]. One common feature of hormones secreted by this system is that they are peptides whose size ranges from 9 to 56 amino acids. To secrete the right amount of hormones at a particular moment, all elements of the translational machinery should be present at optimal levels in these tissues. At the same time, the study was performed in liver, a tissue where protein synthesis is quite active.

Elongation factor-2 catalyzes the movement of the ribosome along the mRNA. One feature of this protein is that it is extremely sensitive to oxidation, mainly to lipid peroxidations [8]. As a potential protective substance to prevent these changes in eEF-2, the focus was on melatonin because of its antioxidant properties, in general, and its effect against lipid peroxidation, in particular. The antioxidant capacity of melatonin has been described extensively. It is

documented that melatonin and its metabolites have direct scavenging actions against the free radicals and related products [35, 36]. Thus, melatonin protects organisms against hydroxyl radicals [26, 37]. Also, it can act indirectly by inducing antioxidant enzymes [35]. Melatonin has the capability of increasing either mRNA levels or the activities of these major antioxidant enzymes [38]. In addition, melatonin stimulate glutathione synthesis [39]. These protective functions of melatonin against oxidative stress are independent of receptor proteins because it can reach all parts within the cell [40, 41]. Melatonin also has been shown to reduce the accumulation of the major products of lipid peroxidation when membranes are exposed to radical-generating agents [38]. Whereas some authors claim that melatonin's ability as a lipoperoxyl radical scavenger is weak [42], others demonstrate that melatonin is twice as effective as vitamin E in interfering with the propagation [43].

Our results show that melatonin prevented partially or totally the effects of LP on eEF-2 induced by CH in both cell cultures and in vivo. Although cells in culture may behave differently from cells in vivo in many ways [44], our results show similar results regarding the effect of the CH and the protective effect of melatonin. The protective effect of melatonin was produced in all tissues studied, probably as a consequence that melatonin quickly gets into cells with only minor differences between organs in terms of the quantity of melatonin taken up [26, 45]. The effect of melatonin on protein oxidative stress agrees with previous reports [46].

It should be noted that melatonin alone did not lower the baseline levels of LP with respect to controls, at least at the dose used in the experiments, but it prevented the increase of these markers when oxidative stress was induced. This fact should be considered for those cases in which the organism is subjected to an oxidative stress-inducing treatment because melatonin, in these cases, would avoid the side effects of such treatments.

Besides this general protective effect of melatonin against oxidative stress, our results show a specific effect of melatonin against the eEF-2 decline in both cultured cells and in tissues of rats treated with CH (1-4). The effect of melatonin was observed in all tissues, which emphasizes its role as a general antioxidant protective compound [40, 41, 47].

In cell cultures, our results show that lower levels of eEF-2 after CH treatment than in control, which is concomitant to the decrease in the rate of protein synthesis. Pretreatment of the cells with melatonin ameliorated the changes in eEF-2 and protein synthesis inhibition caused by CH. The importance of eEF-2 in determining the protein synthesis rate has been demonstrated previously by our group so that alterations in this protein are the cause of the global translation being altered with aging and oxidative stress [48]. In fact, the correlation analysis, made by pooling all the results of Fig. 1B,C, shows that eEF-2 levels and protein synthesis rate correlate positively ($r = 0.985$, $P < 0.05$).

The mechanism by which lipid peroxidation alters eEF-2 is through the formation of adducts with low-molecular weight aldehydes MDA and HNE that are formed by decomposition of LP. This has been shown in vitro where direct treatment of the homogenates with these aldehydes lead to the same effect as CH treatment [8]. Also, the formation of these adducts with eEF-2 has recently been demonstrated in vivo after treatment with CH [13].The formation of these adducts with biomolecules has been described in many conditions and are the cause of many physiological disorders. These aldehydes have the capacity to form adducts with macromolecules [49–51] and are responsible for much of the pathology associated with oxidative stress [52]. In the case of proteins, this is an irreversible reaction between these adducts and certain amino acid residues [53, 54]. Because the consequences of slower rates of protein synthesis are manifold in context of aging and age-related diseases, it would be

interesting to establish interventions that might slow the effects of oxidative stress and adduct formation on translation. In regard to the specific effect on the eEF-2, melatonin prevents the formation of HNE and MDA adducts with this protein (2-4, C and D) and probably will do the same with other proteins. Accordingly, maintaining optimal levels of melatonin may be essential in minimizing in vivo the deleterious consequences of lipid peroxidation. It is well known that a substantial loss of melatonin occurs with age. Obviously, given that melatonin levels decline with age [55, 56], this protection should diminish as the organism ages. Also, because melatonin production exhibits considerable interindividual differences [26], the degree of protection of eEF-2 will depend on each individual melatonin level. In conclusion, our results point out that melatonin supplementation can be an effective treatment to prevent these molecular changes in eEF-2 secondary to lipid peroxidation, which may be the mechanism by which protein synthesis decays during aging. This is corroborated by our experiments in cultured cells, where the rate of global protein synthesis is maintained by cotreatment with melatonin.

One of the biological consequences of eEF-2 alterations in the hypothalamic/pituitary axis would be a lower production of peptide hormones, such as GH. The results show that its serum levels are affected by CH treatment and could be due to the alterations of the protein translation in this axis. Similarly, the same effect is observed in the case of ghrelin, another peptide hormone secreted by the stomach. Besides GH, CH affects other endocrine system, nonpeptide hormones, such as testosterone, melatonin, and thyroxine, all of them being decreased by CH. Obviously, many of these hormones are interdependent and several other factors can contribute to hormone decline. For instance, oxidative stress can damage testosterone secreting Leydig cells and supporting Sertoli cells. Changes in testosterone may also be due to pattern of LH input, for instance. Consequently, it is not possible to establish which percentage of hormone decline is exclusively due to protein synthesis inhibition and adduct formation between aldehydes and eEF-2. However, the results show that melatonin is effective in preventing those changes that occurs on eEF-2 as a consequence of oxidative stress, being this effect accompanied by a total or partial concomitant prevention of hormone decline caused by lipid peroxidation.

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FIGURE LEGENDS

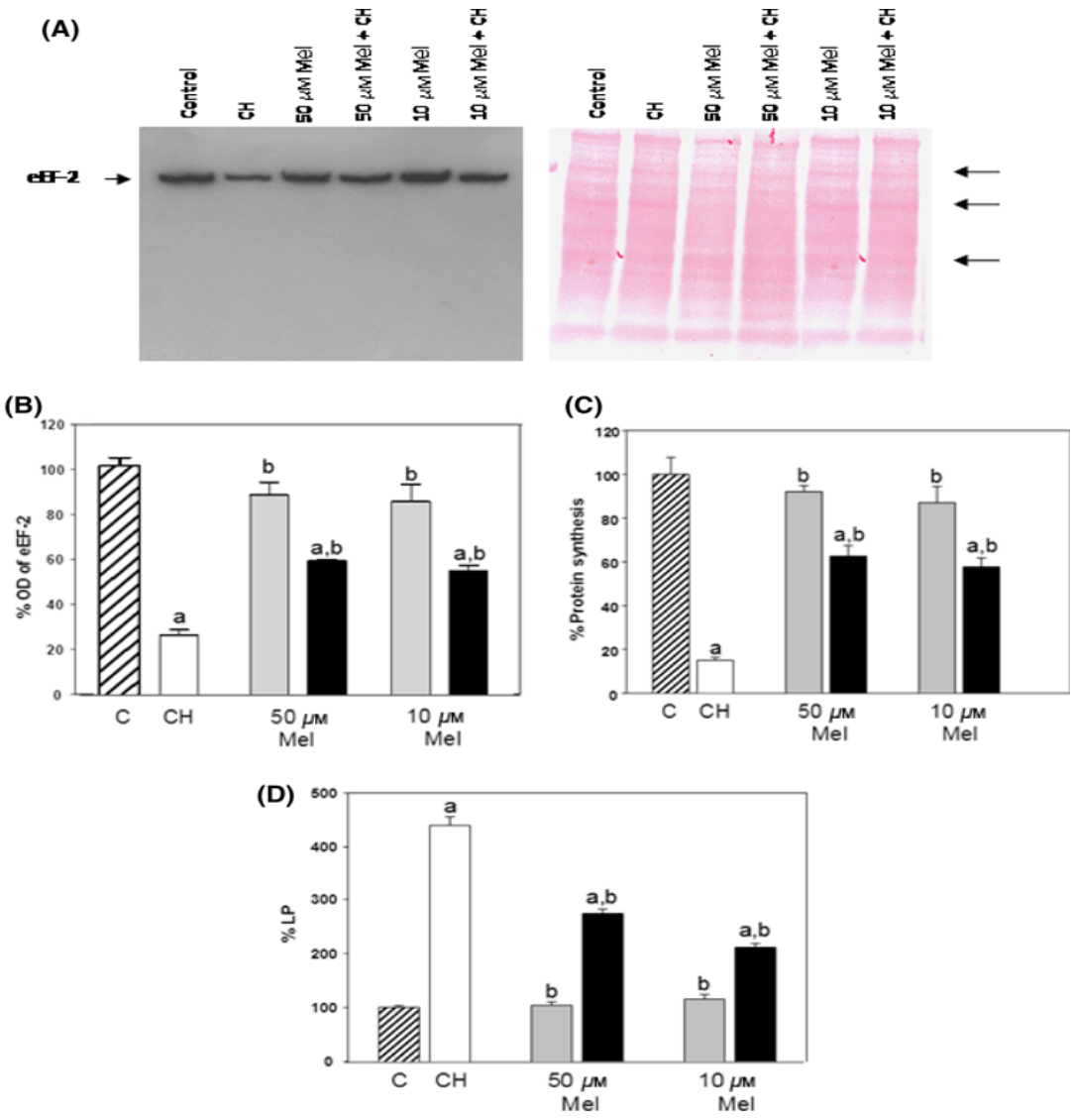

Fig. 1. Effect of cumene hydroperoxide (CH) and CH plus melatonin on elongation factor-2 (eEF-2) levels, protein synthesis, and lipid peroxidation in cell culture. (A) Typical nitrocellulose membrane immunostained with anti-eEF-2 antibody and the same membrane stained with Ponceau red. The arrows show bands used for quantification. (B) Effect of CH and CH plus melatonin on the level of eEF-2. (C) Effect of CH and CH plus melatonin on protein synthesis. (D) Effect of CH and CH plus melatonin on lipid peroxidation. eEF-2, protein synthesis and lipid peroxidation were determined as described in the Materials and methods section. Results are expressed as percentage with respect to control and are the mean ± S.E.M. Gray bars: Cells treated with melatonin alone; Black bars: melatonin plus CH-treated cells. a Significantly different from control (ANOVA followed by Tukeys test, $P < 0.001$), b Significantly different from CH-treated (ANOVA followed by Tukeys test, $P < 0.001$).

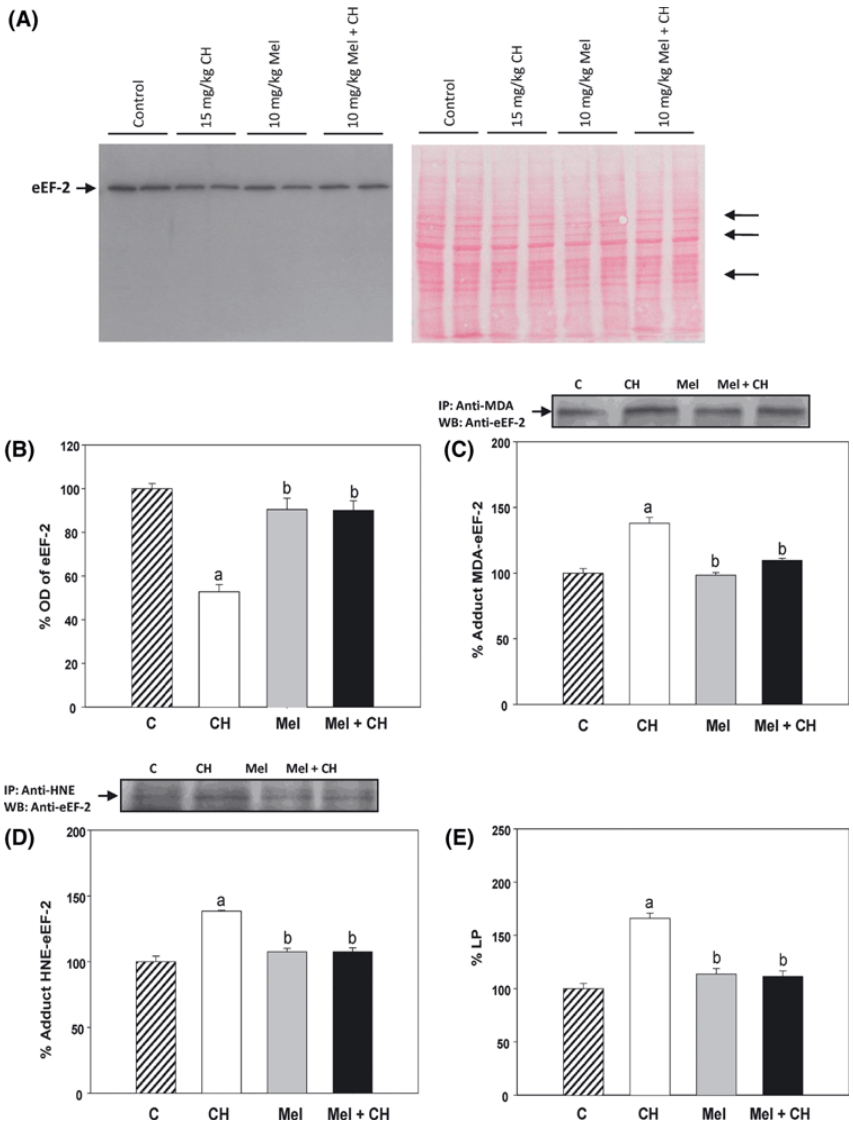

Fig. 2. **In vivo effect of cumene hydroperoxide (CH) and CH plus melatonin on elongation factor-2 (eEF-2) levels, adducts formation, and lipid peroxidation in hypophysis.** (A) Typical nitrocellulose membrane immunostained with anti-eEF-2 antibody and the same membrane stained with Ponceau red. (B) Effect of CH and CH plus melatonin on the level of eEF-2. (C, D) Effect of CH and CH plus melatonin on adducts formation between MDA and HNE with eEF-2. 350 lg of total hypophysis protein were immunoprecipitated with anti-MDA or anti-HNE, and the immune complex was resolved by 10% SDS-PAGE and immunoblotted with anti-eEF-2. (E) Effect of CH and CH plus melatonin on lipid peroxidation. Results are expressed as percentage with respect to the amount found in control and are the mean ± - S.E.M. of five animals. Gray bars: animals treated with melatonin alone; Black bars: Melatonin plus CH-treated rats. a Significantly different from control (ANOVA followed by Tukeys test, $P < 0.001$), b Significantly different from CH-treated (ANOVA followed by Tukeys test, $P < 0.001$).

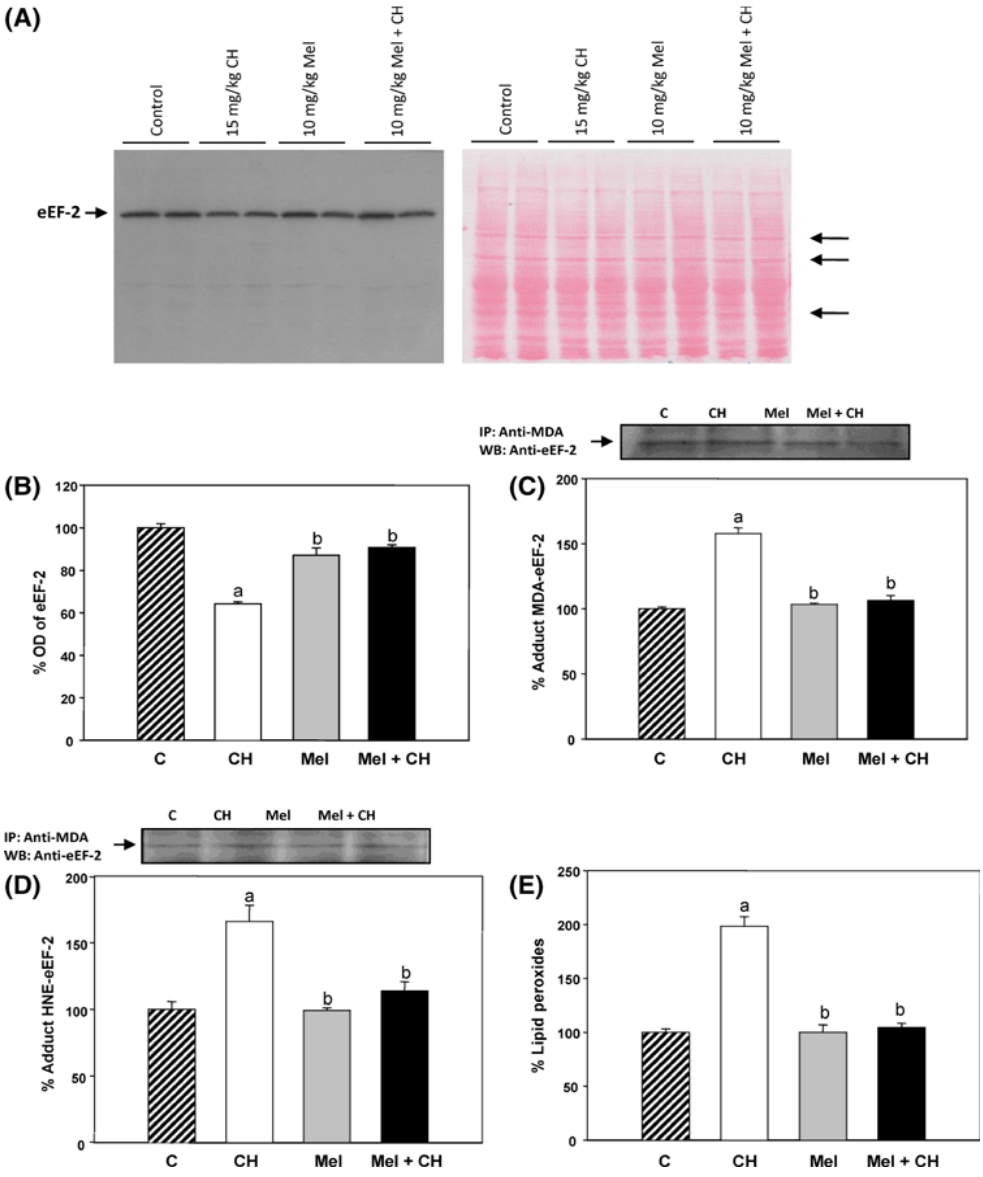

Fig. 3. In vivo effect of cumene hydroperoxide (CH) and CH plus melatonin on elongation factor-2 (eEF-2) levels, adducts formation and lipid peroxidation in hypothalamus. (A) Typical nitrocellulose membrane immunostained with anti-eEF-2 antibody and the same membrane stained with Ponceau red. (B) Effect of CH and CH plus melatonin on the level of eEF-2. (C, D) Effect of CH and CH plus melatonin on adducts formation between MDA and HNE with eEF-2. 350 lg of total hypothalamus protein was immunoprecipitated with anti-MDA or anti-HNE, and the immune complex was resolved by 10% SDS-PAGE and immunoblotted with anti-eEF-2. (E) Effect of CH and CH plus melatonin on lipid peroxidation. Results are expressed as percentage with respect to the amount found in control and are the mean ± - S.E.M. of 5 animals. Gray bars: Rats treated with melatonin alone; Black bars: melatonin plus CH-treated rats. a Significantly different from control (ANOVA followed by Tukeys test, $P < 0.001$), b Significantly different from CH-treated (ANOVA followed by Tukeys test, $P < 0.001$).

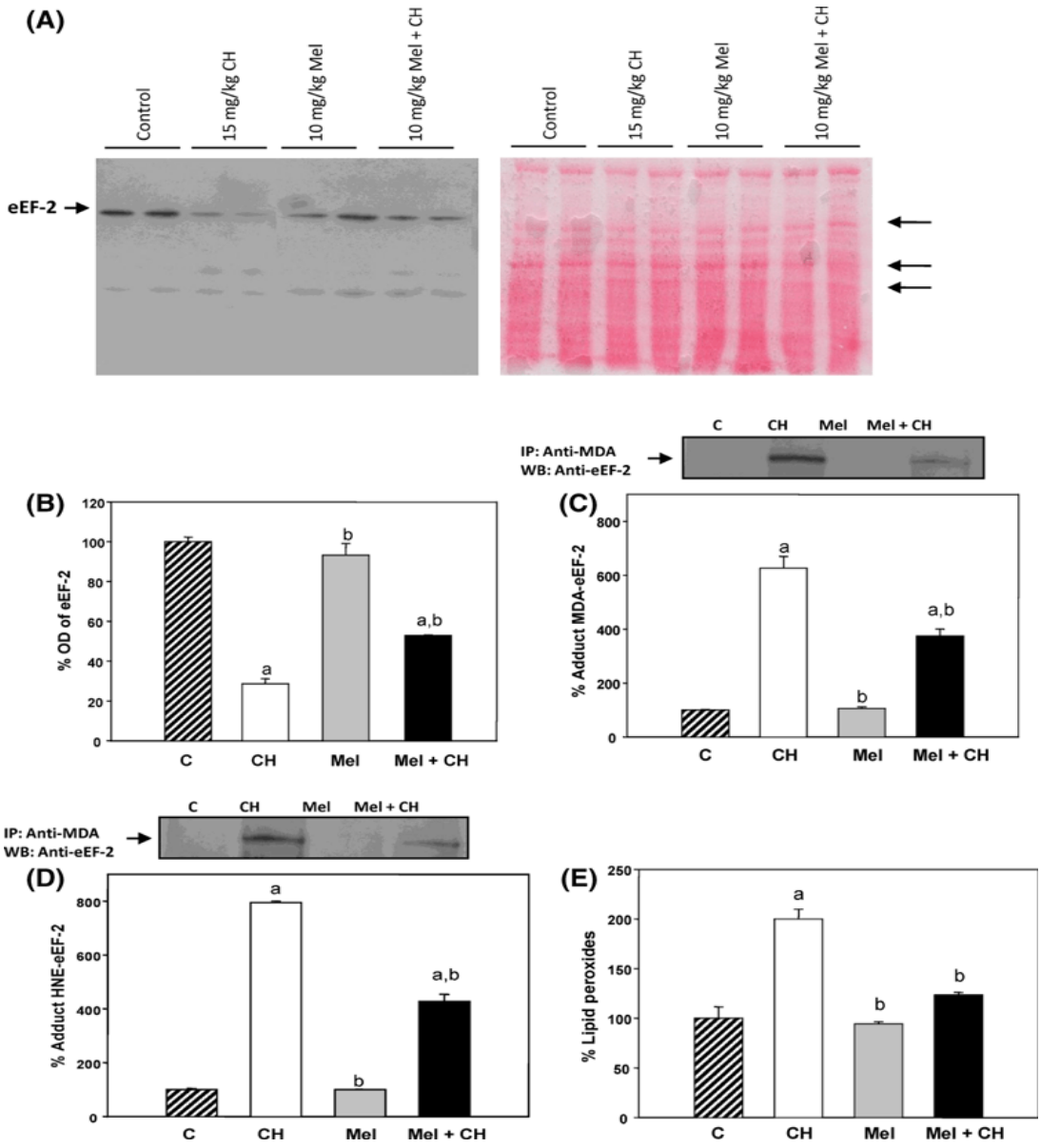

Fig. 4. In vivo effect of cumene hydroperoxide (CH) and CH plus melatonin on elongation factor-2 (eEF-2) levels, adducts formation and lipid peroxidation in liver homogenates. (A) Typical nitrocellulose membrane immunostained with anti-eEF-2 antibody and the same membrane stained with Ponceau red. (B) Effect of CH and CH plus melatonin on the level of eEF-2 in liver homogenates. (C, D) Effect of CH and CH plus melatonin on adduct formation between MDA and HNE with eEF-2. 350 lg of total liver protein was immunoprecipitated with anti-MDA or anti-HNE, and the immune complex was resolved by 10% SDS-PAGE and immunoblotted with anti-eEF-2. (E) Effect of CH and CH plus melatonin on lipid peroxidation. Results are expressed as percentage with respect to the amount found in control and are the mean ± S.E.M. of five animals. Gray bars: rats treated with melatonin alone; Black bars: melatonin plus CH-treated rats. a Significantly different from control (ANOVA followed by Tukeys test, $P < 0.001$), b Significantly different from CH-treated (ANOVA followed by Tukeys test, $P < 0.001$).

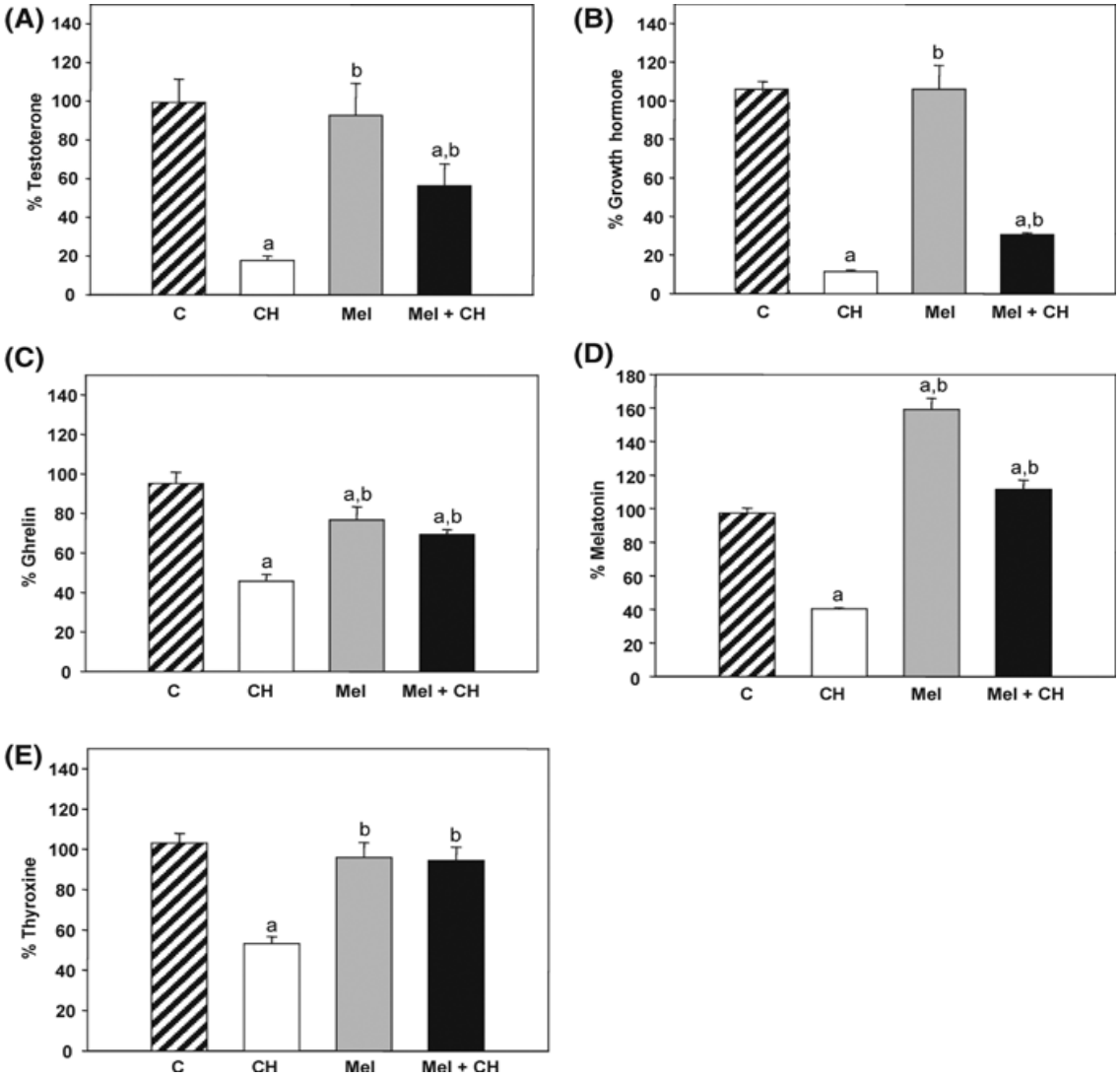

Fig. 5. **In vivo effect of melatonin and cumene hydroperoxide (CH) on serum hormone levels**. (A) serum testoterone levels, (B) serum growth hormone levels, (C) serum ghrelin levels, (D) serum melatonin levels, and (E) serum thyroxine levels. The clear serum was separated from cells by centrifugation at 2000 g for 15 min at 4C, dispensed in 0.5 mL aliquots, and stored at)80C. Results are expressed as percentage with respect to the amount found in control and are the mean ± S.E.M. of five animals. Gray bars represent the results from melatonin-treated rats; Black bars represent the results from melatonin plus CH-treated rats. a Significantly different from control (ANOVA followed by Tukeys test, $P < 0.001$), b Significantly different from CH-treated (ANOVA followed by Tukeys test, $P < 0.001$).