Journal of Cellular Biochemistry 83:426–434 (2001)

Effects of the Circadian Mutation 'Tau' on the Harderian Glands of Syrian Hamsters

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Abstract The Syrian hamster Harderian gland (HG) is an organ continually exposed to oxidative stress caused by high concentrations of porphyric metabolites. According to previous studies, melatonin, which is rhythmically secreted by the pineal gland and tonically produced by the HG, antagonizes the oxidative damage. HGs exhibit a strong gender-dependent correlation between porphyrins, melatonin, and histological appearance. In HGs of both sexes, we have investigated effects of a single gene defect in the circadian clock system (tau mutation) causing a shortened free-running period and an advanced maximum of circulating melatonin. Comparisons were made with wild-type animals, one group of which received daily pharmacological injections of melatonin in late photophase. Changes were observed in histological characteristics, porphyrin content, antioxidant enzyme activities, and damage of proteins and lipids. HGs of tau hamsters showed morphological changes which can be partially interpreted in terms of increased damage. Additionally, tau females exhibited a many-fold augmentation in the percentage of so-called type II cells, which are otherwise typical for the male glands. In tau hamsters of both sexes, major antioxidative enzyme activities (superoxide dismutase, glutathione reductase, and catalase) were markedly enhanced, a presumably compensatory response to increased oxidative stress. Higher oxidative damage in tau HGs was directly demonstrable by a many-fold increase in protein carbonyl. Rises in antioxidative enzymes were also observed upon injections of melatonin; this was, however, not accompanied by changes in protein carbonyl, so that enzyme inductions by the hormone should be understood as protective actions. Our data are not only in accordance with findings on protective effects by melatonin, but also with our earlier observation made in *Drosophila* that perturbations in the circadian system lead to increased oxidative stress. J. Cell. Biochem. 83: 426–434, 2001.

Key words: tau mutation; harderian glands; melatonin

The tau mutation of the Syrian hamster was the first genetic defect detected in the circadian system of a vertebrate. This autosomal semi-dominant mutation is associated with a shortening of the spontaneous period of the circadian activity rhythm from about 24 to 22 h in heterozygotes and to 20 h in homozygotes [Menaker and Refinetti, 1993].

The tau gene was reported to encode casein kinase 1ε, an enzyme also implicated in the turnover of the PER protein in *Drosophila* [Whitmore et al., 2000]. In the *Drosophila* short-period mutant *per*^{s}, we had previously detected increased levels of oxidative damage, as well as in the arrhythmic and melatonin-deficient mutant *per*^{0} [Coto-Montes and Hardeland, 1997, 1999]. The assumption that perturbations in the circadian system and changes in phase positioning of melatonin might lead to tissue damage prompted us to investigate the tau mutant representing another, mammalian system with a similar defect. For this purpose, we studied the Harderian gland (HG), an organ exposed to particularly strong oxidative stress and controlled by melatonin, which enters the HG from the circulation and is also formed in this gland.

The HGs are large orbital lachrymal glands present in most terrestrial vertebrates. Besides lubricating the eye, their precise functions have

Delio Tolivia-Cadrecha is recipient of a predoctoral fellowship from the FICYT (Asturias, Spain).

Grant sponsor: DGICYT; Grant numbers: PB95-1060, PB98-1565.

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Received 10 April 2001; Accepted 31 May 2001

DOI 10.1002/jcb.1240

not been completely identified. In the Syrian hamster, these glands show a strong sexual dimorphism [Payne et al., 1977].

The HG is, by its orbital location, accessible to light. In previous articles [Antolín et al., 1994, 1996b], porphyrin accumulation was reported to cause mitochondrial damage and cell death in the HGs of female and male Syrian hamsters. Nevertheless, the HG from female hamsters is able to survive in a state comparable to permanent porphyria, however, accompanied by cytological alterations and at the expense of frequent necrosis of individual cells [Antolín et al., 1994].

Although effects of photoperiod, blinding, and the pineal gland on porphyrin metabolism in the HG of hamsters have been repeatedly studied [Clabough and Norvell, 1973; Rodríguez-Colunga et al., 1991; Coto-Montes et al., 1994], the actions of melatonin in this gland are still incompletely understood. Melatonin has a dual role in the HG: on the one hand, the HG is influenced by circulating melatonin, as demonstrated by acute melatonin administration, pinealectomy, and presence of melatonin binding sites which are rhythmically modulated; on the other hand, melatonin is produced by the HG in a mainly tonic fashion, showing only a short depression after onset of light and lacking high-amplitude rhythmicity, but being strongly correlated with porphyrin content [Hoffman et al., 1985; Reiter, 1989; Menéndez-Péláez, 1990; Rodriguez-Colunga et al., 1991; Menéndez-Peláez et al., 1993; Guerrero et al., 1994; for reviews, see: Payne, 1994; Chieffi et al., 1996]. According to the marked gender difference in porphyrogenesis, the Syrian hamster HG displays, correspondingly, a considerable difference in melatonin content.

On the background of these findings, we considered the Syrian hamster HG as a suitable model for investigating the effects of perturbations in the circadian system on oxidative stress, compensatory responses of the protection system and related morphological changes. The gender differences of the HG also offered the possibility of comparing different states of exposure to endogenous reactive oxygens as generated by porphyrins and their precursors, processes which are widely compensated in the wild-type by corresponding levels of Harderian melatonin and adapted activities of antioxidative enzymes [Coto-Montes et al., 2001]. Additionally, we investigated the effects of repeated melatonin injections.

MATERIALS AND METHODS

Adult wild type (Charles River Lakeview, Newfield, N.J. Lak/LVG:SYR) and homozygous tau mutant male and female hamsters (*Mesocricetus auratus*) were used in these studies. The mutant hamsters were derived from matings of homozygous hamsters (courtesy of Dr. Michael Menaker, University of Virginia in the animal facilities of Northwestern University. After transport from America, tau hamsters had been kept in Oviedo for one year, under the following standard conditions. Throughout the experiments, all hamsters were maintained under light:dark cycles of 14:10 h (lights on at 07:00 h) and controlled temperature ($20 \pm 2^{\circ}$C). Water and food were provided ad libitum.

Melatonin Injections of Hamsters

Melatonin was pre-dissolved in ethanol and further diluted in saline (final ethanol concentration, 0.5%); it was injected i.p., daily at 17:00 h, using the pharmacological dose of 500 μg/kg body weight, for a treatment period of 2 weeks. Corresponding controls received vehicle i.p., under same conditions.

Morphological Studies

The animals were sacrificed under deep anesthesia. HGs were rapidly removed and immersed in a fixative containing 1.5% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.3. Tissues were kept in the fixative at 4°C overnight. After postfixation in 1% OsO_4, the HG pieces were dehydrated in acetone and embedded in TAAB 812 epoxy resin. Semithin sections (1 μm) were obtained using an LKB ultratome, stained with toluidine blue 0.1% and studied and photographed in a Leitz Orthoplan microscope. The following parameters were evaluated: number of type II cells/mm^2 of tissue and area occupied by intraluminal porphyrins/mm^2 of tissue. In each HG, five different cutting levels separated by 300 μm were analyzed. From these sections, the total area studied was 1 mm^2/animal. For statistical purposes, all determinations from a single gland were used for calculating an individual value as part of a sample. The areal density of porphyrins was estimated by

measuring the area of porphyrins and dividing by the area of the section, using a Videoplan (Kontron). The smallest-sized accretion measured was 50 μm^2. The numbers of type II cells and total nuclei/mm^2 were studied in the microscope at a final magnification of 400×. Data represent means±SEM and were analyzed by Student Newman-Keuls test.

Antioxidant Enzyme Assays

After decapitation at 10:00 h [=zeitgeber time 3:00], HGs were quickly removed, immediately frozen in liquid nitrogen and stored at −70°C. After thawing, tissue was homogenized in 50 mM phosphate buffer pH 7.5 (1:10 w/v), using a Potter-Elvehjem glass homogenizer with motor-driven Teflon pestle. After centrifugation for 10 min at 3000*g*, supernatants were used for assaying catalase (EC 1.11.1.6) [Lubinsky and Bewley, 1979], superoxide dismutase (EC 1.15.1.1) [Martin et al., 1987], glutathione reductase (EC 1.6.4.2) [Kum-Tatt et al., 1975]. Protein concentration was determined as described by Lowry et al. [1951].

Protein Damage

Protein carbonyl was determined, after sacrificing at ZT 3:00, by a variant [Coto-Montes and Hardeland, 1997] of the method of Levine et al. [1990].

RESULTS

Morphological Characteristics

In relation to the morphological differences between groups, HGs from tau hamsters showed certain changes in the adenomeres, especially in females. Some cells appeared lightly stained, displaying a weak basophilia; because of this, they were much paler than the rest of the cells. These 'clear cells' had euchromatic round-shaped nuclei, of generally larger size than those of the other cells (Fig. 1). Some clear cells contained small lipidic vacuoles in their cytoplasm, while others exhibited large ones. Frequently, these cells appeared to be in a phase of detachment from the tubule wall, seemingly preparing for being partially or totally released into the lumen of the tubule (Fig. 2); sometimes they were protruding at the opposite pole toward the connective tissue (Fig. 1). We also observed that the number of clear cells in males was very low compared with females.

Some tubules in the gland were filled with a mass of cellular debris having staining properties resembling those of clear cells.

Occasionally, tubules were observed which contained variable amounts of luminal material composed of lipid droplets, nuclei, etc. The nuclei of secretory cells in such tubules were heterochromatic and often showed a pycnotic aspect (Fig. 3).

The morphological variations described were basically present in all tau hamsters, though with differences between sexes. Male HGs contained more frequently tubules with a large amounts of cytoplasmic debris, with many cells protruding and detaching into the lumen (Fig. 4). In tau females, however, this was confined to very delimited areas of circular profile. In these areas, borders between secretory cells seemed to disappear and may have been lost. The cellular structure of adenomeres was no longer discernable, although the profile of the tubules was not changed and no protrusions into the connective tissue were observed (Fig. 5).

The HGs of tau females showed porphyrin accretions (Fig. 6), as observed in female controls, reaching into the connective tissue (Fig. 5), sometimes accompanied with cellular debris from secretory cells.

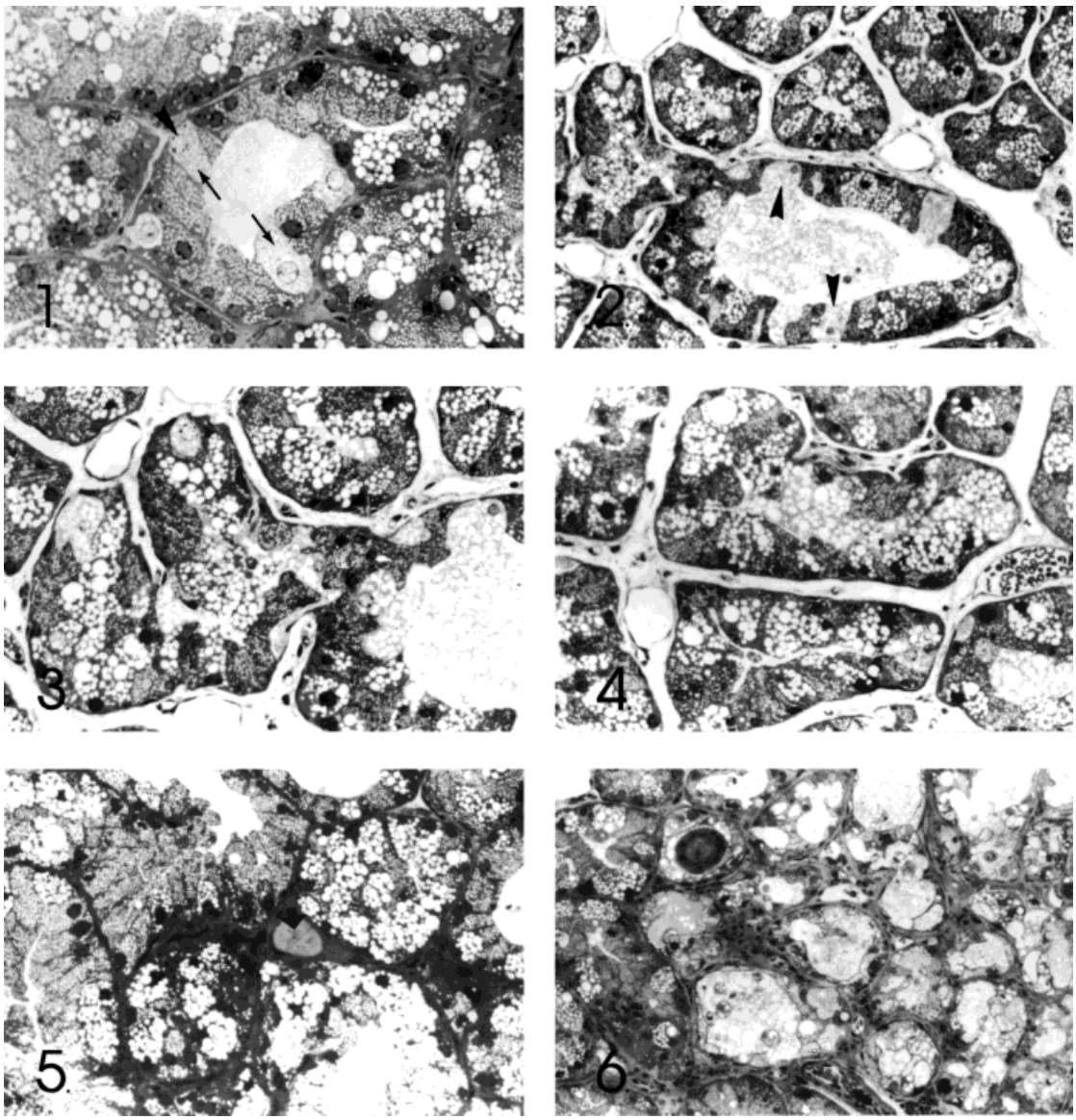
Type II Cells

The percentage of type II cells in the HGs of male Syrian hamsters (control group) was 51% (Fig. 7), whereas it remained in females at the usual low value of 1.3% (Fig. 8). The administration of melatonin, resulted in a significant increase ($P < 0.05$) in the percentage of type II cells in male but not female hamsters (Figs. 7 and 8).

The number of type II cells in female tau hamsters was extremely different from that in wild-type females, whether melatonin-injected or not, and also from anything else previously found in intact females. In these animals, type II cells were by an order of magnitude more abundant than in controls (45.86 vs. 1.3%), the percentage being ever higher than in male controls (53.14%) (Figs. 7 and 8). No significant differences were observed in the number of type II cells between tau and control males.

Porphyrin Deposits

Intraluminal porphyrin was almost undetectable in control and tau males. Also in males



Figs. 1–6. **1**: Section of HG from female tau hamster showing clear cells (arrows). Note one clear cell protuding towards the connective tissue (arrowhead). 340×. **2**: Image from female tau female hamster showing the detachment of clear cells (arrowheads) into the lumen of the tubules. 270×. **3**: Tubules of the HG from male tau mutant showing highly heterochromatic and pycnotic nuclei. 270×. **4**: Section of HG from male tau mutant. Note the cytoplasmic debris of detached cells filling the lumen of some tubules. 270×. **5**: Tubules of the HG from female tau mutant showing the characteristic alterations in the structure and the lack of cellular limits. Intersticial accretion of porphyrin (arrow). 160×. **6**: Toluidine blue-stained semithing section of tau female HG presenting luminal accretion of porphyrins. Note the circular profile areas of cellular damage. 160×.

treated with melatonin, no porphyrin deposits were observed in any of the animals studied. Administration of melatonin to female hamsters resulted in a significant decrease of the area occupied by porphyrin deposits ($P < 0.05$). Wild-type and tau females did not significantly differ from each other with regard to the intraluminal area of porphyrins (Fig. 9).

The presence of normal porphyrin deposits in tau females deserves particular emphasis, since this contrasts to the presence of type II cells. To date, the abundance of type II cells, being characteristic for male HGs, has been thought to be always negatively correlated with porphyrin deposits, which is, in fact, true for the wild-type.

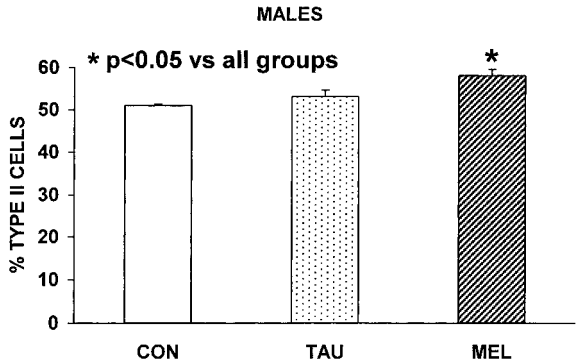


Fig. 7. Percentage of type II cells in male HGs. TAU = tau mutation; MEL = wild-type with daily melatonin injections; CON = wild-type.

Antioxidant Enzymes

In either sex, the activities of the three antioxidant enzymes investigated, superoxide dismutase, glutathione reductase, and catalase, were strongly affected by the tau mutation. Regardless of the basal levels, which differed considerably between genders, the enzymes were enhanced by 2- to 4-fold, compared to the wild-type (Figs. 10 and 11).

Daily injections of melatonin to wild-type hamsters also increased enzyme activities in the majority of cases, but usually to a smaller extent; no rise was demonstrable in glutathione reductase of males (Figs. 10 and 11).

Protein Damage

When compared with the control group, melatonin administration did not lead to deviations from the basal levels of protein carbonyl. However, in both sexes of tau hamsters, protein damage was increased by about 8-fold in males and more than 20-fold in females (Figs. 12 and 13). These remarkable differences demonstrate that the increased activities of antioxidant enzymes are not capable of sufficiently counteracting protein destruction, a result being in accordance with the morphologically apparent cell damage in HGs of tau hamsters.

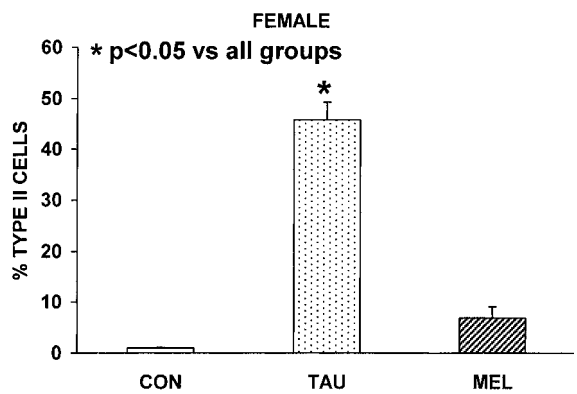


Fig. 8. Percentage of type II cells in female HGs. TAU = tau mutation; MEL = wild-type with daily melatonin injections; CON = wild-type.

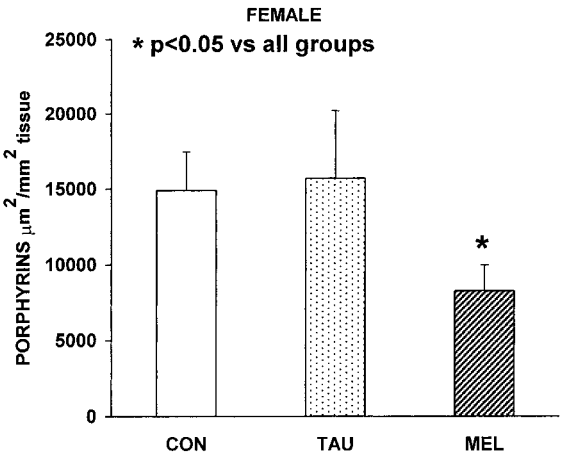


Fig. 9. Changes observed in the area accupied by intraluminal porphyrins in the HGs of intact female syrian hamsters (CON), female tau hamsters (TAU), and female syrian hamsters treated daily with melatonin (MEL).

DISCUSSION

The findings of this study lead to three major conclusions: (1) the observation, previously made in *Drosophila*, that shortening of the circadian period causes increased oxidative stress is confirmed in an entirely different organism, the Syrian hamster, (2) the current view that high levels of porphyrins—being typical for female hamster HGs—are, for fundamental reasons, negatively correlated with the presence of type II cells—normally characteristic for males—is false, and (3) we describe another case showing that melatonin can induce protective enzymes and we, moreover, demonstrate that these increases are not due to enhanced oxidative stress, since protein damage remains basal.

Period shortening by the tau mutation is a well-documented phenomenon [e.g., Menaker and Refinetti, 1993; Loudon et al., 1994; Whitmore et al., 2000]. Such a change in the spontaneous period should be reflected under synchronized conditions by advanced phase positions of rhythmic marker functions relative

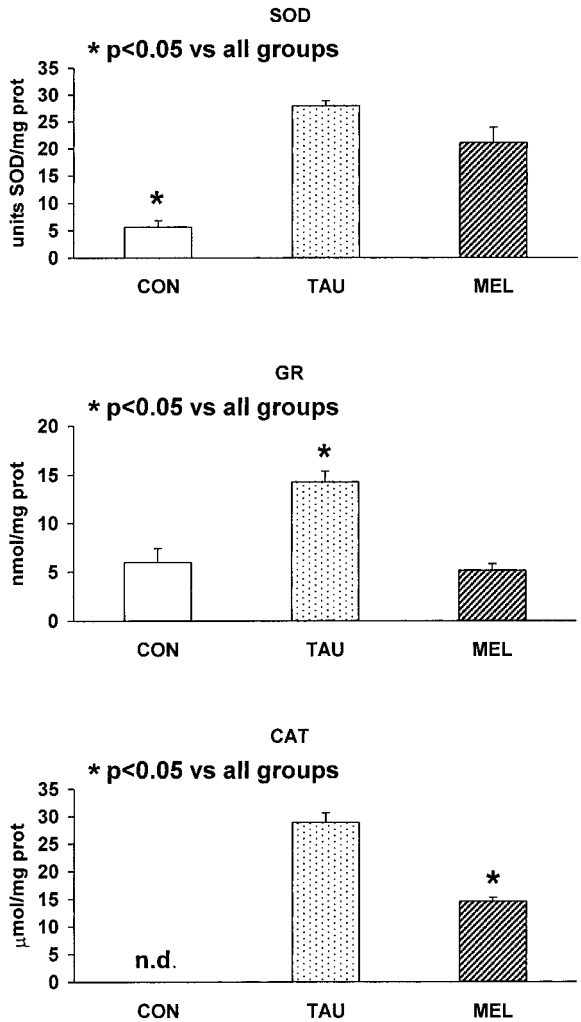


Fig. 10. Effects of tau mutation and melatonin injections on several enzyme activities involved in antioxidative protection in the HG of male hamsters. SOD: superoxide dismutase (top); GR: glutathione reductase (middle); CAT: catalase (bottom); CON: wild-type; TAU: tau mutation; MEL: melatonin injections in wild-type.

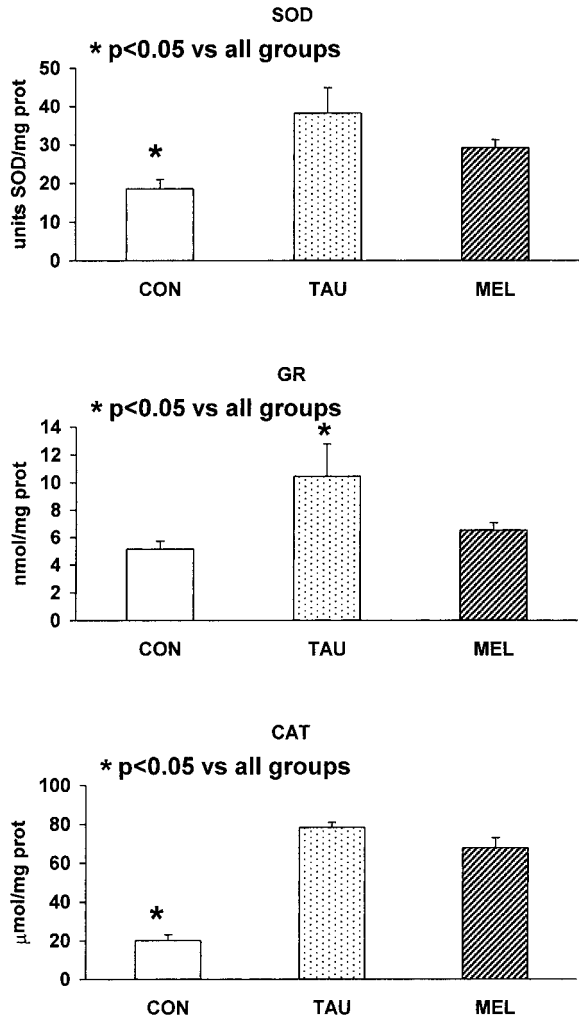


Fig. 11. Effects of tau mutation and melatonin injections on several enzyme activities involved in antioxidative protection in the HG of female hamsters. Details as in Figure 10.

to the light/dark zeitgeber, which has, in fact, been observed [Osiel et al., 1998]. This is basically the same situation as in the short-period *Drosophila* mutant *per*^{s}, in which we had previously stated enhanced levels of oxidative stress [Coto-Montes and Hardeland, 1997, 1999]. The parallel described here for the Syrian hamster HG may be initially interpreted, in a more general way, as the result of temporal malcoordination in which rhythmic functions do not peak in the precise phase positions for minimizing oxidative stress resulting from endogenous energy metabolism or from photocatalytic processes.

Nevertheless, the situation in the HG appears to be more complicated. Apart from the questions of casein kinase 1ε expression and its effects on the hamster per homologue [Whitmore et al., 2000], which would deserve further studies, histological alterations have to be taken into consideration. This is particularly important in females, which are exposed to stronger physiological oxidative stress already in the wild-type, due to higher levels of porphyric metabolites; this correlation between porphyrinogenesis and oxidative stress has been recently demonstrated on the basis of both protein and lipid damage and is also reflected by adaptive alterations in antioxidative enzyme activities [Coto-Montes et al., 2001]. The histo-

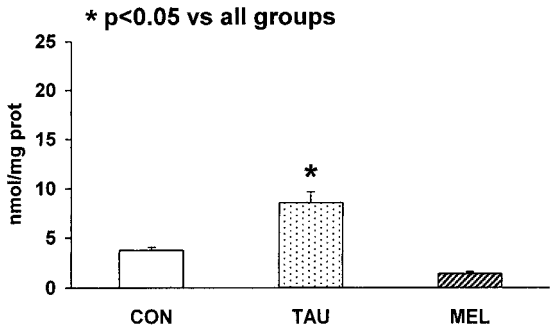


Fig. 12. Effects of tau mutation and melatonin injections on protein damage in the male hamster HG. Protein damage is expressed as nanomoles protein carbonyl per milligram of protein.

logical changes in female HGs caused by tau mainly concern two aspects: (1) the frequency of damaged cells and (2) the percentage of type II cells. Despite these differences from the wild-type, no profound changes were detected in porphyrin levels. This would imply that the increased level of oxidative stress, as demonstrated by cell damage and protein carbonyl, is not mainly caused by alterations of porphyrin concentrations in tau hamsters, but rather by temporal malcoordination. This would be in line with the findings in males, in which tau also resulted in increased oxidative stress, although the histological appearance was less affected and no rise in porphyrins was observed.

Regarding histologically demonstrable damage, the invasive processes seen in the female HGs of tau hamsters are qualitatively similar those known from wild-type females [Tolivia et al., 1996], but the quantitative extent observed in tau females differed from any

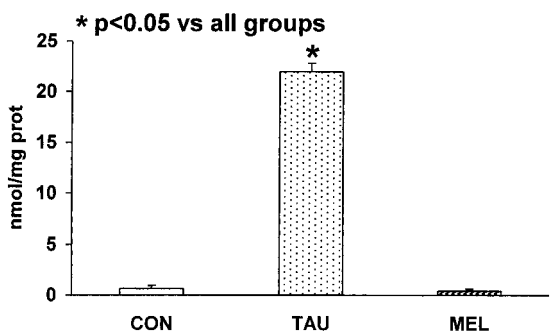


Fig. 13. Effects of tau mutation and melatonin injections on protein damage in the female hamster HG. Details as in Figure 12.

previous experience, sometimes even reminding of histopathological features in HG tumors [Coto-Montes et al., 1997].

The histological changes concerning type II cells in tau females are highly unexpected. In all previous studies, type II cells were low in HGs of untreated wild-type females and inversely correlated with porphyrin accumulation. Never had such a considerable percentage of type II cells been found in normal females [Rodríguez-Colunga et al., 1991, 1992, 1993; Menéndez-Peláez et al., 1992]. Only treatments affecting gonadosteroids, leading to virilization, caused rises in type II cells, though in conjunction with decreases in porphyrins; therefore, induced interconversions between feminine and masculine phenotypes always maintained this inverse correlation [Payne et al., 1977; McMasters and Hoffmann, 1984; Payne, 1994]. The findings presented here break this rule, and it remains to be studied how a change in the phase position relative to the zeitgeber, or, perhaps, in casein kinase 1ε expression, can lead to a high percentage of type II cells, in the presence of accumulated porphyrins.

The high number of type II cells in tau females should have consequences for the composition of the secretions, in particular, with regard to the lipids, which differ between genders. Seyama et al. [1996] proposed a pheromone function of those lipids which are typical for females, by which mothers communicate with pups. Moreover, methyl-branched lipids, being typical for females, were shown to depend on photoperiod [Buzzell et al., 1997] and may, therefore, relate to the reproduction.

The differences in antioxidative enzyme activities between tau and wild-type controls should not have resulted from measurements made at different phase positions of the respective enzyme rhythms. We have recently described the temporal patterns of these enzymes in the HGs of both sexes [Coto-Montes et al., 2001], and, in the present study, animals were sacrificed at or very close to the respective diurnal peaks. Advances in the phase position, as expected for tau, would have resulted in a deviation from the peaks, i.e., in lower activities. The higher values measured, on the contrary, have, thus, to be explained in a different way, namely, in terms of an adaptive response to increased oxidative stress. This conclusion would be in aggreement with the considerably augmented damage in proteins,

as shown by the concentrations of protein carbonyl.

The effects of repeated melatonin injections have to be interpreted in a different way. Changes in antioxidative enzyme activities can be induced via multiple pathways, not only by those related to redox sensing. Melatonin was shown to stimulate various protective enzymes in different tissues [reviews: Reiter et al., 1995; Hardeland, 1997], including the HG [Antolín et al., 1996a], although the mRNA levels determined in that study did not fully reflect the activities at protein levels and experiments were not designed for considering rhythmic changes. Moreover, melatonin decreases porphyrogenesis, so that oxidative stress is diminished also from this side. Taken together, the effects of melatonin cannot be interpreted in terms of rises in oxidative stress, and protein carbonyl levels have remained at basal levels in animals treated with the hormone. In summary, melatonin should have mainly acted as an antioxidant agent, including its documented additional radical scavenging properties [Reiter et al., 1995; Hardeland, 1997]. As to whether the effects of melatonin described here for the HG had an additional chronobiological dimension, i.e., via influencing the circadian system, remains to be analyzed. At least, one can state that the parallels to the effects of the tau allele were confined to antioxidant enzyme activities, but did not concern tissue or protein damage.

ACKNOWLEDGMENTS

The authors are grateful to Dr. M. Menaker from University of Virginia for generously providing the tau mutants. The technical assistance of Gloria Menéndez from the Morphology and Cellular Biology Department is greatly appreciated.

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