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Journal of Cellular Biochemistry 110:1219–1225 (2010)

Journal of Cellular Biochemistry

Melatonin Protects Against Taurolithocholic-Induced Oxidative Stress in Rat Liver

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ABSTRACT

Cholestasis, encountered in a variety of clinical disorders, is characterized by intracellular accumulation of toxic bile acids in the liver. Furthermore, oxidative stress plays an important role in the pathogenesis of bile acids. Taurolithocholic acid (TLC) was revealed in previous studies as the most pro-oxidative bile acid. Melatonin, a well-known antioxidant, is a safe and widely used therapeutic agent. Herein, we investigated the hepatoprotective role of melatonin on lipid and protein oxidation induced by TLC alone and in combination with $FeCl_3$ and ascorbic acid in rat liver homogenates and hepatic membranes. The lipid peroxidation products, malondialdehyde and 4-hydroxyalkenals (MDA + 4-HDA), and carbonyl levels were quantified as indices of oxidative damage to hepatic lipids and proteins, respectively. In the current study, the rise in MDA + 4-HDA levels induced by TLC was inhibited by melatonin in a concentration-dependent manner in both liver homogenates and in hepatic membranes. Melatonin also had protective effects against structural damage to proteins induced by TLC in membranes. These results suggest that the indoleamine melatonin may potentially act as a protective agent in the therapy of those diseases that involve bile acid toxicity. J. Cell. Biochem. 110: 1219–1225, 2010. © 2010 Wiley-Liss, Inc.

KEY WORDS: MELATONIN; BILE ACID; TAUROLITHOCHOLIC ACID; OXIDATIVE STRESS; CHOLESTASIS; LIPID PEROXIDATION; PROTEIN CARBONYLS

Cholestatic liver disorders, among the leading reasons for liver transplantation [Neuberger, 2003], are a heterogeneous group of clinical diseases with the common finding of impaired bile flow [Javitt et al., 1982]. Although the various causes of chronic cholestasis include structural, genetic, immunologic, and inflammatory processes, one of the putative final common pathways leading to cholestatic liver injury is the intracellular accumulation of toxic bile acids [Greim et al., 1972]. Bile acids are well-established as being cytotoxic [Perez and Briz, 2009]. These acids contribute to hepatocellular dysfunction during cholestasis; the toxicity of bile acids is related, at least in part, to their ability to produce free radicals. Several studies have documented that oxidative stress plays an important role in the pathogenesis of bile acid hepatotoxicity [Sokol et al., 1993; Fuentes-Broto et al., 2009]. Furthermore, oxidative stress in cholestatic liver disease is a systemic phenomenon [Ljubuncic et al., 2000], probably involving all tissues and organs, even those normally isolated by the blood–brain barrier [Chroni et al., 2006]. In the current work, we selected taurolithocholic acid (TLC), a secondary monohydroxylated bile acid conjugated with taurine, which we have previously shown to have the most potent pro-oxidant effect of all the bile acids [Fuentes-Broto et al., 2009]. We also induced free radical generation with a model consisting of $FeCl_3$ and ascorbic acid; this combination of reagents promotes the Fenton reaction which generates the highly toxic hydroxyl radical [Halliwell and Gutteridge, 1999].

Cells have developed complex mechanisms to maintain redox homeostasis and to cope with the excess of oxygen and nitrogen-based reactants produced by free radicals. These antioxidant protective mechanisms either scavenge and/or detoxify free radicals, block their production, sequester transition metals that are a source of donated electrons and stimulate antioxidative enzymes. Collectively, the molecular damage that accumulates is

Grant sponsor: Gobierno de Aragón; Grant number: B40 and PI036/09; Grant sponsor: Instituto de Salud Carlos III; Grant number: RD06/0013/1017.

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Received 7 January 2010; Accepted 30 March 2010 • DOI 10.1002/jcb.22636 • © 2010 Wiley-Liss, Inc.
Published online 17 May 2010 in Wiley InterScience (www.interscience.wiley.com).

referred to as oxidative stress; this damage accumulates when there is an imbalance between oxidants and antioxidants in favor of the oxidants.

N-acetyl-5-methoxytryptamine or melatonin, mainly synthesized in the pineal gland of vertebrates, participates in many important physiological functions including endocrine, neural, immune, and antioxidant [Korkmaz et al., 2009]. In both in vitro and in vivo experiments melatonin and its metabolites detoxifies a variety of free radicals and reactive oxygen species (ROS), including the hydroxyl radical, singlet oxygen, peroxyl radical, peroxynitrite anion, nitric oxide, and hydrogen peroxide [Tan et al., 2007; Peyrot and Ducrocq, 2008; Gitto et al., 2009; Hardeland et al., 2009]. Melatonin also has been reported to stimulate the activities of enzymes and increase gene expression that improve the total antioxidative defense capacity of the organism, that is, superoxide dismutase, glutathione peroxidase, and glutathione reductase [Antolin et al., 1996; Rodriguez et al., 2004; Tengattini et al., 2008]. In addition, the efficacy of melatonin as an antioxidant may relate to the ease with which it crosses biological membranes including the blood–brain barrier [Costa et al., 1995]. Finally, the products derived from the interaction of melatonin with free radicals also possess antioxidant activity; thus, these derivates are believed to augment the direct protective actions of melatonin against free radicals [Tan et al., 2007; Peyrot and Ducrocq, 2008; Reyes-Gonzales et al., 2009].

During the last decade, it has been shown that in animals and tissues exposed to agents which induce lipid peroxidation (LPO) or protein damage, melatonin provides substantial protection against this oxidative damage [Piñol-Ripoll et al., 2006; Albendea et al., 2007; Catala, 2007; Ortega-Gutiérrez et al., 2007; Reiter et al., 2009]. Its beneficial effects against hepatic oxidative stress have been demonstrated in liver cirrhosis [Cruz et al., 2005], and following cholestasis [Montilla et al., 2001; Ohta et al., 2003; Padillo et al., 2004; Esrefoglu et al., 2005]. Moreover to date, no serious toxicity has been reported for melatonin and it is widely used in clinical conditions [Korkmaz et al., 2009].

The aim of the present work was to test whether melatonin protects against damage from lipid and protein oxidation caused by TLC, in the absence and presence of $FeCl_3$ and ascorbic acid, in hepatic cell membranes and homogenates.

MATERIALS AND METHODS

CHEMICALS

Taurolithocholic acid, $FeCl_3$, ascorbic acid, ethylenediaminetetraacetic acid disodium (EDTA-Na_2), Tris (hydroxymethyl) aminomethane (TRIS), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), and melatonin were purchased from Sigma–Aldrich (Madrid, Spain). Malondialdehyde and 4-hydroxyalkenals (MDA + 4-HDA) levels were measured with the Bioxytech LPO-586 Assay Kit (Portland, OR). Other chemicals were of the highest quality available. Melatonin was diluted in absolute ethanol and the incubation buffer; the ethanol concentration was 2% (v/v) in the final mixture. TLC, $FeCl_3$ and ascorbic acid were diluted in the incubation buffer (Tris–HCl 20 mM; pH 7.4). All reagents were prepared fresh just prior to use.

ANIMALS, LIVER HOMOGENATES AND MEMBRANES ISOLATION

The handling and animal procedures were performed in strict compliance with the recommendations of the European Economic Community (86/609/CEE) for the care and use of laboratory animals. Sprague–Dawley rats weighing 225–250 g were purchased from Harlan Iberica (Barcelona, Spain), and received standard chow and water ad libitum. After being acclimated for 2 weeks, animals were anesthetized with sodium thiopental given intraperitoneally (50 mg/kg) and perfused through the heart with 0.9% ice-cold saline, in order to minimize the excess of extracellular iron and other metallic ions that could artificially increase free radical damage. Immediately after perfusion, the liver was quickly removed, washed in saline solution (0.9% NaCl), frozen, and stored at −80°C prior to use. To obtain homogenates, livers were homogenized 1/5 (w/v) in Tris–HCl buffer (20 mM; pH 7.4). To isolate membranes, livers were homogenized 1/7 (w/v) in 140 mM KCl/20 mM HEPES buffer (pH 7.4). The resulting suspension was centrifuged at 1,000*g* for 10 min at 4°C to clear cell debris and nuclei by centrifugation. The supernatant was centrifuged at 50,000*g* for 20 min at 4°C. The pellet obtained was re-suspended in 140 mM KCl/20 mM HEPES buffer (pH 7.4) and centrifuged at 10,000*g* for 10 min at 4°C. The supernatant and the buffycoat, which contained the membranes, were homogenated and re-centrifuged at 50,000*g* for 20 min at 4°C. The resulting pellet was suspended 1/2 (v/v) in 140 mM KCl/20 mM HEPES buffer (pH 7.4), frozen, and stored at −80°C until assay.

EFFECTS OF MELATONIN IN TAUROLITHOCHOLIC HEPATOTOXICITY

Aliquots of liver homogenates and aliquots of hepatic membranes (0.5 mg protein/ml) suspended in 20 mM Tris–HCl buffer (pH = 7.4) were incubated in a water bath with shaking at 37°C either in the absence or presence of 1 mM TLC and different concentrations of melatonin (0.1, 0.5, 1, 2, 3, 5 mM). Incubations with the same concentrations of melatonin were performed either in absence or presence of 1 mM TLC, 0.1 mM $FeCl_3$ and 0.1 mM ascorbic acid. After 120 min, the aliquots of liver homogenates or hepatic membranes were assayed to determine melatonin's protective effect against TLC-induced lipid and protein oxidation.

MEASUREMENTS OF PROTEIN CONCENTRATIONS

The protein concentrations in incubation media were determined using the method of Bradford [1976], in which bovine serum albumin served as standard.

MEASUREMENT OF MALONDIALDEHYDE AND 4-HYDROXYALKENALS LEVELS

MDA and 4-HDA are the end-products of peroxidation of polyunsaturated fatty acids. Therefore, MDA + 4-HDA levels are widely used as an index of the oxidative breakdown of lipid in the preparations [Janero, 1990]. Total MDA + 4-HDA levels in incubation media were measured using a spectrophotometric assay [Esterbauer and Cheeseman, 1990]. In short, the assay was based on the reaction of a chromogenic reagent, *N*-methyl-2-phenylindole with MDA or 4-HDA, yielding a stable chromophore with a peak of maximum absorbance at 586 nm. 1,1,3,3-Tetramethoxypropane was used as standard. Results were expressed as nmol MDA + 4-HDA per mg of protein.

MEASUREMENT OF CARBONYL CONTENT

The carbonyl content of proteins was quantified by the reaction with 2,4-dinitrophenylhydrazine (DNPH), using the method previously described by Levine et al. [1990]. Briefly, 1 ml of samples were mixed with 100 μl of 20 mM Tris–HCl buffer and 200 μl of 10 mM DNPH and reacted for 1 h at 37°C to give final concentration of 2 mM of DNPH. Next, proteins were precipitated by adding 325 μl of 50% ice-cold trichloroacetic acid. After 10 min of incubation on ice, samples were centrifuged at 3,000*g* for 10 min. Pellets containing the proteins were washed three times with ethanol/ethyl acetate (1:1 v/v). Each wash was followed by centrifugation at 11,000*g* for 3 min. Finally, the pellets were dissolved in 700 μl of 6 M guanidine in 2N HCl (pH = 2.0) by vortexing, and incubated at 37°C for 15 min. After this interval, samples were clarified by centrifugation at 12,000*g* for 10 min and the absorbance of supernatants was read at 375 nm. Protein carbonyl content, expressed in nanomoles per milligram of protein, was estimated by using the molar absorption coefficient of 22,000 M^{-1} cm^{-1} for DNPH derivates. Guanidine–HCl solution was used as a blank.

STATISTICAL ANALYSIS

The results are expressed as means with their corresponding standard errors. Distribution of the groups was analyzed using the Kolmogorov–Smirnov test. Groups showed normal distribution, then, parametric statistical methods were used to analyze the data. Homogeneity of variance was assessed with Levene's test. Comparisons were conducted by either one-way analysis of variance (ANOVA) or two-tailed Student's *t*-tests paired as appropriate. Statistical significance was set at $P \leq 0.05$.

The concentration that inhibits oxidation by 50% (IC_{50}) was determined by calculating the concentration of melatonin required to inhibit by a 50% the formation due to TLC, induced or not by iron of MDA + 4-HDA or carbonyl content in liver homogenates and hepatic membranes.

RESULTS

TLC increased LPO and carbonyl content in liver homogenates and in hepatic membranes (Figs.1A,B and 2A,B). The aim of this study was to test whether melatonin would prevent TLC-induced oxidative stress. In the absence of iron, the co-incubation of the liver homogenates and hepatic membranes with melatonin reduced the levels of the products of LPO which were induced by TLC (Fig. 1A,B). The concentration of melatonin required to inhibit the formation of MDA + 4-HDA in liver homogenates by a 50%, that is, IC_{50} was 4.73 mM (Table I). No significant differences were found in the reduction of the carbonyl content in liver homogenates caused by melatonin (Fig. 2A). In hepatic membranes, 5 mM melatonin reduced LPO and carbonyl content induced by TLC (Figs. 1B and 2B). It was calculated that only 0.15 mM melatonin was needed to inhibit the formation of carbonyl content by a 50% in hepatic membranes (Table I).

Incubation of the liver homogenates and hepatic membranes with $FeCl_3$ and ascorbic acid raised MDA + 4-HDA concentrations, indicating the oxidation of membrane lipids. The addition of TLC to the incubation mixture further increased levels of MDA + 4-HDA such that the values where significantly elevated over those induced by $FeCl_3$ and ascorbic acid only (Fig. 1C,D). In the presence of iron, melatonin's inhibitory effect on TLC-induced LPO was concentration-dependent as shown in Figure 1C,D. The lowest studied concentration of melatonin (0.1 mM) or greater significantly reduced levels of MDA + 4-HDA below those treated with TLC in liver homogenates and hepatic membranes. Additionally, with 1 mM melatonin or greater, MDA + 4-HDA levels were lower than those measured in membranes incubated with $FeCl_3$ and ascorbic acid in the absence of TLC. In the presence of iron and TLC, IC_{50} of melatonin for MDA + 4-HDA levels in liver homogenates and hepatic membranes were 0.40 and 1.06 mM, respectively (Table I).

Our previous studies showed that incubation of the liver homogenates with $FeCl_3$ and ascorbic acid raised carbonyl content, but the addition of TLC to the incubation mixture did not further increase protein carbonylation over that induced by $FeCl_3$ and ascorbic acid alone [Fuentes-Broto et al., 2009]. Thus, no studies of carbonyl content in the presence of iron and TLC were made in liver homogenates.

Hepatic membranes incubated with $FeCl_3$ and ascorbic acid raised carbonyl content. The addition of TLC to the hepatic membranes further increased levels of carbonyls such that the values where significantly elevated over those induced by $FeCl_3$ and ascorbic acid only (Fig. 2C).

Melatonin concentrations of 0.5 mM or greater significantly reduced the carbonyl content in relation to the samples treated with TLC in a concentration-dependent manner. At 3 mM melatonin reduced carbonyl content below that of the control basal samples (no melatonin or TLC; Fig. 2C). IC_{50} of carbonyl content in the presence of iron and TLC in hepatic membranes was 0.79 mM (Table I).

DISCUSSION

The mechanisms by which bile acids are toxic to the liver are not fully understood. Several studies have suggested a role for ROS in bile acid-mediated toxicity [Sokol et al., 1993; Fuentes-Broto et al., 2009]. During cholestasis, accumulation of these acids alters essential processes in the hepatocyte including energy production by mitochondria and formation of additional ROS capable of oxidizing lipids, proteins, and nucleic acids. This results in compromised cellular function, and, consequently, hepatocellular necrosis and apoptosis [Sokol et al., 1993; Perez and Briz, 2009]. The increased ROS may also cause remote organ damage (e.g., brain, heart, and kidney) via systemic circulation [Ljubuncic et al., 2000; Chroni et al., 2006].

In liver tissue of patients with cholestatic disease, such as primary biliary cirrhosis, the intrahepatic concentration of total bile acids may increase to 600 nmol/g of hepatic tissue [Poupon and Poupon, 1995]. In the current experiments, homogenates and hepatic membranes were incubated with 1 mM TLC. Assuming that 1 g liver is equivalent to 1 ml, these concentrations approximate the bile acid concentrations found in cholestatic livers. In the current

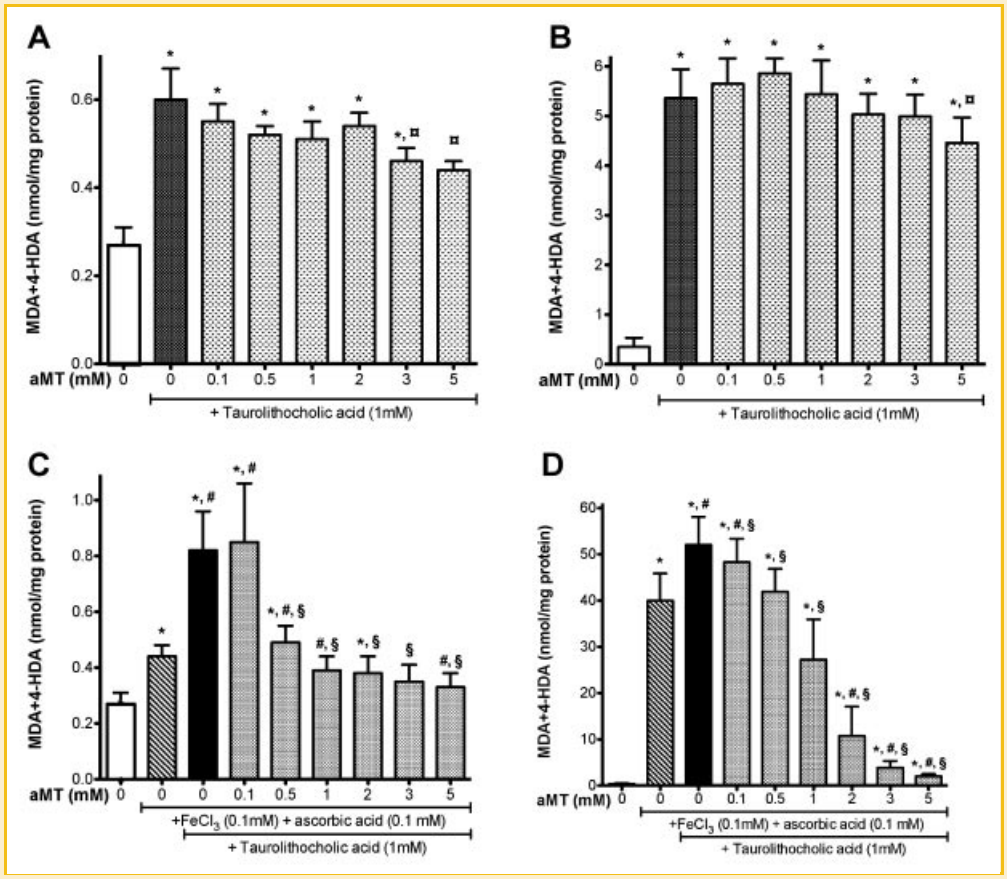

Fig. 1. Protective effect of melatonin (aMT) on lipid peroxidation induced by taurolithocholic acid (TLC) in absence of iron in rat liver homogenates (A) and hepatic membranes (B). Protective effect of melatonin against TLC promotion of lipid peroxidation induced by $FeCl_3$ and ascorbic acid in rat liver homogenates (C) and hepatic membranes (D). Incubation for 2 h at 37°C. Values are means ± SEM (n = 6). $P \leq 0.05$ versus control (*), versus control in the presence of iron (#), versus TLC (¤), or versus TLC in the presence of iron (§).

experiments TLC increased MDA + 4-HDA and carbonyl content levels, both in homogenized tissues and in membranes, reflecting its pro-oxidative effect.

Physiological concentrations of bile acids are apparently tolerated without causing damaging effects. However, under conditions in which free iron is available, even low concentrations of bile acids may become cytotoxic [Halliwell and Gutteridge, 1999]. Iron readily promotes the highly toxic hydroxyl radical from hydrogen peroxide or superoxide via the Fenton and Haber-Weiss reactions [Halliwell and Gutteridge, 1999]. Thus, iron ions are responsible for most of the LPO that occurs in vivo. In this study, we used $FeCl_3$ and ascorbic acid since ascorbate is a strong reducing agent and it has been classically used to reduce transition metals such as Fe^{3+} or Cu^{2+} [García et al., 2005]. The present study demonstrated the ability of TLC to promote the effect of iron, consequence presumed to be due to the increased production of ROS with oxidative stress being a major component of hepatocyte damage in cholestasis. Through a different mechanism, these results are in agreement with the concept that cholestasis is associated with an overproduction of reactive radical species and oxidative products, which are then responsible for several intracellular derangements [Sokol et al., 1993; Fuentes-Broto et al., 2009]. In this regard, LPO of the hepatic mitochondria correlates with the severity of cholestatic injury [Bergendi et al., 1999].

The proposed mechanisms of toxicity of bile acids, involve mitochondria as important targets [Sokol et al., 1993], and at the same time, as free radical generators [Sokol et al., 1995]. Also reduced endogenous antioxidant defenses in bile duct-ligated rats [Krahenbuhl et al., 1995], and in the liver of patients with diverse hepatopaties [Togashi et al., 1990], are documented. This suggests that antioxidants may play an important hepatoprotector role against bile acid-induced liver toxicity.

Melatonin can be synthesized in many extra-pineal tissues as in enterochromaffin cells of intestine mucosa [Messner et al., 2001]. Moreover, bile has 10 times higher concentrations of melatonin than plasma [Tan et al., 1999]. It is proposed that these high endogenous levels of melatonin, have a hepatoprotective function [Messner et al., 2001].

In the current report, the concentrations of MDA + 4-HDA were measured as an index of lipid breakdown by free radicals. Herein, melatonin treatment, in a concentration-dependent manner, abolished the increase of LPO products induced by TLC in both homogenates and hepatic membranes, both in the presence or absence of iron. As TLC caused higher damage in the presence of

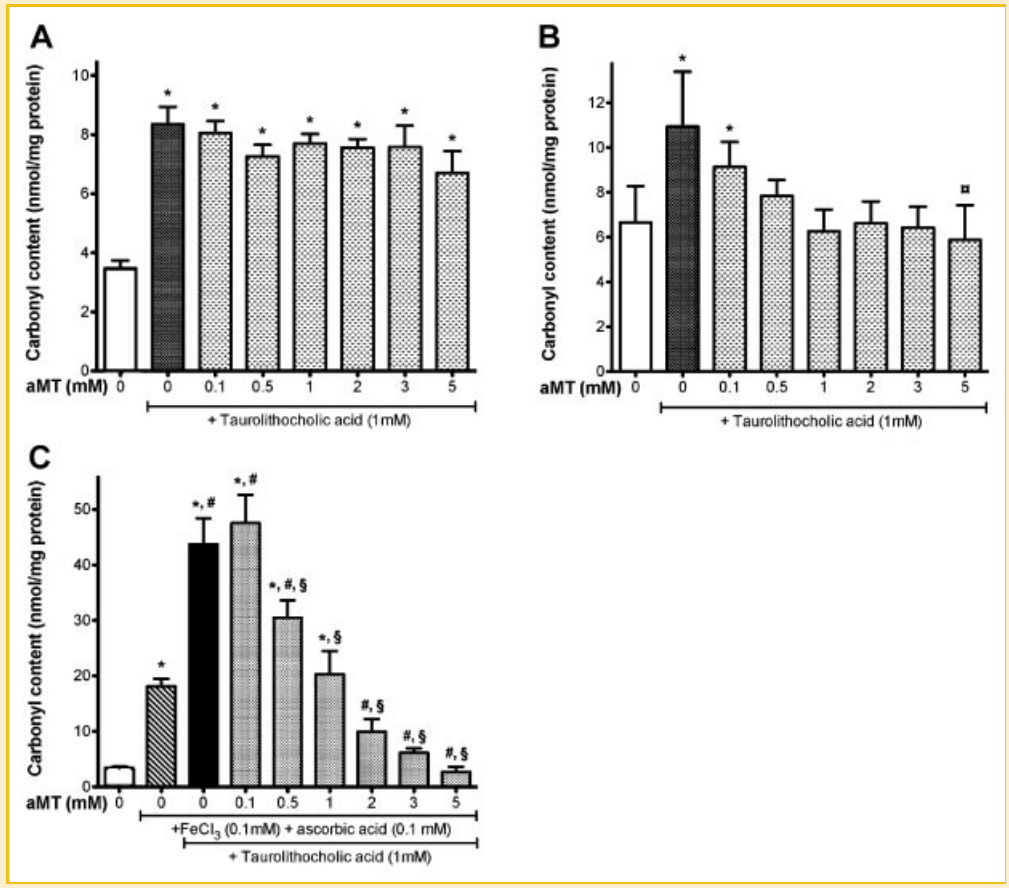

Fig. 2. Protective effect of melatonin (aMT) on protein oxidation induced by taurolithocholic acid (TLC) in absence of iron in rat liver homogenates (A) and in rat hepatic membranes (B). Protective effect of melatonin against TLC promotion of protein oxidation induced by $FeCl_3$ and ascorbic acid in hepatic membranes (C). Values are means ± SEM (n = 6). $P \leq 0.05$ versus control (*), versus control+ (#), versus TLC (¤), or versus TLC+ (§).

iron, IC_{50} of melatonin for MDA + 4-HDA levels are lower in the presence of $FeCl_3$ and ascorbic acid than in absence of iron. But in homogenates, either in the presence or absence of iron, melatonin recovered LPO values similar to control basal samples (in absence of TLC, $FeCl_3$, or ascorbic acid). Consequently, we speculate that the reduction of LPO, at least in part, was a consequence of the antioxidant properties of melatonin, probably involving scavenging of the highly reactive hydroxyl and peroxyl radicals [Antolin et al., 1996; Tan et al., 2007; Peyrot and Ducrocq, 2008; Gitto et al., 2009; Hardeland et al., 2009]. These observations agree with numerous studies in which melatonin was protective against LPO in several experimental models in vivo and in vitro [Catala, 2007].

Whereas several reports have documented the protective actions of melatonin on DNA and lipids, few have focused on its effectiveness in reducing oxidative damage to proteins. In a previous report, Kim et al. [2000] demonstrated that melatonin prevented protein oxidation due to free radicals generated by ascorbate-Fe^{3+}–EDTA. Data presented herein, reports the protective effects of melatonin against structural damage to proteins induced by TLC in membranes. Melatonin protection against TLC-induced oxidative stress was stronger in proteins than in lipids. Melatonin

TABLE I. Melatonin (aMT) Concentrations Required to Inhibit by a 50% (IC_{50}) the Lipid Peroxidation or Protein Oxidation Due to Taurolithocholic Acid (TLC) in the Absence or Presence of $FeCl_3$ and Ascorbic Acid (AA) in Rat Liver Homogenates and in Rat Hepatic Membranes

	aMT IC_{50} (mM)			
	Homogenates		Membranes	
	Lipid peroxidation	Protein oxidation	Lipid peroxidation	Protein oxidation
TLC	4.73	>5	>5	0.15
TLC + $FeCl_3$ + AA	0.40	–	1.06	0.79

reverted the levels of carbonyl content to the basal levels, because no significant differences were observed between membranes with 0.5 mM or higher concentration of melatonin added to TLC and the controls in the absence of TLC.

Several other studies have also demonstrated melatonin beneficial effects against hepatic oxidative damage in cholestasis [Montilla et al., 2001; Ohta et al., 2003; Padillo et al., 2004; Esrefoglu et al., 2005]. Systemically, the treatment with melatonin increased the total antioxidant status, as well as reduced the MDA concentrations of the serum, beside improving the parameters of the damage in remote organs [Chen et al., 2001].

Another possible mechanism of action of melatonin is increasing the activity of antioxidant enzymes including: glutathione peroxidase, glutathione reductase, glutathione transferase, catalase, and superoxide dismutase in the hepatic tissue, a mechanism that in our experimental model can only have special interest in those cases in which we have used homogenized tissues, since in the isolated membranes the enzymatical activity is almost completely lost during the purification.

Finally, the findings of our present study extend previous experiences with melatonin against the LPO that takes place during the cholestatic pathology and demonstrate for the first time that melatonin, a safe and widely used therapeutical agent, with its potent free radical scavenging and antioxidant properties, can counteract the TLC-induced oxidative hepatic damage. This study and the previous results, relative to the antioxidant activity of melatonin against bile acids, suggest that melatonin may represent a new therapeutic strategy against cholestatic pathologies. This fact reinforces the need for further pharmacological studies to evaluate the role of melatonin in the prevention of these hepatic pathologies of wide incidence in our society.

ACKNOWLEDGMENTS

This work was supported by the Gobierno de Aragón (Aging and Oxidative Stress Physiology, Grant No. B40 and PI036/09) and by F.I.S. from Instituto de Salud Carlos III (Grant No. RD06/0013/1017).

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