0013-7227/04/$15.00/0
Printed in U.S.A.

Endocrinology 145(1):418–425

doi: 10.1210/en.2003-0764

Involvement of Bax Protein in the Prevention of Glucocorticoid-Induced Thymocytes Apoptosis by Melatonin

ESTEBAN HOIJMAN, LUCIANA ROCHA VIEGAS, MARÍA INÉS KELLER SARMIENTO, RUTH E. ROSENSTEIN, AND ADALI PECCI

Departamento de Química Biológica (E.H., L.R.V., A.P.), Facultad de Ciencias Exactas y Naturales, Ciudad Universitaria, C1428EGA; and Laboratorio de Neuroquímica Retiniana y Oftalmología Experimental (M.I.K.S., R.E.R.), Departamento de Bioquímica Humana, Facultad de Medicina, Universidad de Buenos Aires, Consejo Nacional de Investigaciones Científicas y Técnicas, Buenos Aires 1428, Argentina

The antiapoptotic effect of melatonin has been described in several systems. In this study, the antagonistic effect of the methoxyindole on dexamethasone-induced apoptosis in mouse thymocytes was examined. Melatonin decreased both DNA fragmentation, and the number of annexin V-positive cells incubated in the presence of dexamethasone. Analysis of the expression of the members of the Bcl-2 family indicated that the synthetic glucocorticoid increased Bax protein levels without affecting the levels of Bcl-2, Bcl-X_L, Bcl-X_S, or Bak. This effect correlated with an increase in thymocytes *bax* mRNA levels. Dexamethasone also increased the release of cytochrome C from mitochondria. All of these effects were reduced in the presence of melatonin, which was ineffective *per se* on these parameters. In addition, the involvement of cAMP on glucocorticoid/melatonin antagonism was examined. Both melatonin and dexamethasone decreased the levels of this nucleotide in mouse thymocytes, indicating that the antagonistic action between both hormones involves a cAMP-independent pathway. In summary, the present results suggest that the antiapoptotic effect of melatonin on glucocorticoid-treated thymocytes would be a consequence of an inhibition of the mitochondrial pathway, presumably through the regulation of Bax protein levels. (*Endocrinology* 145: 418–425, 2004)

APOPTOSIS, OR PROGRAMMED cell death, is an intricately regulated process. It is well known that glucocorticoid-mediated thymus involution is a consequence of apoptosis provoked by the direct action of glucocorticoids on thymocytes. *In vivo* treatment with glucocorticoids induces 90% of cell death in the rat thymus, mainly of immature cells that are localized in the thymus cortex (1).

The molecular mechanism of apoptosis is a matter of an active debate. However, it is accepted that morphological changes observed during programmed cell death are the consequence of an activation of caspases cascade (2). At least two main signaling pathways have been postulated to participate in this process. The first one involves membrane receptors called death receptors (*i.e.* TNF receptor-1 and Fas/Apo-1) (3, 4), and the second one relies on the cell's ability to sense changes in the ratio between the protein levels of the members of the Bcl-2 family. Bcl-2 prevents apoptosis induced by a wide range of stimuli, suggesting that different pathways of transduction signals converge at this point (for review, see Refs. 5 and 6). Several authors have identified a variety of proteins related with Bcl-2, such as Bax, Bak, Bid, and the different Bcl-X isoforms, which can either promote or prevent apoptosis (7).

Many attempts have been made to isolate steroid-regulated genes involved in controlling cell death. In fact, a link between glucocorticoids and the Bcl-2 family has been demonstrated in several systems (8–10). On the other hand, a growing number of gene products seem to be implicated in blocking glucocorticoid-induced apoptosis; for example, the classic inhibitors of mitochondrion-dependent cell death *bcl-2* and *bcl-X_L* (11, 12) as well as the inhibitors of apoptosis proteins that inhibit the activity of some caspases (13).

Mitochondria plays a key role in the apoptotic pathway through the release of several factors from the intermembrane space to the cytoplasm, such as cytochrome C (14). It has been suggested that this pathway could be regulated by the relative levels and subcellular distribution of Bcl-2 family proteins (15). The antiapoptotic members (*i.e* Bcl-2 or Bcl-X_L) are mostly associated to the outer membrane of mitochondria and inhibit cytochrome C release. On the other hand, the proapoptotic molecules such as Bax, Bad, or Bid are cytosolic proteins; they translocate to the mitochondria and trigger cytochrome C release on apoptosis induction (16). Although cytochrome C release has been demonstrated in apoptotic thymocytes, little is known about the involvement of mitochondrial events in the glucocorticoid-mediated cell death signal. Bcl-2 family proteins are factors involved in this process (12). Bcl-2 knockout mice show fulminant apoptosis in the thymus and hypersensitivity to glucocorticoids (17). On the other hand, thymus hyperplasia was observed in Bax knockout mice, whereas Bax overexpression increases thymus glucocorticoid-induced apoptosis (18).

The hormone melatonin (*N*-acetyl-5-methoxytryptamine),

Abbreviations: *gapdh*, Glyceraldehyde-3-phosphate dehydrogenase; GR, glucocorticoid receptor; IBMX, 3-isobutyl-1-methylxanthine; ROR, retinoid acid-related orphan receptor; SDS, sodium dodecyl sulfate.

***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

which is synthesized mainly in the pineal gland, is thought to be involved in several physiological functions, including the entrainment of seasonal and circadian rhythms (19). In humans, melatonin is believed to be involved in the regulation of sleep, seasonal disorders, depression, and aging. Besides, antitumoral properties of melatonin, as well as its involvement in the responsiveness of the immune system, have been described (for a review, see Refs. 20 and 21). Most of the melatonin actions have been attributed to its interaction with membrane receptors acting through a cAMP-dependent pathway. On the other hand, several evidences suggest that melatonin, being a highly lipophilic molecule with pleiotropic non-receptor-mediated functions, may also influence peripheral tissues as direct targets. In this context, experimental data support that melatonin, as an antioxidant, can protect tissues against damage caused by free radicals (22). Moreover, it was shown that melatonin is able to bind and activate nuclear receptors of the retinoid acid-related orphan receptor (ROR) family (23).

Several reports support a link between melatonin and glucocorticoids. Melatonin or 2-Iodo-melatonin administration was associated with changes in the density and the affinity of cytosolic and nuclear forms of glucocorticoid receptors (GRs) in rat brain, pituitary, thymus, and liver (24), although melatonin does not seem to be a competitive glucocorticoid antagonist (25). Many lines of evidence indicate an effect of melatonin on thymus apoptosis. In this sense, it was shown that both melatonin and pineal grafts partially prevent thymic involution in very old mice (26), and that the methoxyindole decreases DNA fragmentation induced by glucocorticoids in cultured thymocytes of 25-d-old rats (27). In addition, an age-related decrease in 2-[^{125}I] iodomelatonin binding to mouse thymus that correlates with thymus involution was demonstrated (28). Several hypotheses have been postulated to explain the antiapoptotic effect of melatonin in the thymus: a direct interaction of melatonin with thymus GRs, stimulation of IL-4 release, a direct genomic action that modulates the expression of apoptosis-inhibiting genes, an effect on nitric oxide synthase, and finally, the antioxidant action of melatonin (27, 29). Recently, it was demonstrated that melatonin down-regulates GR mRNA levels in thymocytes (30).

Although it was shown that glucocorticoids modulate the expression of several members of the Bcl-2 family proteins (8–10), their involvement as well as the participation of cytochrome C release in the antagonistic effect of melatonin on glucocorticoid-induced apoptosis is still lacking. Therefore, we considered it worthwhile to analyze the effect of melatonin on the levels of these pro- and antiapoptotic proteins in mouse thymocytes. In addition, the involvement of the cAMP pathway in the prevention of the dexamethasone-induced apoptosis by melatonin was studied.

Materials and Methods

Reagents and drugs

Dexamethasone, RU 38486, melatonin, 3-isobutyl-1-methylxanthine (IBMX), concanavalin A, and RPMI 1640 medium were purchased from Sigma (St. Louis, MO). Fetal calf serum was purchased from Life Technologies, Inc. (Carlsbad, CA). Hormones were dissolved in absolute ethanol; for *in vitro* assays ×100 solutions were prepared. Stock solution of Concanavalin A (2 mg/ml) was prepared in water and added to RPMI 1640 medium just before incubations.

Animals and thymocytes preparation

CF-1 male mice (21 d old) were housed in a standard animal room, with food and water *ad libitum*, under controlled conditions of humidity and temperature (21 ± 2 C). Fluorescent lights were turned on automatically every 12 h, from 0600–1800 h. All animals were treated and cared for in accordance with standard international animal care protocols (31). Mice were killed by cervical dislocation at fixed hours (between 1100 and 1200 h) to avoid diurnal variations in serum melatonin levels. Thymuses were immediately removed and minced with scissors extensively into ice-cold RPMI 1640 medium. The cell suspension was filtered through Nytex, and viable cells were counted in a Neubauer hemocytometer in the presence of 0.04% Trypan-Blue. Aliquots of the cell suspension were used for the different assays as indicated below.

In vitro incubation of thymocytes

Thymocytes were incubated in plastic dishes in 1 ml of RPMI 1640 medium containing 10% charcoal-stripped fetal calf serum and concanavalin A (2 μg/ml), in the presence of 10^{-8} M dexamethasone with or without 10^{-8} M melatonin. The corresponding volume of ethanol was added to control cells. Cells were incubated at 37 C for different periods of time in a water bath under a normal atmosphere. After incubation, cells were gently resuspended, placed in a 1.5-ml tube, and centrifuged at 2000 rpm for 5 min at room temperature.

DNA isolation and apoptosis analysis

DNA fragmentation. In each experiment, cell suspension was prepared from a pool of six thymuses, and DNA from 10^6 cells was isolated according to the method described by Herrmann *et al.* (32). Briefly, cells were gently resuspended in 0.1 ml lysis buffer (50 mM Tris, pH 7.5, 20 mM EDTA, and 1% Nonidet P-40) for 1 min. After centrifugation at 2000 rpm for 5 min at room temperature, the supernatant, containing the fragmented DNA, was collected in another tube. The pellet (nuclear DNA) was then resuspended in 0.1 ml lysis buffer. Both fractions were incubated at 56 C for 2 h in the presence of 5 μg/μl ribonuclease A and 1% sodium dodecyl sulfate (SDS). After ribonuclease A treatment, 5 μg/μl proteinase K was added to each sample and further incubated at 37 C overnight. DNA was precipitated by the addition of 75 μl of 10 M ammonium acetate and 450 μl absolute ethanol. After precipitation, DNA was resuspended in 100 μl water, electrophoresed in 1.5% agarose gels, and visualized under UV light. The negative was scanned, and the density was quantified with ImageQuant software (Molecular Dynamics, Inc., Sunnyvale, CA). The fragmentation index was defined as the ratio: fragmented DNA/(fragmented DNA + nuclear DNA), in which fragmented DNA was calculated as the area corresponding to fragments smaller than 2000 bp.

Flow cytometry. An annexin V-fluorescein isothiocyanate apoptosis detection kit from Clontech Products (Palo Alto, CA) (catalog no. K2025–2) was used, following the instructions recommended by the manufacturer. In each experiment, cell suspension was prepared from a pool of six thymuses, and samples of 5×10^5 cells were analyzed by flow cytometry in a Cytoron Absolute cytometer (Ortho Diagnostic Systems, Johnson & Johnson, New Brunswick, NJ). Data were processed by a Wimdi 2.7 software (Windows Multiple Document Interface Flow Cytometry Application, The Scripps Research Institute, La Jolla, CA).

Confocal microscopy

For indirect immunofluorescence, cell suspension employed in each experiment was prepared from a pool of six thymuses, and 10^4 thymocytes were incubated with dexamethasone, melatonin, or both for 4 h at 37 C. After the treatments, a drop of the cell culture was extended on a glass cover slide, which was waved over a flame for 5 sec. Thymocytes were finally fixed with methanol for 5 min at −20 C. Cells were incubated with 100 nM Mitotracker Red CMXRos (Molecular Probes, M-7512, 579-nm excitation wavelength and 599-nm emission wavelength) in PBS for 10 min at room temperature in the dark, followed by permeabili-

zation with 1% SDS in PBS for 5 min and washing three times with PBS. After this treatment, thymocytes were incubated with an anti-cytochrome-C antibody (Cytochrome C H-104, catalog no. sc-7159 from Santa Cruz Biotechnology, Inc., Santa Cruz, CA), diluted (1:50) in PBS containing 3% BSA (PBS-BSA) for 2 h at 37 C in a humid chamber and were washed three times with PBS-BSA. Then, cells were incubated with a Cy2-conjugated secondary antibody antirabbit IgG (492 nm excitation wavelength and 510 nm emission wavelength, catalog no. 711-225-152, Jackson ImmunoResearch, West Grove, PA) diluted 1:200 in PBS-BSA. Cells were finally washed five times with PBS and mounted on a glass slide by adding one drop of 50% glycerol in PBS. Fluorescence was detected with a Zeiss (Berlin, Germany) LSM 510 laser scanning confocal microscope, and images were analyzed with a LSM 510 Image Browser software.

Western blot

In each experiment, cell suspension was prepared from a pool of 16 thymuses, and protein extracts were prepared by lysing 2×10^7 cells in 5 vol lysis buffer (20 mM Tris-Cl, pH 8.0; 137 mM NaCl; 1% Nonidet P-40; and 10% glycerol) supplemented with protease inhibitors (10 μg/ml leupeptin, 1 mM phenylmethylsulfonylfluoride, and 2 μg/ml pepstatin A). The lysates were centrifuged at 4 C for 10 min at 13,000 rpm, and the pellets were discarded. Protein concentration in the supernatant was determined by the Bradford assay (33). After boiling for 5 min, 30 μg of protein was electrophoresed on a 15% SDS-polyacrylamide gel at 120 mV for 1.5 h. The proteins were transferred onto PVDF membranes in transfer buffer containing 20% methanol (vol/vol), 0.19 M glycine, 0.025 M Tris-base (pH 8.3) at 80 mV for 2 h at 4 C. Blots were blocked for 1 h in TBS (4 mM Tris-Cl, pH 7.5; 100 mM NaCl) containing 3% low-fat powdered milk and 0.05% Tween 20 at room temperature. Rabbit polyclonal anti-Bax (N-20), anti-Bcl-2 (N-19), anti-Bcl-x (S-18) (catalog nos. 493, 492, and 634, respectively, from Santa Cruz Biotechnology, Inc.), anti-Bak (catalog no. A3538, from Dako Corp, CA) diluted 1:300 in TBS, as well a mouse monoclonal anti-Glucocorticoid-Receptor antibody BuGR2 (Affinity Bioreagents, Golden, CO, diluted 1:500) were used as primary antibodies and incubated with the membranes overnight. Protein bands were visualized by incubating with a peroxidase-conjugated secondary antibody antirabbit IgG or antimouse IgG (1:3000, 1 h) followed by enhanced chemiluminescence with detection system, ECL (Amersham-Pharmacia, Little Chalfont, Buckinghamshire, UK). Densitometric analysis of protein levels was performed with ImageQuant software. The proper loading was evaluated by staining the membranes with Ponceau-S.

Isolation of total RNA and RT-PCR analysis

In each experiment, cell suspension was prepared from a pool of 16 thymuses, and 2×10^7 thymocytes were resuspended in denaturing solution (4 M guanidinium thiocyanate; 25 mM sodium citrate, pH 7; 0.1 M β-mercaptoethanol, and 0.5% sarcosyl). Total RNA was extracted by the single-step method (34). For reverse-transcription, 4 μg of total RNA was used. The first cDNA strand was synthesized with superscript-reverse transcriptase (Life Technologies, Inc.) and 25 ng/μl oligo dT (Life Technologies, Inc.) as reverse complementary primer. For PCR amplification the following oligonucleotides: 5′-Bax: 5′-GGAATTCCAAGAAGCTGAGCGAGTGT-3′ and 3′-Bax: 5′-GGAATTCTTCTTCCA GATGGTGAGCGAG-3′ of *bax* coding region were used as forward and reverse primers, respectively. The reaction yielded a 394-bp length cDNA fragment.

All PCRs were normalized against glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) expression. Primers *gapdh* forward 5′-TGATGACATCAAGAAGGTGGTGAAG-3′ and *gapdh* reverse 5′-TCCTTGGAGGCCA TGTAGGCCAT-3′, which specifically hybridize with *gapdh* mRNA, were used. The reaction yielded a 146-bp-length cDNA fragment. To achieve semiquantitative conditions, RT-PCRs were terminated, and the products were quantified when all the samples were in the linear range of amplification. The cDNA pool (2 μl), 1.25 U Thermus aquaticus polymerase (Life Technologies, Inc.) and amplification primers (20 pmol each) in 50 μl of PCR cocktail ($\times$ polymerase buffer, 2 mM MgCl2, 200 μM each deoxynucleotide triphosphate, 50,000 dpm [^{32}P]deoxycytidine triphosphate) denatured 3 min at 96 C followed by 8, 15, 25, and 30 cycles of amplification by using a step program (96 C, 40 sec; 65 C (for *gaphd*), 58 C (for *bax*), 30 sec, and 72 C, 1 min), and a final extension at 72 C, 10 min. Ten microliters of PCR products were analyzed by electrophoresis in 8% nondenaturing polyacrylamide gels. Radioactivity was quantified with a phosphorimager (Fuji Photo Film Co. Ltd., Cypress, CA).

cAMP assessment

In each experiment, cell suspension was prepared from a pool of six thymuses, and 5×10^5 thymocytes were incubated for 30 min at 37 C with dexamethasone, melatonin, or both in the presence of 0.5 mM IBMX. After centrifuging for 10 min at 900 $\times$ *g*, pellets were resuspended in distilled water and boiled for 2 min. cAMP levels were assessed as previously described (35). Briefly, homogenates were centrifuged at 5000 $\times$ *g* for 5 min at 4 C, and cAMP content was assessed in the supernatants by RIA after acetylation with acetic anhydride/triethylamine. The acetylated products were incubated with [^{125}I]cAMP (15,000–20,000 dpm, specific activity 140 mCi/mmol) and a rabbit antiserum, kindly supplied by the National Institute of Diabetes and Digestive and Kidney Disease, diluted 1:5000, and incubated overnight at 4 C. After adding 2 ml ethanol with 2% BSA, the antigen-antibody complexes were precipitated by centrifugation at 2000 $\times$ *g* for 15 min. The supernatants were separated by aspiration, and the radioactivity was measured in a γ-counter. The range of the standard curves was 10–5000 fmol cAMP. Three independent experiments were performed in duplicate.

Statistical analysis

Statistical analysis was performed by the two-way ANOVA followed by Tukey's or Dunnett's test as stated.

Results

Figure 1A depicts the effect of 10 nM melatonin on genomic DNA fragmentation of thymocytes induced by 10 nM dexamethasone. After 3 h of incubation with the glucocorticoid, a significant increase in the DNA fragmentation index was observed (control = 0.19 $\pm$ 0.02; dexamethasone = 0.58 $\pm$ 0.04). When cells were incubated in the presence of both dexamethasone and melatonin, a significant reduction of this parameter was detected (dexamethasone + melatonin = 0.33 $\pm$ 0.03). In these conditions, melatonin showed no effect *per se* (melatonin = 0.28 $\pm$ 0.04). A similar pattern was obtained when the number of apoptotic cells was quantified by flow cytometry after staining with FITC-annexin V, as shown in Fig. 1B. In this case, the percentages of annexin-V-positive cells were: control = 19 $\pm$ 1, dexamethasone = 29 $\pm$ 1, dexamethasone + melatonin = 18 $\pm$ 1, and melatonin = 19 $\pm$ 2.

To evaluate the involvement of cytochrome C release in the antagonism melatonin/dexamethasone, the intracellular localization of this protein was analyzed by immunofluorescence confocal microscopy. In control conditions, cytochrome C was predominantly localized in mitochondria, as confirmed by its colocalization with Mitotracker Red CMXRos, a specific dye for this organelle (Fig. 2, A–C). When thymocytes were incubated with dexamethasone, the cytochrome C signal segregated from the mitochondrial dye (Fig. 2, D–F), suggesting its translocation to the cytosol. This effect of dexamethasone was blocked in the presence of melatonin (Fig. 2, G–I). Melatonin *per se* did not change the subcellular localization of cytochrome C (data not shown).

The relative abundance of the members of the Bcl-2 family is one of the key points in the control of cytochrome C release from mitochondria. Therefore, the effect of dexamethasone,

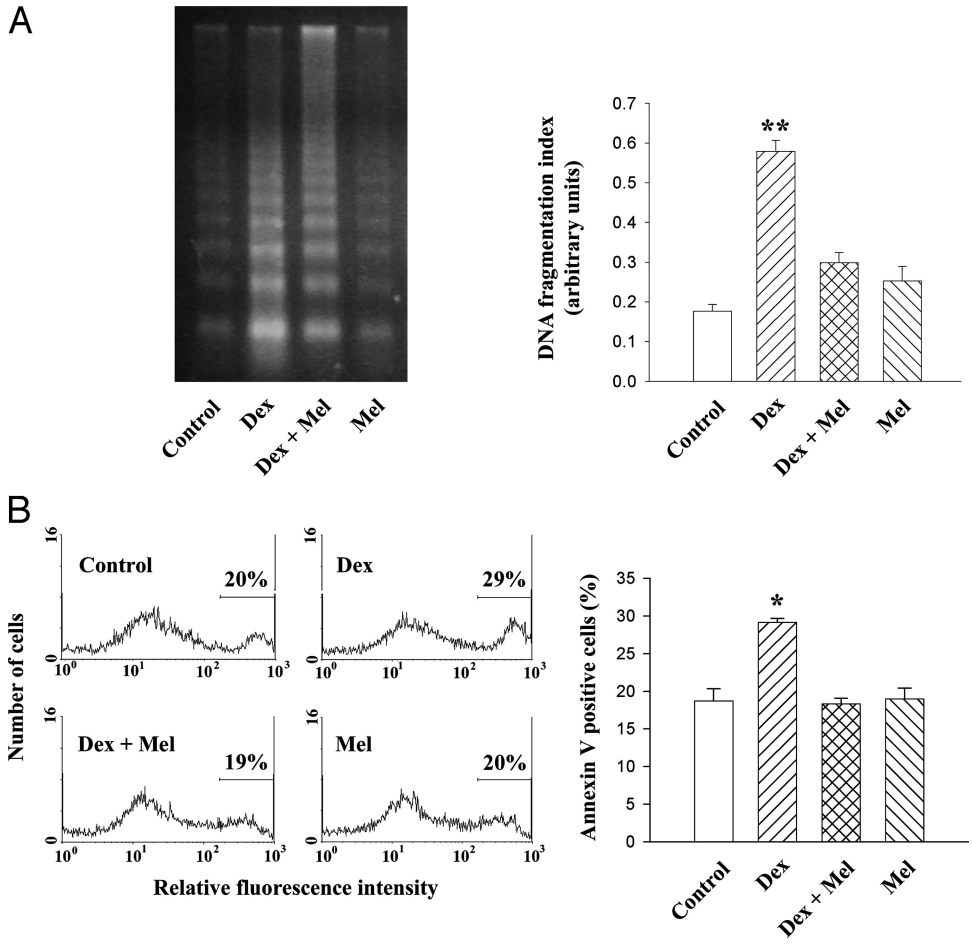

FIG. 1. Effect of melatonin (Mel) on apoptosis of mouse thymocytes induced by dexamethasone (Dex). A, DNA fragmentation. Cells were incubated for 3 h with or without 10^{-8} M Dex or 10^{-8} M Mel. DNA density was quantified with Image Quant software, and the fragmentation index was defined as described in *Materials and Methods*. The gel is representative of three independent experiments. The *right panel* shows mean ± SEM values (n = 3). Dex significantly increased this parameter, whereas Mel reverted the effect of the glucocorticoid. **, $P < 0.01$ *vs.* control, Dex + Mel, and Mel, by Tukey's test. B, Positive annexin V cells. Cells were incubated for 6 h with or without Dex (10^{-8} M) or Mel (10^{-8} M) and analyzed by flow cytometry as described in *Materials and Methods*. The histograms are representative of three independent experiments. The *right panel* shows mean ± SEM values (n = 3). *, $P < 0.05$ *vs.* control (n = 3), Dex + Mel, and Mel by Tukey's test.

melatonin, or both, on the levels of these proteins was examined. Figure 3 shows representative Western blot analyses of some of these proteins. The levels of Bax were increased by dexamethasone (control: 2.03 ± 0.22 arbitrary units dexamethasone: 4.28 ± 0.48 arbitrary units), its effect being abolished by the specific antagonist RU 38486 [1.94 ± 0.25 arbitrary units, $P < 0.001$ *vs.* dexamethasone (n = 3), by Tukey's test]. In the presence of melatonin, which showed no effect *per se*, Bax levels were reduced almost to control values (dexamethasone + melatonin, 2.52 ± 0.35 arbitrary units; melatonin, 2.24 ± 0.30 arbitrary units). The levels of Bcl-2, Bcl-X_L, Bcl-X_S, and Bak did not significantly change as a consequence of any of these treatments (Fig. 3, B–D).

We next examined *bax* mRNA levels by semiquantitative RT-PCR analysis. Dexamethasone provoked a 70% increase of this parameter, being this effect partially blocked by melatonin (Fig. 4). RU 38486 completely reduced the effect of dexamethasone (RU 38486: 2.21 ± 0.36 arbitrary units, $P < 0.01$ *vs.* dexamethasone (n = 3), by Tukey's test), whereas melatonin showed no effect *per se* on *bax* mRNA levels (melatonin: 1.98 ± 0.22 arbitrary units). These results suggest that glucocorticoids could regulate *bax* expression, at least partially, in a melatonin-sensitive manner.

The levels of GR in thymocytes incubated in the presence of dexamethasone, melatonin, or both were assessed. As shown in Fig. 5, neither dexamethasone nor melatonin affected this parameter.

Figure 6 depicts the effect of melatonin, dexamethasone, or both on thymocytes cAMP accumulation in the presence of IBMX. Although melatonin decreased this parameter, the antagonistic action between both hormones was not observed. In fact, dexamethasone significantly decreased the nucleotide levels to a similar extent as that observed in the presence of both compounds.

Discussion

The present results indicate that melatonin, which prevented thymocyte apoptosis mediated by dexamethasone, also inhibits both the glucocorticoid-induced cytochrome C release and the increase in the levels of Bax protein and *bax* mRNA.

The antiapoptotic activity of melatonin was previously described in several systems, including cerebellar neurons (36) and rat brain astrocytes (37), among others. As for the thymus, a well-known model for apoptosis studies, our results are in agreement with a previous report by Sainz *et al.* (30), who showed that the daily administration of melatonin, during 40 d, reduces glucocorticoid-induced apoptosis in thymocytes from 65-d-old rats. As mentioned before, it was shown that preincubation with melatonin for 3 h significantly decreases the percentage of DNA fragmentation (assessed by a spectrophotometric assay) in thymocytes from 25-d-old rats, further incubated for an additional 6 h-period

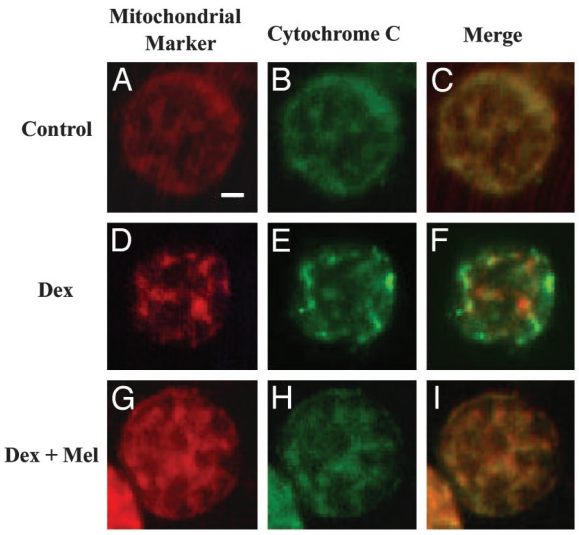

FIG. 2. Cytochrome C release from mitochondria. Cells were incubated with Dex (10^{-8} M) (D–F), Dex (10^{-8} M) + Mel (10^{-8} M) (G–I) for 4 h at 37 C as described in *Materials and Methods*. Control: A–C. Then, they were treated with Mitotracker Red CMXRos to stain mitochondria, immunolabeled for Cytochrome C, and analyzed by laser fluorescence confocal microscopy as described in *Materials and Methods*. Each field was independently visualized with the appropiate wavelength for Mitochondrial Marker (*red*; A, D, and G) and for Cytochrome-C antibody plus Cy2 dye-conjugated secondary antibody (*green*; B, E, and H), and then the two images were overlaid (C, F, and I). Magnification, ×2000. *Bar*, 2 μm. Results are representative of three independent experiments.

with dexamethasone (27). In our hands, the antiapoptotic effect of melatonin was also evident when it was coincubated with dexamethasone without preincubation and for only 3 h. Although our results confirm that melatonin reduces mouse genomic DNA fragmentation induced by dexamethasone, we extend this conclusion by showing that the methoxyindole also reduced the glucocorticoid-induced phosphatidylserine translocation to the outer layer of the cytoplasmatic membrane as shown by the annexin V assay.

During apoptosis, cytochrome C translocates from mitochondria to cytosol, where it triggers a cascade of events that involves caspases activation that, in turn, cleave multiple cytoplasmic and nuclear substrates. The link between apoptosis/mitochondria/glucocorticoids is a matter of active debate. As shown herein by immunofluorescence confocal microscopy, dexamethasone induced the segregation of the cytochrome C signal from the mitochondria. This result agrees with the demonstration that dexamethasone induces cytochrome C release in mouse thymocytes assessed by Western blotting (38). In addition, glucocorticoids induce loss of mitochondrial membrane potential in thymocytes and in T cell hybridoma cells (39, 40). In contrast, apoptosis induced by dexamethasone has no detectable effect on cytochrome C release in multiple myeloma cells (41). In this sense, Distelhorts (42) suggested that this step seems to occur downstream of the commitment to cell death, and may be induced by caspases activity. However, more experiments are still needed to clarify this point. Because melatonin prevented both apoptosis and cytochrome C release induced by dexamethasone, it seems possible that a target for melatonin in the glucocorticoid pathway could be located upstream from the mitochondrial dysfunction.

For the first time, the present results show an increase in thymocyte Bax levels induced by dexamethasone *in vitro*. This glucocorticoid action seems to be specific, because it was reverted by the specific antagonist RU 38486 and because the other tested members of the Bcl-2 family were unaffected by dexamethasone. Apoptosis of thymocytes induced by dexamethasone is an extensively examined process. It is thus surprising that the mechanism described herein remained largely unknown, although an increase in Bax-like (but not in Bcl-2-like) immunoreactivity was described in thymocytes after a treatment with hydrocortisone *in vivo* (43).

Because an increase in *bax* mRNA levels was also evident, the effect of dexamethasone on the Bax content in thymocytes is presumably mediated by a transcriptional mechanism. Moreover, *in silico* analysis showed the presence of putative GREs in the *bax* promoter (unpublished data). Although a direct link between the increase in Bax levels and apoptosis is still lacking in this system, it is tempting to infer a causal relationship between both phenomena. Several lines of evidence support this hypothesis. In effect, a correlation between dexamethasone-induced apoptosis and the increase in *bax* mRNA and protein levels in rat hippocampus granule cells was demonstrated (44). Moreover, the same cells from Bax knockout mice did not undergo apoptosis upon dexamethasone treatment (44). It has further been shown that the overexpression of Bax accelerates dexamethasone-mediated cell death in the thymus (18). In addition, as shown herein, melatonin reduced the increase in Bax levels and reverted the proapoptotic effect of dexamethasone. Taken together, these evidences support the consensus that Bax may play a key role in corticosteroid-induced apoptosis and suggest the involvement of this protein in the antagonism melatonin/dexamethasone.

The ability of Bax to translocate to mitochondria in mouse thymocytes has been reported (16). The intracellular events that account for the redistribution of Bcl-2 family proteins are still unclear. However, the abundance of these proteins could be a necessary (although not sufficient) condition. It is possible that an increase in Bax levels could increase the probability of homo- or heterodimerization that seems necessary for the translocation (45). In this way, the increase in Bax levels observed after the incubation with dexamethasone could be an initial step in its destination to mitochondria.

So far, three molecular mechanisms have been described for the melatonin effect: (1) its antioxidant effect, and its specific interaction with (2) membrane and (3) nuclear receptors. Many evidences suggest that the antiapoptotic effect of melatonin in different tissues could be related to its antioxidant activity. In this sense, it was reported that 200 μM melatonin prevents cell death induced by hydroxyl radicals (OH•) in primary culture of mouse thymocytes (29). Based on the fact that the proapoptotic effect of glucocorticoids has been related to oxidative stress (46, 47), this hypothesis was examined in rat thymocytes by Sainz *et al.* (30). These authors concluded that melatonin does not regulate the mRNA levels of antioxidant enzymes in mouse thymocytes. High concentrations of melatonin are needed to effectively scavenge free radicals, thus it seems unlikely that an antioxidant effect

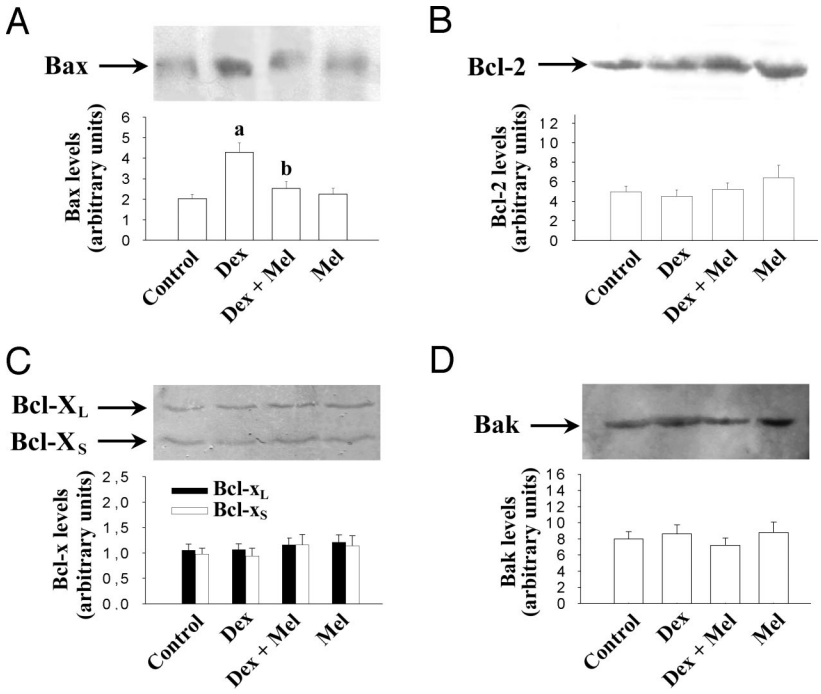

FIG. 3. Effect of Dex and Mel on the levels of Bcl-2-related proteins. Cells were incubated for 2 h with or without Dex (10^{-8} M) or Mel (10^{-8} M). After incubation proteins were processed and Western blot analysis was performed as described with a rabbit polyclonal anti-Bax (A), anti-Bcl-2 (B), anti-Bcl-$X_{(S/liter)}$ (C), or anti-Bak (D), densitometric analysis of protein levels was performed with Image Quant software. The proper loading was evaluated by staining the membranes with Ponceau-S. The gels correspond to one representative experiment. Mean ± SEM values from three independent experiments are shown below each gel. a, $P < 0.01$ *vs.* control; b, $P < 0.01$ *vs.* Dex, by Tukey's test.

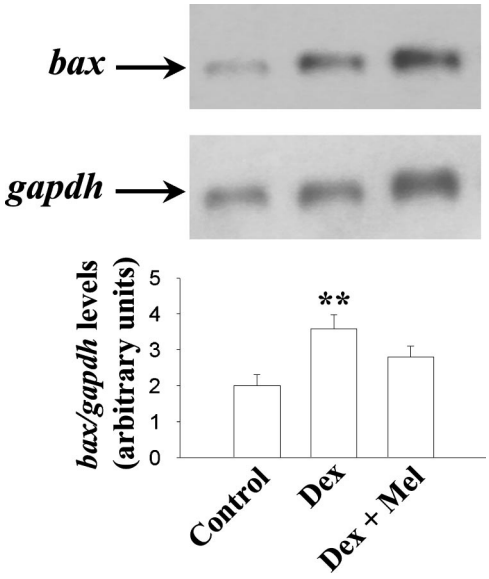

FIG. 4. Effect of Dex and Mel on bax mRNA levels. Cells were incubated with 10^{-8} M Dex, 10^{-8} M Mel, or both for 1 h. After incubation, total RNA was obtained, and semiquantitative RT-PCR with specific primers for *bax* and *gapdh* coding region were performed as described in *Materials and Methods*. PCR products were resolved by electrophoresis in 8% nondenaturing polyacrylamide gel. Radioactivity was quantified with a phosphoimager (Fuji Photo Film Co. Ltd.). Dex significantly increased *bax* mRNA levels, and this effect was reverted in the presence of Mel. Data are mean ± SEM values (n = 3); **, $P < 0.01$ *vs.* control, by Tukey's test.

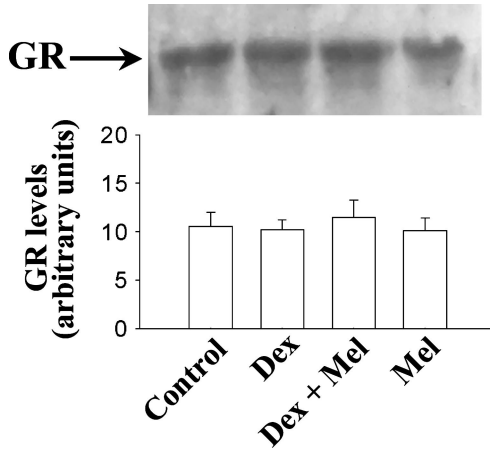

FIG. 5. Effect of Dex, Mel, or both on thymocytes GR levels. Cells were incubated and processed as described in Fig. 3. No changes in this parameter were observed after any of these treatments. Results are representative of three independent experiments. Data are mean ± SEM values (n = 3). Image corresponds to one representative experiment. No significant differences were detected by Tukey's test.

could account for the antiapoptotic action of melatonin at a 10-nM concentration. However, because it was reported that nanomolar concentrations of melatonin increase mitochondrial electron transport chain activity and ATP production, counteracting mitochondrial oxidative stress, the possibility that melatonin increases mitochondrial membrane potential and, hence, may counteract the mechanism(s) involved in transition pore opening and cytochrome C release cannot be formally ruled out (48, 49). Sainz *et al.* (30) suggested that melatonin negatively regulates the expression of thymocyte GRs as previously described in other systems (36). Because, in our conditions, no changes in mouse thymocyte GR levels were found in the presence of melatonin, it seems unlikely that this mechanism may explain the effects of melatonin described herein.

Mel 1a melatonin membrane receptors have been described in rat T and B lymphocytes (50). Accordingly, the present results demonstrate that melatonin decreases cAMP accumulation in mouse thymocytes. As shown herein, dexamethasone also decreased thymocyte cAMP levels. Although

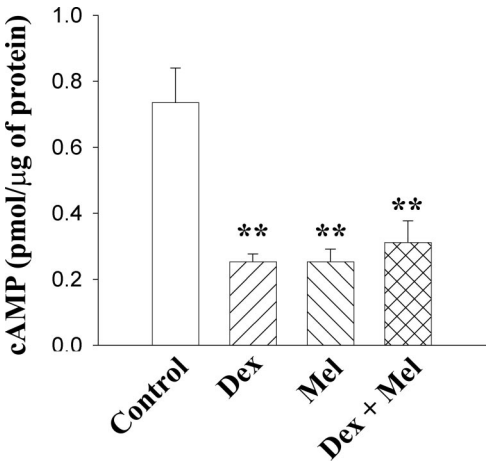

FIG. 6. Effect of Dex, Mel, or both on thymocytes cAMP accumulation. Cells were incubated for 30 min with or without 10^{-8} M Dex or 10^{-8} M Mel in the presence 0.5 mM IBMX. cAMP content was assessed in the supernatants by RIA after acetylation. Both Dex and Mel significantly decreased this parameter. Data are mean ± SEM values from three independent experiments performed in duplicate. **, $P < 0.01$, by Dunnett's test.

an inhibitory effect of dexamethasone on the increase in cAMP levels induced by adrenergic agonists in rat thymic epithelial cells was reported (51), the effect *per se* of the glucocorticoid on this parameter in thymus has not been previously described. Because both melatonin and dexamethasone reduced cAMP levels, and no reversion of this effect was observed in the presence of both compounds, the role of cAMP as a mediator of melatonin/glucocorticoid antagonism seems unlikely.

Melatonin binds to purified thymocyte nuclei (52), suggesting the existence of functional nuclear receptors of the ROR family in the thymus. Through this receptor, melatonin regulates the expression of several genes in other tissues (53, 54). Because the effect of melatonin in thymus does not seem to involve membrane receptors or an antioxidant mechanism, RORα receptors emerge as possible candidates to account for this effect. In fact, besides the glucocorticoid-responsive elements, several putative RORα-responsive elements were found in *bax* promotor (unpublished data). In addition, the GR-interacting protein-1 has been shown to be a coactivator for RORα (55). There are several possibilities to explain the antagonism between melatonin and glucocorticoids involving this type of receptors: 1) the RORα/melatonin complex may affect the dexamethasone/receptor complex translocation to the nucleus; 2) it may modulate the binding of the dexamethasone/receptor complex to its DNA-responsive element, or 3) both complexes may bind to the *bax* promotor and regulate the expression of this gene in opposite directions. These mechanisms are currently under study.

The physiological relevance of the effect of melatonin described herein still remains an open question. The dose of melatonin used in this study (10 nM) is slightly above its normal plasma concentration measured during its nocturnal peak (1 nM). However, it was proposed that melatonin may accumulate in some tissues, and cellular structures such as cell membrane, cytosol, and nuclei to about 10^3 times higher than circulating levels (56). In addition, Martín *et al.* (48) reported intramitochondrial melatonin levels 100 times higher than those of plasma. Altogether, these data support a physiological role of melatonin in the prevention of thymocytes apoptosis.

In summary, the present results suggest the involvement of the Bax protein in the prevention of glucocorticoid-induced apoptosis in thymocytes by melatonin. Because glucocorticoids are well known as pleiotropic regulators, the existence of signals able to modulate their functions may contribute to prevent an all-or-none type of response, providing specific targets (such as the thymus) with a higher capacity to respond to a wide range of physiological demands. This is in accordance with reports on the effect of melatonin on age-related thymic involution (57). In this way, melatonin could well provide a "break" to adjust the timing of thymus apoptosis.

Acknowledgments

The authors thank Dr. Carlos P. Lantos, Dr. Diego A. Golombek, and Dr. Cora B. Cymeryng for helpful discussion of this manuscript; Dr. Omar Pignataro for iodination of [^{125}I]cAMP; and Dr. Tomás Santa Coloma and Dr. H. Eduardo Chuluyan for confocal microscopy and flow cytometry studies.

Received June 18, 2003. Accepted September 11, 2003.

Address all correspondence and requests for reprints to: Adalí Pecci, Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Pab. II, 4° P, Ciudad Universitaria (C1428EGA), Buenos Aires 1428, Argentina. E-mail: apecci@qb.fcen.uba.ar.

This work was supported by grants from Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT), University of Buenos Aires, Fundación Antorchas and by the fellowship "Ramón Carrillo-Arturo Oñativia," Ministerio de Salud de la Nación, Argentina.

References

1. **Cidlowski JA, King KL, Evans-Storms RB, Montague JW, Bortner CD, Hughes Jr FM** 1996 The biochemistry and molecular biology of glucocorticoid-induced apoptosis in the immune system. Recent Prog Horm Res 51:457–490; discussion 490–491
2. **Green DR** 1998 Apoptotic pathways: the roads to ruin. Cell 94:695–698
3. **Mak TW, Yeh WC** 1999 Genetic analysis of apoptotic and survival signals. Cold Spring Harb Symp Quant Biol 64:335–342
4. **Hengartner MO** 2000 The biochemistry of apoptosis. Nature 407:770–776
5. **Adams JM, Cory S** 1998 The Bcl-2 protein family: arbiters of cell survival. Science 281:1322–1326
6. **Zornig M, Hueber A, Baum W, Evan G** 2001 Apoptosis regulators and their role in tumorigenesis. Biochim Biophys Acta 1551:F1–F37
7. **Cory S** 1995 Regulation of lymphocyte survival by the bcl-2 gene family. Annu Rev Immunol 13:513–543
8. **Pecci A, Scholz A, Pelster D, Beato M** 1997 Progestins prevent apoptosis in a rat endometrial cell line and increase the ratio of bcl-XL to bcl-XS. J Biol Chem 272:11791–11798
9. **Vicent GP, Pecci A, Ghini A, Piwien-Pilipuk G, Galigniana MD** 2002 Differences in nuclear retention characteristics of agonist-activated glucocorticoid receptor may determine specific responses. Exp Cell Res 276:142–154
10. **Yamamoto M, Fukuda K, Miura N, Suzuki R, Kido T, Komatsu Y** 1998 Inhibition by dexamethasone of transforming growth factor β1-induced apoptosis in rat hepatoma cells: a possible association with Bcl-xL induction. Hepatology 27:959–966
11. **Sentman CL, Shutter JR, Hockenbery D, Kanagawa O, Korsmeyer SJ** 1991 bcl-2 inhibits multiple forms of apoptosis but not negative selection in thymocytes. Cell 67:879–888
12. **Boise LH, Gonzalez-Garcia M, Postema CE, Ding L, Lindsten T, Turka LA, Mao X, Nunez G, Thompson CB** 1993 bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. Cell 74:597–608
13. **Duckett CS, Li F, Wang Y, Tomaselli KJ, Thompson CB, Armstrong RC** 1998 Human IAP-like protein regulates programmed cell death downstream of Bcl-xL and cytochrome c. Mol Cell Biol 18:608–615
14. **Liu X, Kim CN, Yang J, Jemmerson R, Wang X** 1996 Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. Cell 86:147–157

15. **Shimizu S, Narita M, Tsujimoto Y** 1999 Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC. Nature 399:483–487
16. **Hsu YT, Wolter KG, Youle RJ** 1997 Cytosol-to-membrane redistribution of Bax and Bcl-X(L) during apoptosis. Proc Natl Acad Sci USA 94:3668–3672
17. **Veis DJ, Sorenson CM, Shutter JR, Korsmeyer SJ** 1993 Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. Cell 75:229–240
18. **Brady HJ, Salomons GS, Bobeldijk RC, Berns AJ** 1996 T cells from baxα transgenic mice show accelerated apoptosis in response to stimuli but do not show restored DNA damage-induced cell death in the absence of p53. EMBO J 15:1221–1230
19. **Casone V** 1991 Melatonin and suprachiasmatic nucleus function. In: Klein DC, Moore RYR, SM, eds. Suprachiasmatic nucleus. The mind's clock. Oxford: Oxford University Press; 309–323
20. **Bubenik GA, Blask DE, Brown GM, Maestroni GJ, Pang SF, Reiter RJ, Viswanathan M, Zisapel N** 1998 Prospects of the clinical utilization of melatonin. Biol Signals Recept 7:195–219
21. **Cos S, Sanchez-Barcelo EJ** 2000 Melatonin and mammary pathological growth. Front Neuroendocrinol 21:133–170
22. **Reiter RJ** 1995 Functional pleiotropy of the neurohormone melatonin: antioxidant protection and neuroendocrine regulation. Front Neuroendocrinol 16:383–415
23. **Becker-Andre M, Wiesenberg I, Schaeren-Wiemers N, Andre E, Missbach M, Saurat JH, Carlberg C** 1994 Pineal gland hormone melatonin binds and activates an orphan of the nuclear receptor superfamily. J Biol Chem 269:28531–28534
24. **Persengiev SP, Kondova II** 1993 Tissue-specific modulation of rat glucocorticoid receptor binding activity by melatonin. Experientia 49:332–334
25. **Familari M, Funder JW** 1988 Melatonin and glucocorticoid hormones. Jpn J Exp Med 58:73–77
26. **Provinciali M, Di Stefano G, Bulian D, Tibaldi A, Fabris N** 1996 Effect of melatonin and pineal grafting on thymocyte apoptosis in aging mice. Mech Ageing Dev 90:1–19
27. **Sainz RM, Mayo JC, Uria H, Kotler M, Antolin I, Rodriguez C, Menendez-Pelaez A** 1995 The pineal neurohormone melatonin prevents *in vivo* and *in vitro* apoptosis in thymocytes. J Pineal Res 19:178–188
28. **Liu Z, Zhao Y, Peng S** 1995 Identification of 2-[125I] iodomelatonin binding sites in the thymus of mice and its significance. Sci China B 38:1455–1461
29. **Tian YM, Li PP, Jiang XF, Zhang GY, Dai YR** 2001 Rejuvenation of degenerative thymus by oral melatonin administration and the antagonistic action of melatonin against hydroxyl radical-induced apoptosis of cultured thymocytes in mice. J Pineal Res 31:214–221
30. **Sainz RM, Mayo JC, Reiter RJ, Antolin I, Esteban MM, Rodriguez C** 1999 Melatonin regulates glucocorticoid receptor: an answer to its antiapoptotic action in thymus. FASEB J 13:1547–1556
31. **CCAC** 1980 Guide to the care and use of experimental animals. Ottawa: Canadian Council of Animal Care
32. **Herrmann M, Lorenz HM, Voll R, Grunke M, Woith W, Kalden JR** 1994 A rapid and simple method for the isolation of apoptotic DNA fragments. Nucleic Acids Res 22:5506–5507
33. **Bradford MM** 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
34. **Chomczynski P, Sacchi N** 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159
35. **Faillace MP, Sarmiento MI, Siri LN, Rosenstein RE** 1994 Diurnal variations in cyclic AMP and melatonin content of golden hamster retina. J Neurochem 62:1995–2000
36. **Persengiev SP** 2001 The neuroprotective and antiapoptotic effects of melatonin in cerebellar neurons involve glucocorticoid receptor and p130 signal pathways. J Steroid Biochem Mol Biol 77:151–158
37. **Baydas G, Reiter RJ, Nedzvetskii VS, Yasar A, Tuzcu M, Ozveren F, Canatan H** 2003 Melatonin protects the central nervous system of rats against toluene-containing thinner intoxication by reducing reactive gliosis. Toxicol Lett 137:169–174
38. **Yoshino T, Kishi H, Nagata T, Tsukada K, Saito S, Muraguchi A** 2001 Differential involvement of p38 MAP kinase pathway and Bax translocation in the mitochondria-mediated cell death in TCR- and dexamethasone-stimulated thymocytes. Eur J Immunol 31:2702–2708
39. **Camilleri-Broet S, Vanderwerff H, Caldwell E, Hockenbery D** 1998 Distinct alterations in mitochondrial mass and function characterize different models of apoptosis. Exp Cell Res 239:277–292
40. **Castedo M, Hirsch T, Susin SA, Zamzami N, Marchetti P, Macho A, Kroemer G** 1996 Sequential acquisition of mitochondrial and plasma membrane alterations during early lymphocyte apoptosis. J Immunol 157:512–521
41. **Chauhan D, Pandey P, Ogata A, Teoh G, Krett N, Halgren R, Rosen S, Kufe D, Kharbanda S, Anderson K** 1997 Cytochrome c-dependent and -independent induction of apoptosis in multiple myeloma cells. J Biol Chem 272:29995–29997
42. **Distelhorst CW** 2002 Recent insights into the mechanism of glucocorticosteroid-induced apoptosis. Cell Death Differ 9:6–19
43. **Denis G, Humblet C, Verlaet M, Boniver J, Defresne MP** 1998 p53, Bax and Bcl-2 *in vivo* expression in the murine thymus after apoptogenic treatments. Anticancer Res 18:3315–3321
44. **Almeida OF, Conde GL, Crochemore C, Demeneix BA, Fischer D, Hassan AH, Meyer M, Holsboer F, Michaelidis TM** 2000 Subtle shifts in the ratio between pro- and antiapoptotic molecules after activation of corticosteroid receptors decide neuronal fate. FASEB J 14:779–790
45. **Gross A, Jockel J, Wei MC, Korsmeyer SJ** 1998 Enforced dimerization of BAX results in its translocation, mitochondrial dysfunction and apoptosis. EMBO J 17:3878–3885
46. **Beaver JP, Waring P** 1995 A decrease in intracellular glutathione concentration precedes the onset of apoptosis in murine thymocytes. Eur J Cell Biol 68:47–54
47. **Behl C, Lezoualc'h F, Trapp T, Widmann M, Skutella T, Holsboer F** 1997 Glucocorticoids enhance oxidative stress-induced cell death in hippocampal neurons *in vitro*. Endocrinology 138:101–106
48. **Martin M, Macias M, Escames G, Leon J, Acuña-Castroviejo D** 2000 Melatonin but not vitamins C and E maintains glutathione homeostasis in t-butyl hydroperoxide-induced mitochondrial oxidative stress. FASEB J 14:1677–1679
49. **Acuña-Castroviejo D, Escames G, Carazo A, León J, Khaldy H, Reiter RJ** 2002 Melatonin, mitochondrial homeostasis and mitochondrial-related diseases. Curr Top Med Chem 2:133–151
50. **Pozo D, Delgado M, Fernandez-Santos JM, Calvo JR, Gomariz RP, Martin-Lacave I, Ortiz GG, Guerrero JM** 1997 Expression of the Mel1a-melatonin receptor mRNA in T and B subsets of lymphocytes from rat thymus and spleen. FASEB J 11:466–473
51. **Kurz B, Feindt J, von Gaudecker B, Kranz A, Loppnow H, Mentlein R** 1997 β-adrenoceptor-mediated effects in rat cultured thymic epithelial cells. Br J Pharmacol 120:1401–1408
52. **Rafii-El-Idrissi M, Calvo JR, Harmouch A, Garcia-Maurino S, Guerrero JM** 1998 Specific binding of melatonin by purified cell nuclei from spleen and thymus of the rat. J Neuroimmunol 86:190–197
53. **Schrader M, Danielsson C, Wiesenberg I, Carlberg C** 1996 Identification of natural monomeric response elements of the nuclear receptor RZR/ROR. They also bind COUP-TF homodimers. J Biol Chem 271:19732–19736
54. **Steinhilber D, Brungs M, Werz O, Wiesenberg I, Danielsson C, Kahlen JP, Nayeri S, Schrader M, Carlberg C** 1995 The nuclear receptor for melatonin represses 5-lipoxygenase gene expression in human B lymphocytes. J Biol Chem 270:7037–7040
55. **Atkins GB, Hu X, Guenther MG, Rachez C, Freedman LP, Lazar MA** 1999 Coactivators for the orphan nuclear receptor RORalpha. Mol Endocrinol 13:1550–1557
56. **Menendez-Pelaez A, Poeggeler B, Reiter RJ, Barlow-Walden L, Pablos MI, Tan DX** 1993 Nuclear localization of melatonin in different mammalian tissues: immunocytochemical and radioimmunoassay evidence. J Cell Biochem 53:373–382
57. **Fabris N, Mocchegiani E, Provinciali M** 1997 Plasticity of neuroendocrine-thymus interactions during aging. Exp Gerontol 32:415–429

***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**