Melatonin decreases apoptosis and expression of apoptosis-associated proteins in acute puromycin aminonucleoside nephrosis

Adriana Pedreañez\(^1\), Jaimar Rincón\(^2\), Maritza Romero\(^2\), Ninoska Viera\(^3\) and Jesús Mosquera\(^2\)

\(^1\)Catedra de Inmunologia, Escuela de Bioanalisis, \(^2\)Instituto de Investigaciones Clinicas ‘Dr Americo Negrette’, Facultad de Medicina and \(^3\)Instituto de Investigaciones Odontologicas, Facultad de Odontologia, Universidad del Zulia, Maracaibo, Venezuela

Abstract

Background. The anti-apoptotic properties of melatonin have been demonstrated previously in several in vivo and in vitro studies. Previous reports have shown increased apoptosis during puromycin aminonucleoside nephrosis (PAN). The aim of this study was to determine if melatonin (MEL) can prevent apoptosis and modify oxidative stress, an apoptosis inducer, in this experimental model.

Methods. Rats were injected intraperitoneally with puromycin aminonucleoside. In addition, by the intragastric route they received 1 mg/kg/day of MEL or vehicle 3 days before puromycin injection and throughout the experiment. Animals were sacrificed at weeks 1 and 2 of nephrosis and frozen renal sections were studied for apoptosis by TUNEL, for apoptosis-associated proteins by monoclonal and polyclonal antibodies, and for superoxide anion (O\(_2\)-) by a histochemical method. Nitric oxide (NO), malondialdehyde (MDA) and reduced glutathione (GSH), and the activities of superoxide dismutase (SOD) and catalase were measured in homogenized kidney tissue by appropriate biochemical and enzymatic methods.

Results. Increases in apoptosis, p53, Fas and Fas-ligand were observed in nephrotic animals. MEL treatment decreased apoptosis at weeks 1 and 2 in the glomerular, interstitial and tubular compartments. This was accompanied by decreased expression of p53 (glomerulus, week 1; tubules, weeks 1 and 2), Fas (glomerulus and interstitium, week 2; tubules, weeks 1 and 2) and Fas-ligand (interstitium and tubules, week 2). Increased expression of Bel-2-positive cells was observed at week 2 in all renal compartments in MEL-treated animals. High levels of O\(_2\)- and NO generation and lipid peroxidation (MDA) were found in nephrotic animals. SOD and GSH remained unchanged, and only decreased catalase activity (week 1) was observed in PAN animals. Tendencies toward decreased values of O\(_2\)- and MDA content along with recovery of catalase activity (week 1) were observed in MEL-treated nephrotic animals, but were insignificant in magnitude. MEL, however, did significantly downregulate pro-apoptotic genes and upregulated anti-apoptotic genes.

Conclusions. The data demonstrate that, in PAN, melatonin has anti-apoptotic effects, which might in part be independent of the modulation of the oxidative status.

Keywords: aminonucleoside nephrosis; apoptosis; Bel-2; Fas; Fas-L; p53; melatonin

Introduction

Melatonin (MEL), a product of tryptophan metabolism, is the principal pineal hormone. The main physiological function of MEL is to transduce variations in the light–darkness cycle and the diurnal and seasonal periodicity of various physiological functions [1]. More recently, it has been demonstrated that MEL may exert a modulatory role on apoptosis. The data available indicate that MEL treatment has an inducer or blocking effect on apoptosis in several biological systems [2–4] and its anti-apoptotic effect has been attributed to its antioxidant properties [5]. Apoptosis and oxidative stress have been observed previously in acute puromycin aminonucleoside nephrosis (PAN) [6,7]. Therefore, the aim of this work is to examine the effect of MEL on the incidence of apoptosis and oxidative stress during the course of PAN. Our results indicate that MEL treatment reduces apoptosis in PAN, accompanied by downregulation of pro-apoptotic and upregulation of anti-apoptotic genes.
proteins as well as by a tendency toward decreased oxidative stress.

**Subjects and methods**

**Reagents**

Apoptosis was evaluated using an *in situ* apoptosis detection kit (Promega Corporation, WI). The anti-rat p53 tumour suppressor protein monoclonal antibody (mAb), and anti-rat Bcl-2 protein mAb were purchased from Biosource International Inc. (Camarillo, CA). Fluorescein isothiocyanate (FITC)-conjugated F(ab')2 fragment rat anti-mouse IgG was obtained from Accurate Chemical & Scientific Corporation (Westbury, NY). Rabbit anti-rat Fas, rabbit anti-rat Fas-ligand (Fas-L) and FITC-labelled F(ab')2 fragment sheep anti-rabbit IgG antibodies were obtained from Calbiochem-Novabiochem Corp. (San Diego, CA). Rabbit anti-rat Fas, rabbit IgG was obtained from Accurate Chemical & Scientific Corporation (Westbury, NY). Puromycin aminonucleoside (PA), MEL and Calbiochem-Novabiochem (La Jolla, CA). Tetramethylrhodamine isothiocyanate (TRITC)-labelled goat anti-mouse immunoglobulins were obtained from Biosource International. Puromycin aminonucleoside (PA), MEL and an mAb against smooth muscle myosin were obtained from Sigma Chemical Co. (St Louis, MO).

**Experimental protocol**

Male Sprague–Dawley rats, weighing 250–300 g were made nephrotic by a single intraperitoneal (i.p.) injection of PA, 15 mg/100 g body weight. The puromycin group was divided into two. Group 1 received 1 mg/kg/day of MEL by the intragastric route using an orogastric tube during the 3 days prior to and every day after the injection of PA. Group 2 received by a similar route a 0.9% NaCl solution instead of MEL throughout the experiment. Controls of PA-injected animals were rats injected i.p. with 0.9% NaCl solution. Experimental and control rats were sacrificed on weeks 1 and 2 after PA injection (n=5–6 per group). Before their sacrifice, 24-h urine samples were collected from all rats and proteinuria was determined by the sulfosalicylic acid method. At sacrifice, kidneys were removed after being perfused with 20 ml of 0.9% saline solution via the abdominal aorta and were used for histological and biochemical assays. For immunofluorescence studies, pieces of the perfused kidneys were immersed in OCT compound (Tissue Tek, Miles Inc., Diagnostics Division, Kankakee, IL), frozen in dry ice and acetone and stored at −70°C until use.

**Identification of apoptosis (TUNEL)**

To measure apoptosis, we utilized a histological assay that allows selective labelling of cells with fragmented DNA in tissue sections. Fragments of DNA in apoptotic cells were nick end-labelled using an *in situ* apoptosis detection kit following the manufacturer’s instructions. To detect developmental cell deletion, forelimb buds from embryonic rats were used as positive controls. Negative controls were represented by renal and embryonic rat forelimb bud tissues incubated with distilled water, instead of terminal deoxynucleotidyl transferase, in the reaction buffer. Slides were mounted with p-phenylenediamine, to delay fluorescence quenching, and were examined with an epifluorescence microscope (Axioskop Zeiss, Germany).

**Determination of oxidative stress, antioxidant enzymes and reduced glutathione**

Superoxide anion production in the different renal compartments was determined at the cellular level by a cytochemical method described previously [8]. The results in the glomeruli were expressed as positive cells per glomerular cross-section and in the tubulointerstitial area as positive cells per 0.025 mm². Nitric oxide (NO) formation was detected as nitrite/nitrate accumulation in the renal homogenates by the Griess reaction [9] and expressed as nmol/l. Malondialdehyde (MDA) content was measured in renal homogenates by the thiobarbituric acid assay [10] and was expressed as nmol/mg of protein. Reduced glutathione (GSH) content was measured in renal homogenates as previously described [11] and the results are expressed as nmol/mg of renal protein. To determine enzymatic activities, renal slides from normal and experimental animals were homogenized in 50 mM phosphate buffer, pH 7.2. Catalase activity was determined according to the method described by Aebi [12]. The change in absorbance was read at 250 nm and the rate constant of a first-order reaction (k) was used. Results are expressed as k/mg of homogenate proteins. Superoxide dismutase (SOD) activity in renal homogenates was measured using a commercial kit, following the instructions of the manufacturer (Calbiochem-Novabiochem Corp., San Diego, CA). The results are expressed as U/mg of kidney proteins. Total protein content was measured in renal homogenates by the method of Lowry.

**Determination of Fas, Fas-L, p53 and Bcl-2 in renal tissues**

Frozen renal sections (4 μm) from both control and nephrotic animals were divided into two groups: one group was fixed in acetone at −20°C for 15 min, washed and incubated with rabbit anti-rat Fas or rabbit anti-rat Fas-L for 1 h at room temperature. The other group was fixed in 10% neutral buffered formalin for 15 min and treated with anti-rat p53 mAb for 24 h at 4°C or with anti-rat Bcl-2 mAbs for 1 h at room temperature. Indirect immunofluorescence was performed using an FITC-conjugated F(ab')2 fragment rat anti-mouse IgG for 1 h at room temperature or an FITC-conjugated F(ab')2 fragment goat anti-rabbit antibody to determine, respectively, the presence of mAbs or rabbit anti-bodies on renal tissues. Controls included sections subjected to a non-immune rabbit IgG fraction or mAbs with the same isotype but against non-relevant antigens as primary antibodies. Sections were mounted in a solution of p-phenylenediamine in phosphate-buffered saline (PBS)–glycerol and inspected by epifluorescence microscopy.

**Double staining for leukocytes, smooth muscle myosin-positive cells and proapoptosis proteins**

Frozen renal sections from PAN rats at 2 weeks were fixed in acetone at −20°C for 15 min and treated with rabbit anti-rat Fas or rabbit anti-rat Fas-L for 1 h at room temperature. Thereafter, sections were incubated with an FITC-conjugated F(ab')2 fragment goat anti-rabbit antibody for
an additional hour. After washing, tissues were incubated with an mAb against leukocyte common antigen (LCA) or against smooth muscle myosin for 1 h at room temperature. Tissues were washed and incubated with TRITC-labelled rabbit anti-mouse immunoglobulins antibody for 1 h at room temperature. Renal sections were mounted in a solution of p-phenylenediamine in PBS–glycerol and observed under a fluorescence microscope.

Calculations and statistical analysis
Positive cells for TUNEL, Bcl-2, p53, Fas and Fas-L were counted in at least 20 glomerular cross-sections per renal tissue. Positive interstitial and tubular cells were counted in at least 20 tubulointerstitial areas of 0.0625 mm² in each renal sample, except for tubular Fas and Fas-L, where the number of positive tubular cross-sections per 0.0625 mm² was counted. These inspections were performed using an ocular piece fitted with a grid. The results for each group are shown as mean ± SEM. The Mann–Whitney test was used to analyse differences between groups. Multiple comparisons were done using the analysis of variance (ANOVA) test followed by Dunn’s post-test. For correlations between two variables, Spearman’s correlation was used. A two-tailed \( P < 0.05 \) was considered statistically significant.

Results

Effect of MEL on renal apoptosis and apoptosis-associated protein expression in PAN
Expression of apoptosis was observed in all renal compartments in non-nephrotic animals. The glomerular incidence of apoptosis was found to be significantly high at weeks 1 and 2, accompanied by increased expression of p53 (week 1) and Fas (week 2). MEL treatment reduced the number of cells positive for TUNEL, p53 and Fas (Figures 1 and 2). In the interstitium, the increased number of apoptotic nuclei and Fas- and Fas-L-positive cells during week 2 was reduced in rats treated with MEL (Figures 1 and 2). MEL treatment also reduced the increased number of apoptotic tubular cells and the increased expression of p53, Fas and Fas-L found at weeks 1 and 2 of nephrosis (Figures 1 and 2). Increased Bcl-2 expression was found in the glomerular, interstitial and tubular compartments at week 2 in the nephrotic animals treated with MEL (Figure 3). Immunofluorescence staining of MEL-treated and non-treated animals is shown in Figure 4.

Effect of MEL on oxidative stress in PAN
Superoxide-producing cells were increased at week 1 in tubules and at week 2 in glomeruli. An increment of NO was evident at weeks 1 and 2 of nephrosis. Lipid peroxidation products (MDA) were increased at week 2. In general, antioxidant mechanisms, such as SOD activity and GSH content, remained unchanged during PAN; however, the activity of catalase was reduced at week 1 (Table 1). We found a significant correlation between glomerular apoptosis and \( \text{O}_2^- \) (\( r = 0.6701; P = 0.003 \)), NO (\( r = 0.5225; P = 0.02 \)), MDA (\( r = 0.4902; P = 0.03 \)) and decreased activity of catalase (\( r = 0.5093; P = 0.03 \)); between interstitial apoptosis and NO (\( r = 0.5638; P = 0.01 \)) and MDA (\( r = 0.5747; P = 0.01 \)); and between tubular apoptosis and \( \text{O}_2^- \) (\( r = 0.5918; P = 0.009 \)), NO (\( r = 0.5300; P = 0.02 \)) and MDA (\( r = 0.5437; P = 0.01 \)). Decreased numbers of \( \text{O}_2^- \)-expressing cells and MDA levels were observed in nephrotic animals treated with MEL; however, statistical significance was absent (Table 1). Significant recovery of catalase activity was observed in MEL-treated animals at week 1 of nephrosis (Table 1).
Double staining studies

Frozen renal sections from animals at week 2 of PAN were double stained for LCA/Fas, LCA/Fas-L, smooth muscle myosin/Fas and smooth muscle myosin/Fas-L (Figure 5). As shown in Table 2, a high percentage of LCA/Fas cells was found in glomeruli; however, minimal double staining for Fas and Fas-L was observed in smooth muscle myosin-positive cells (presumably mesangial cells). In contrast, ~25% of the interstitial leukocytes or smooth muscle-positive cells (presumably myofibroblasts or myoepithelial cells) stained for Fas or Fas-L.

Proteinuria

All animals injected with PA showed a significant increment of proteinuria at weeks 1 and 2 of nephrosis (control, 3.56 ± 2.54; PAN week 1, 192 ± 81.52; PAN week 2, 201.30 ± 57.18; PAN + MEL week 1, 162 ± 107.39; PAN + MEL week 2, 222.60 ± 27.06 mg/24 h).

Discussion

The increased incidence of apoptosis in PAN has been described previously [7]. In this study, PA administration brought about a significant increase in apoptosis accompanied by an increased expression of pro-apoptotic proteins Fas, Fas-L and p53. MEL is able to prevent or induce apoptosis in several biological systems [2–4], and several hypotheses have been advanced to explain the inhibitory effect of MEL on apoptosis, among them: the induction of interleukin (IL)-4 release, a direct genomic action modulating the expression of apoptosis-inhibiting genes, an effect on nitric oxide synthase, a direct interaction of MEL with glucocorticoid receptors, and the hormone’s
Fig. 4. Expression of renal cellular markers in nephrotic animals untreated (A, C and E) and treated (B, D and F) with melatonin. (A and B) TUNEL-positive cells in tubules (large arrows) and interstitium (small arrows). (C and D) Tubular expression of p53 (arrows). (E and F) Fas-positive interstitial cells. (G) and (H) from MEL-treated nephrotic animals show tubular (G) and interstitial (H) Bcl-2-positive cells (arrows) ×400.
Antioxidant action [13,14]. In this regard, treating nephrotic animals with MEL resulted in a decreased expression of apoptosis and Fas, Fas-L and p53 proteins in the different renal compartments, suggesting that the apoptosis-inhibiting effect of MEL could involve the control of Fas, Fas-L and p53 pathways. Changes in the expression of p53 induced by MEL include an increased expression, associated with an anti-proliferative effect of MEL on cellular cultures [15]. However, the expression of p53 was unchanged when the antioxidant properties of MEL were put to use as anti-apoptotic treatment in vitro [16]. In this study, MEL induced a stronger downregulation of p53 expression, compared with its antioxidant properties. This difference could be related to different pathways by which MEL prevents apoptosis or the presence of MEL-induced mediators during nephrosis. Little is known about the effect of MEL on Fas and its ligand by

Table 1. Oxidative stress in PAN: effect of melatonin on oxidative stress

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>PA (week 1)</th>
<th>P</th>
<th>PA (week 2)</th>
<th>P</th>
<th>PA + MEL (week 1)</th>
<th>P</th>
<th>PA + MEL (week 2)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>O₂</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glom</td>
<td>0.17±0.04</td>
<td>0.80±0.28</td>
<td>NS</td>
<td>0.99±0.37*</td>
<td>&lt;0.05</td>
<td>0.39±0.10</td>
<td>NS</td>
<td>0.37±0.15</td>
<td>NS</td>
</tr>
<tr>
<td>Int</td>
<td>0.24±0.04</td>
<td>0.54±0.17</td>
<td>NS</td>
<td>0.48±0.14</td>
<td>NS</td>
<td>0.33±0.06</td>
<td>NS</td>
<td>0.20±0.05</td>
<td>NS</td>
</tr>
<tr>
<td>Tub</td>
<td>0.13±0.06</td>
<td>0.69±0.21*</td>
<td>&lt;0.05</td>
<td>0.32±0.10</td>
<td>NS</td>
<td>0.23±0.08</td>
<td>NS</td>
<td>0.14±0.04</td>
<td>NS</td>
</tr>
<tr>
<td>NO</td>
<td>1.97±0.27</td>
<td>5.42±1.18*</td>
<td>&lt;0.01</td>
<td>4.67±0.50*</td>
<td>&lt;0.05</td>
<td>5.54±0.83</td>
<td>NS</td>
<td>3.72±0.37</td>
<td>NS</td>
</tr>
<tr>
<td>GSH</td>
<td>8.30±1.32</td>
<td>12.7±1.13</td>
<td>NS</td>
<td>11.6±1.85</td>
<td>&lt;0.05</td>
<td>16.2±2.29</td>
<td>NS</td>
<td>7.80±1.48</td>
<td>NS</td>
</tr>
<tr>
<td>MDA</td>
<td>0.83±0.24</td>
<td>1.48±0.41</td>
<td>NS</td>
<td>4.19±1.32*</td>
<td>&lt;0.01</td>
<td>0.80±0.40</td>
<td>NS</td>
<td>2.48±1.29</td>
<td>NS</td>
</tr>
<tr>
<td>Catalase</td>
<td>0.28±0.10</td>
<td>0.03±0.01*</td>
<td>&lt;0.05</td>
<td>0.10±0.03</td>
<td>NS</td>
<td>0.07±0.01**</td>
<td>0.008</td>
<td>0.15±0.04</td>
<td>NS</td>
</tr>
<tr>
<td>SOD</td>
<td>6.35±1.17</td>
<td>6.53±2.72</td>
<td>NS</td>
<td>8.39±2.18</td>
<td>NS</td>
<td>4.64±0.83</td>
<td>NS</td>
<td>7.36±1.19</td>
<td>NS</td>
</tr>
</tbody>
</table>

Glom, glomerulus (positive cells per glomerular cross-section); Int, interstitium (positive interstitial cells per 0.025 mm²); Tub, tubule (positive tubular cells per 0.025 mm²); PA, puromycin aminonucleoside; MEL, melatonin.

*P-values comparing PA with control; **P-values comparing PA with PA + MEL at week 1.

Fig. 5. Double-stained frozen renal sections from nephrotic rats (week 2) for smooth muscle myosin/Fas (A and B) and for LCA/Fas-L (C and D). An interstitial smooth muscle myosin-positive cell in (A) (arrow) corresponds to a Fas-positive cell in (B) (arrow); ×400. In the same way, an interstitial LCA-positive cell in (C) (arrow) corresponds to a Fas-L-positive cell in D; ×400. Note that double staining is not observed in glomeruli. Renal tissues in (A) and (C) were treated with a TRITC-labelled antibody, and tissues in (B) and (D) with an FITC-labelled antibody.
MEL in vivo. In vitro studies have shown MEL to modulate apoptosis via the Fas and Fas-L pathways. In this regard, a high concentration of MEL increased the level of Fas-L in human neuroblastoma cells [17] and the prooxidant activity of MEL promotes Fas-induced cell death in human leukaemic Jurkat cells [18]. Bcl-2 is the first mammalian regulator protein that was identified to have an anti-apoptotic potential in a variety of cell systems [1]. MEL increased expression of Bcl-2 at week 2 of PAN. This finding could represent the presence of a powerful anti-apoptotic mechanism during this nephrosis. Bcl-2 has been shown to prevent apoptosis via an antioxidant mechanism [20]; however, little down-regulation of oxidative stress was found in our study, suggesting that other mechanisms are involved in the anti-apoptotic effect of Bcl-2 in PAN. In this regard, Bcl-2 can heterodimerize with Bax (a pro-apoptotic member of the Bcl-2 family) leading to the maintenance of organelle integrity, prevention of the release of mitochondrial cytochrome c, and inhibition of the association of Apaf-1 with pro-caspase-9, thereby preventing caspase-9 activation and apoptosis [19]. In support of this, it has been reported that Bcl-2 inhibits the apoptosis induced by various stimuli, including heat shock, serum depletion and chemotherapeutic agents [21], and alternative mechanisms for the inhibition of apoptosis after MEL induction of Bcl-2 such as blocking of caspase 3 activation and inhibition of cytochrome c release have been suggested [3]. The upregulation of Bcl-2 by MEL, associated with the decreased apoptosis observed in this study, has been reported in neural tissues and pineal cells under ischaemic and oxidative conditions, respectively [2,3]. Conversely, when MEL acts as an apoptosis inducer, downregulation of Bcl-2 expression with increased expression of Bax has been reported [4].

Reactive oxygen species may induce apoptosis [20] and may be important mediators of tissue injury in PAN [6]. MEL is one of the most potent physiological free radical scavengers known. It has been reported that the anti-apoptotic effect of MEL could be due to its antioxidant properties [5]. In a previous report, MEL at the dose used in this study (1 mg/kg/day) was able to diminish renal oxidative stress and apoptosis in rats treated with mercuric chloride [5]. Our results showed increased oxidative stress during PAN, and, after MEL treatment, decrease in the numbers of $O_2^-$-expressing cells and in MDA content were observed. Although not statistically significant, the tendency was toward decreased oxidative activity; and since MEL induced a significant recovery of the decreased activity of catalase in PAN, it is possible that this enzyme decreases the concentration of $H_2O_2$. Taking these data together, we cannot rule out the involvement of the anti-oxidant pathways of MEL; however, the magnitude of the changes of apoptosis-associated proteins compared with those observed in oxidative stress suggests that, in this model of nephrosis, the anti-apoptotic effect of MEL is mediated at least in part by other mechanisms.

In conclusion, MEL treatment of PAN rats markedly diminished renal apoptosis. Since the anti-oxidant effects of the treatment did not reach statistical significance, diminished apoptosis could at least in part result from alternative, as yet unidentified pathways.

Acknowledgements. This study was supported by Consejo Científico y Humanístico de la Universidad del Zulia, CONDES (Grant: 137000).

Conflict of interest statement. None declared.

References


Table 2. Glomerular and interstitial double staining for leukocytes, smooth muscle myosin and pro-apoptosis proteins during week 2 of PAN

<table>
<thead>
<tr>
<th>Rat</th>
<th>Glomerulus</th>
<th>Interstitium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LCA/Fas</td>
<td>SM/Fas</td>
</tr>
<tr>
<td>54</td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td>27</td>
<td>30</td>
<td>0</td>
</tr>
<tr>
<td>52</td>
<td>23.1</td>
<td>0</td>
</tr>
<tr>
<td>53</td>
<td>36.8</td>
<td>0.625</td>
</tr>
<tr>
<td>43</td>
<td>37.5</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>33.5±6.9</td>
<td>0.13±0.28</td>
</tr>
</tbody>
</table>

Data represent the percentage of leukocytes or smooth muscle myosin-positive cells positive for Fas or Fas-ligand.
Melatonin decreases apoptosis in aminonucleoside nephrosis

14. Ciesla W. Can melatonin regulate the expression of prohormone convertase 1 and 2 genes via monomeric and dimeric forms of RZR/ROR nuclear receptor, and can melatonin influence the processes of embryogenesis or carcinogenesis by disturbing the proportion of cAMP and cGMP concentrations? Theoretic model of controlled apoptosis. Med Hypotheses 2001; 56: 181–193

Received for publication: 5.5.03
Accepted in revised form: 29.10.03