Melatonin ameliorates oxidative stress, inflammation, proteinuria, and progression of renal damage in rats with renal mass reduction

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1Centro de Investigaciones Biomédicas, Instituto Venezolano de Investigaciones Cientı ´ficas-Zulia and 2Renal Service Hospital Universitario and Universidad del Zulia, Maracaibo, Venezuela; and 3Division of Nephrology and Hypertension, Department of Medicine, University of California, Irvine, California

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Quiroz Y, Ferrebuz A, Romero F, Vaziri ND, Rodriguez-Iturbe B. Melatonin ameliorates oxidative stress, inflammation, proteinuria, and progression of renal damage in rats with renal mass reduction. Am J Physiol Renal Physiol 294: F336–F344, 2008. First published December 12, 2007; doi:10.1152/ajprenal.00500.2007.—The progressive deterioration of renal function and structure resulting from renal mass reduction are mediated by a variety of mechanisms, including oxidative stress and inflammation. Melatonin, the major product of the pineal gland, has potent antioxidant and anti-inflammatory properties, and its production is impaired in chronic renal failure. We therefore investigated if melatonin treatment would modify the course of chronic renal failure in the remnant kidney model. We studied rats followed 12 wk after renal ablation untreated (Nx group, n = 7) and treated with melatonin administered in the drinking water (10 mg/100 ml) (Nx + MEL group, n = 8). Sham-operated rats (n = 10) were used as controls. Melatonin administration increased 13–15 times the endogenous hormone levels. Rats in the Nx + MEL group had reduced oxidative stress (malondialdehyde levels in plasma and in the remnant kidney as well as nitrotyrosine renal abundance) and renal inflammation (p65 nuclear factor-κB-positive renal interstitial cells and infiltration of lymphocytes and macrophages). Collagen, α-smooth muscle actin, and transforming growth factor-β renal abundance were all increased in the remnant kidney of the untreated rats and were reduced significantly by melatonin treatment. Deterioration of renal function (plasma creatinine and proteinuria) and structure (glomerulosclerosis and tubulointerstitial damage) resulting from renal ablation were ameliorated significantly with melatonin treatment. In conclusion, melatonin administration improves the course of chronic renal failure in rats with renal mass reduction. Further studies are necessary to define the potential usefulness of this treatment in other animal models and in patients with chronic renal disease.

oxidative stress; renal inflammation; renal ablation; renal failure; melatonin; antioxidants

SIGNIFICANT REDUCTION of nephron mass by subtotal nephrectomy in experimental animals or by various diseases in humans triggers a chain of events that lead to glomerulosclerosis, tubulointerstitial injury, proteinuria, and progression to end-stage renal disease. Progressive deterioration of kidney function and structure in the remnant/diseased kidney is mediated by a constellation of renal hemodynamic abnormalities, namely glomerular hypertension and hyperfiltration, as well as oxidative stress and inflammation.

Oxidative stress may cause inflammation and fibrosis by direct toxic effects of reactive oxygen species (ROS) and by inducing activation of redox-sensitive proinflammatory transcription factors and signal transduction pathways. Therefore, oxidative stress and its constant companion, inflammation, play a major role in the pathogenesis of the progression of renal injury (32) as well as in the development of various complications of chronic renal failure, such as hypertension, atherosclerosis, and anemia, among others (9–11, 14, 16, 44, 47, 49).

Oxidative stress in chronic kidney disease (CKD) is caused by a combination of excessive ROS production and antioxidant depletion. Significant upregulation of NAD(P)H oxidase, a main source of ROS, has been demonstrated in the remnant kidney and in cardiovascular tissues in the 5/6 nephrectomized rats (48), and spontaneous production of superoxide and hydrogen peroxide has been shown in circulating leukocytes of patients with end-stage kidney disease (52).

Increased production of ROS and inflammation in CKD are compounded by impaired antioxidant/anti-inflammatory systems. For instance, tissue abundance and activities of antioxidant enzymes are altered in CKD animals; plasma high-density lipoprotein, apolipoprotein A-1, and reduced thiols are depressed in patients with advanced renal failure (10, 46, 47).

The available data on the effect of antioxidant therapy on progression of renal disease are limited. Administration of the superoxide dismutase mimetic drug tempol for 2 wk has been shown to ameliorate hypertension without altering renal function in 5/6 nephrectomized rats (48). In a recent study, Tain et al. (40) found significant reduction of glomerulosclerosis but no change in creatinine clearance or proteinuria with vitamin E supplementation for 15 wk in 5/6 nephrectomized rats. Similar findings were reported by Van den Branden et al. (43) and Hahn et al. (7). It should be noted that the use of high doses of antioxidant vitamins in humans has been hampered by the general lack of significant demonstrable benefit and the emerging evidence of adverse outcome found in clinical trials (18).

This is not entirely surprising since oxidative stress and the accompanying inflammation in renal disease, cardiovascular disorders, and cancer are not usually caused by deficiencies of the given vitamins.

Melatonin (N-acetyl-5-methoxytryptamine) is the major product of the pineal gland that functions as a modulator of sleep, sexual behavior, immune function, and circadian rhythm. In addition, melatonin has a potent ROS-scavenger activity chiefly because of its capacity to act as an electron donor (25, 29, 30, 41). Melatonin and its metabolites have powerful anti-inflamm-
MELATONIN AMELIORATES PROGRESSION OF CHRONIC RENAL FAILURE

Table 1. Baseline data

<table>
<thead>
<tr>
<th></th>
<th>Sham (n = 10)</th>
<th>Nx (n = 7)</th>
<th>56 Nx + MEL (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBP, mmHg</td>
<td>130.8±3.68</td>
<td>128.0±5.43</td>
<td>131.2±3.91</td>
</tr>
<tr>
<td>Body wt. g</td>
<td>302.8±20.85</td>
<td>311.2±17.72</td>
<td>306.8±27.64</td>
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<tr>
<td>Pcr, mg/dl</td>
<td>0.40±0.02</td>
<td>0.36±0.05</td>
<td>0.40±0.07</td>
</tr>
<tr>
<td>Proteinuria, mg/24 h</td>
<td>5.7±2.15 (n = 9)</td>
<td>5.6±2.14</td>
<td>5.7±3.79</td>
</tr>
<tr>
<td>MDA plasma, mmol/ml</td>
<td>1.54±0.14 (n = 5)</td>
<td>1.49±0.08 (n = 5)</td>
<td>1.44±0.09 (n = 5)</td>
</tr>
<tr>
<td>Right kidney, g</td>
<td>—</td>
<td>1.31±0.07</td>
<td>1.38±0.10</td>
</tr>
<tr>
<td>Two poles of left kidney, g</td>
<td>—</td>
<td>0.30±0.04</td>
<td>0.33±0.03</td>
</tr>
</tbody>
</table>

Data are means ± SE. No. of animals (n) is indicated for each group and, when different, is indicated in the corresponding determination. Nx, nephrectomy; MEL, melatonin; SBP, systolic blood pressure; Pcr, plasma creatinine; MDA, malondialdehyde.
thymocytes and T lymphocytes CD5 (Biosource); rabbit anti-human collagen IV (accurate Chemical Scientific, Westbury, NY); and rabbit anti-NF-κB (p65) (Zymed, San Francisco, CA).

Secondary antibodies used were goat anti-rabbit IgG horseradish peroxidase conjugated, goat anti-mouse IgG horseradish peroxidase conjugated (Stressgen Bioreagents, Glandford, Canada), and donkey anti-rabbit IgG biotin conjugate (Accurate Chemical Scientific, Westbury, NJ).

Other immunostaining reagents were the Dako fast red substrate system for immunohistochemistry (catalog no. K-0597; Dako), Extravidin alkaline phosphatase conjugate (E-2636; Sigma), and Levamisol solution (SP-5000; Vector Laboratories).

MDA determinations. Thiobarbituric acid-reactive substances were determined by the method of Ohkawa et al. (22) as described in previous investigations (33, 37). MDA was determined in plasma at the beginning of the study and at the end of the experiments, and in the kidney tissue harvested at the end of the study.

Melatonin determinations. Plasma melatonin levels were determined by enzyme-linked immunosorbent assay with commercially available kits (ICN Biomedical Research Products, Costa Mesa, CA). As described previously (20), melatonin was extracted from the samples with reverse-phase extraction columns provided with the kit. Extracts were stored at −20°C. The samples of the animals that received melatonin in the drinking water were diluted 20 times for the assay. The sensitivity of the assay is 3 pg/ml, and intra-assay variability was 7.3 ± 4.2% (SD; see Ref. 20).

Western blot analysis. Whole kidney homogenates were prepared in lysis buffer (50 mM Tris·HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, and 0.1% SDS) containing protease inhibitor. After centrifugation (3,000 g, 30 min, 4°C), the supernatant was recovered, and protein concentration was determined by a modified Coomassie blue method using BSA as standard. Samples (10–20 μg) were resuspended in 25 μl of 60 mM Tris·HCl (containing 25% glycerol, 2% SDS, 14.4 mM 2-mercaptoethanol, and 0.1% bromophenol blue), heated at 65°C for 2 min, centrifuged at 3,000 g for 1 min, loaded on a 10% separating gel, and electrophoresed at 100 mV. Separated proteins were then transferred to a nitrocellulose membrane (Bio-Rad). After being blocked with nonfat dry milk (5%) in TBS containing 0.1% Tween-20 for 1 h at room temperature, the membranes were incubated overnight at 4°C with the following primary antibodies: polyclonal antibody (1:1,000) against β-actin (Santa Cruz Biotechnology), anti-TGF-β (Promega), anti-human collagen IV (Accurate Chemical Scientific), α-actin (Cedarlane Laboratories Limited), and anti-nitrotyrosine. Thereafter, the membranes were washed in TBS containing 0.1% Tween 20 and incubated with secondary antibody [horseradish peroxidase-conjugated rabbit anti-mouse IgG antibody (Promega)] at 1:1,000 dilution in TBS containing 5% dry milk and 0.1% Tween 20] for 1 h at room temperature. Peroxidase activity was developed using 3,3′-diaminobenzidine with ion metal enhancement as recommended (2), and then protein expression levels were quantified using the Image J program.

Statistical analysis. Statistical analyses were done by multigroup ANOVA, and significant differences (P < 0.05) were analyzed by Tukey-Kramer posttests. Serial changes were evaluated with repeated-measures ANOVA and changes with respect to baseline with Dunnett tests. Data are given as means ± SE.

RESULTS

General data and renal function. Data obtained at baseline and 12 wk after renal ablation are summarized in Tables 1 and 2, respectively. No significant differences were found in baseline values among the study groups (Table 1).

Plasma melatonin levels in samples obtained at 7:00–8:00 A.M. were significantly higher in the melatonin-treated group [522 ± 102.4 (SE) pg/ml] than in the sham-operated [44 ± 6.78 (pg/ml)] and untreated Nx [40.6 ± 11.1 pg/ml] groups.

Table 2. Data at the end of the experiment (12 wk)

<table>
<thead>
<tr>
<th>Data at the end of the experiment (12 wk)</th>
<th>Sham (n = 10)</th>
<th>Nx (n = 7)</th>
<th>5/6 Nx + MEL (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body wt, g</td>
<td>408.6 ± 8.91a</td>
<td>361.0 ± 10.41</td>
<td>344.2 ± 16.34</td>
</tr>
<tr>
<td>SBP, mmHg</td>
<td>132.6 ± 4.53b</td>
<td>197.0 ± 4.42</td>
<td>178.6 ± 3.77</td>
</tr>
<tr>
<td>Pcr, mg/dl</td>
<td>0.42 ± 0.03 (n = 8)</td>
<td>2.06 ± 0.12b</td>
<td>1.02 ± 0.05b</td>
</tr>
<tr>
<td>Proteinuria, mg/24 h</td>
<td>6.3 ± 1.88 (n = 8)</td>
<td>184.1 ± 19.50b</td>
<td>66.7 ± 3.13</td>
</tr>
<tr>
<td>MDA plasma, nmol/ml</td>
<td>1.9 ± 0.28</td>
<td>6.3 ± 1.22c</td>
<td>2.8 ± 0.60</td>
</tr>
<tr>
<td>MDA/g protein (kidney)</td>
<td>0.78 ± 0.05 (n = 5)</td>
<td>1.23 ± 0.14 (n = 5)</td>
<td>0.61 ± 0.13 (n = 5)</td>
</tr>
<tr>
<td>Wt of the remnant kidney, g</td>
<td>4.14 ± 0.1</td>
<td>1.36 ± 0.16</td>
<td>1.36 ± 0.16</td>
</tr>
<tr>
<td>Hypertrophy of the remnant kidney, %</td>
<td>42.3 ± 17.84</td>
<td>34.9 ± 13.23</td>
<td>34.9 ± 13.23</td>
</tr>
</tbody>
</table>

Data are means ± SE. No. of animals (n) is indicated for each group as in Table 1 and, when different, is indicated in the corresponding determination. Hypertrophy of the remnant kidney was estimated as described in METHODS. *P < 0.05 vs. the rest; †P < 0.001 vs. the rest; ‡P < 0.01 vs. sham and P < 0.05 vs. MEL; §P < 0.05 vs. sham and P < 0.01 vs. MEL; ¶P < 0.05 vs. 5/6 Nx; and ‡P < 0.01 vs. sham.
Renal ablation resulted in an ~50% reduction of the mean plasma melatonin levels during the midnight surge (sham = 228 ± 17.1 pg/ml; Nx = 104.6 ± 9.8 pg/ml, P < 0.001). Plasma melatonin level obtained at midnight in the Nx MEL group (3,580 ± 201) was significantly higher (P < 0.001) than in the untreated NX and sham-operated control groups.

Table 2 shows the findings resulting from 12 wk of melatonin treatment. Renal ablation resulted in hypertension that was ameliorated by melatonin treatment.

Renal ablation resulted in hypertrophy of the remnant kidney that was not significantly different in the untreated rats (42.3 ± 17.8%) and the rats treated with melatonin (34.0 ± 13.2%).

**Oxidative stress.** Oxidative stress was evaluated by determination of MDA levels and nitrotyrosine abundance in plasma and in the remnant kidney. As shown in Table 2, plasma MDA levels and renal MDA contents were increased in the untreated NX group and were normalized by melatonin treatment. Nitrotyrosine abundance was also significantly (P < 0.001) increased in rats with renal ablation and was reduced by melatonin treatment. Nitrotyrosine adducts in the remnant kidney were detected in two faint and one main band. Figure 3 shows the main band in kidneys treated and untreated with melatonin.

Plasma creatinine and proteinuria were increased in rats with renal ablation and were significantly reduced by melatonin. The longitudinal measurements of plasma creatinine and proteinuria are shown in Figs. 1 and 2, respectively. The untreated rats showed progressive increases in plasma creatinine and proteinuria following renal mass reduction. In contrast, plasma creatinine and proteinuria remained stable during the observation period and did not increase beyond those seen shortly in the renal ablation (Figs. 1 and 2).

The mean weight of the removed right kidney and the two poles of the left kidney are shown in Table 1. It may be appreciated that, assuming a similar weight of both kidneys, the excised renal mass was approximately two-thirds (62–66%) of the total. It is also demonstrated in Table 1 that the renal ablation procedure removed similar renal mass in the animals from in the melatonin-treated and the untreated groups. As shown in Table 2, the hypertrophy of the remnant kidney was not significantly different in the untreated rats (42.3 ± 17.8%) and the rats treated with melatonin (34.0 ± 13.2%).

Fig. 3. Nitrotyrosine abundance in representative rats (numbers) of the corresponding groups. Graphic representation shows integrated optical density (OD) normalized for β-actin control. Open bar, sham; filled bar, Nx; shaded bar, Nx + MEL. Data are means ± SE. ***P < 0.001 and **P < 0.01.

Fig. 4. Collagen IV estimated by Western blot in Sham, Nx, and Nx + MEL groups. Each no. represents a rat in the corresponding group. Data are means ± SE. ***P < 0.001 and **P < 0.01. Bottom: immunostaining for collagen IV in kidney sections of the Nx group and Nx + MEL group. The increment in collagen in the Nx group contrasts with the findings in the Nx + MEL group (alkaline phosphatase staining).
Collagen, α-SMA, and TGF-β. Figures 4, 5, and 6 show the abundance (Western blots) of collagen IV, α-SMA, and TGF-β, respectively, in the panels on top and the corresponding immunohistological staining in the panels on the bottom. Collagen IV, α-SMA, and TGF-β were increased markedly in the remnant kidney of the untreated NX rats and were reduced to baseline levels by melatonin treatment.

Renal histology and immunohistology. As expected, the remnant kidneys in the untreated rats showed significant glomerulosclerosis and tubulointerstitial damage. Melatonin administration significantly reduced the severity of glomerulosclerosis and tubulointerstitial damage. As shown in Fig. 7, glomerulosclerosis and the extension of tubular injury were reduced by approximately one-half in the melatonin-treated group.

The number of p65 NF-κB-positive cells was increased in the untreated rats with renal ablation and was reduced significantly by administration of melatonin. The latter finding points to an inhibitory effect of melatonin on activation of this proinflammatory transcription factor (Fig. 8). Lymphocyte and macrophage infiltration in tubulointerstitial areas was increased correspondingly in the untreated Nx rats and was decreased by ~1.8-fold with melatonin treatment (Fig. 9). Unlike the tubulointerstitial region, infiltration of lymphocytes and macrophages in the glomeruli was scarce (~1 positive cells/glomerular cross section in all instances).

DISCUSSION

The present study shows that the administration of melatonin attenuates hypertension and retards deterioration of renal function and structure caused by renal mass reduction in rats. The kidney removed by the surgical procedures in this study represented about two-thirds of the total functioning renal mass. This was sufficient to trigger the progression of CKD in the remnant kidney as evidenced by the rising creatinine and proteinuria, as well as the structural damage observed in the remnant kidney. Because the amount of excised renal mass (Table 1) was similar in the untreated and melatonin-treated Nx groups, it is reasonable to assume that adaptive and pathogenetic mechanisms derived from renal mass reduction were comparable in the two groups.

The favorable results seen with melatonin administration are likely related to the antioxidant and anti-inflammatory properties of this compound resulting from its strong ROS scavenger properties. Indeed, abundant evidence has accumulated indicating that CKD causes oxidative stress (11, 48), and oxidative stress accelerates the progression of renal injury directly by inducing cytotoxicity and indirectly by promoting inflammation (11, 32, 44, 48). Oxidative stress in uremia is not only the result of increased generation of ROS but also because of the depletion of antioxidant defenses (10, 47). Among those, the deficiency of melatonin (23, 50, 51) may contribute to the reduction of antioxidant capacity in renal insufficiency. Therefore, we tested the hypothesis that, by attenuating oxidative stress and inflammation, melatonin supplementation may retard progression of renal disease. To this end, rats with renal ablation were treated with melatonin in their drinking water in a manner and amount described in our previous studies (20). As reported in other studies (13, 50, 51), the nocturnal surge of melatonin was impaired in the untreated rats with chronic renal failure. In
addition to the impairment in melatonin production, endogenous melatonin is likely consumed by the elevated free radical levels in the uremic rats. Melatonin administration resulted in a marked rise in plasma melatonin concentration that far exceeded those found in the other groups (Table 1).

Other investigators have used antioxidant therapy in rats with renal ablation. Early studies (7, 43) showed that vitamin E supplementation reduces glomerulosclerosis in the remnant kidney model. These observations were confirmed in a recent study by Tain et al. (40) who found amelioration of glomerulosclerosis without improvement in renal function or proteinuria in 5/6 nephrectomized rats. Our results showed not only a marked structural improvement in the remnant kidney but also an impressive preservation of renal function and reduction of glomerulosclerosis.

Fig. 6. Transforming growth factor-β (TGF-β) abundance evaluated by Western blots and by immune histology (bottom) in the sham, Nx, and Nx + MEL groups. Each no. represents a rat in the corresponding group. Data are means ± SE. ***P < 0.001. Increased TGF-β staining is present in tubular cells of a renal section of a rat in the Nx group in contrast with a renal section of a rat treated with melatonin (immunoperoxidase staining).

Fig. 7. Light microscopic histology findings in the sham (n = 8), Nx (n = 7), and Nx + MEL (n = 8) are shown in top as the glomerulosclerosis (GS) index and extent of tubulointerstitial (TI) damage. Data are means ± SE. Bottom: contrasting appearance of a renal section of a rat in the Nx group and a rat in the Nx + MEL group (periodic acid-Schiff stainings).
proteinuria with melatonin administration in this model. Several reasons may explain the superior benefits obtained with melatonin. First, as mentioned earlier, impaired melatonin production is a feature of CKD, whereas vitamin E deficiency is not. Second, the ROS scavenger and antioxidant activities of melatonin are much broader than those of vitamin E (1). In addition, unlike vitamin E whose interaction with ROS leads formation of potentially cytotoxic tocopheroxyl radical, by-products of melatonin interaction with ROS are safe and lack free radical activity (25, 29). Finally, as a fat-soluble substance, vitamin E can accumulate in the body when administered at high doses for extended periods. In contrast, melatonin is water soluble and is readily metabolized, and excreted and as such its use is not associated with cumulative toxicity.

Melatonin has been shown to be effective in protecting against severe free radical-mediated toxicity in a variety of conditions, including, chemotherapy (6), ischemia-reperfusion injury (15, 39), nitrogen mustard toxicity (42), acute renal failure caused by mercuric chloride (21), and gentamycin (38), among others. Plasma melatonin levels of the Nx and control rats were comparable to those reported in previous studies (12, 20). It is of note that the plasma melatonin levels achieved with its addition to the drinking water were 13-fold higher in the morning (7:00–8:00 A.M.) and 15- to 30-fold higher at mid-
night than the corresponding values found in the control and untreated uremic rats. Although the range of the effective dosage of melatonin in animals with renal insufficiency is not known, high levels are likely required to counteract the heavy burden of oxidative stress and inflammation in this condition.

Melatonin administration resulted in significant reduction of the lipid peroxidation product MDA in the plasma and remnant kidney tissues of the Nx rats (Figs. 8 and 9). Oxidative stress and inflammation play a major role in the pathogenesis of hypertension (reviewed in Ref. 45). In a previous communication, we reported that melatonin improves hypertension in the spontaneously hypertensive rat (20). Amelioration of hypertension observed in the present study is most likely the result of both the reduction in oxidative stress and preservation of renal function and structure; in turn, the reduction in blood pressure levels is renoprotective. The present studies do not allow discrimination between these beneficial effects. Nevertheless, the direct anti-inflammatory and antioxidant effects of melatonin likely play a critical role because other antioxidant strategies that ameliorate hypertension, such as administration of the superoxide dismutase mimetic drug tempol, are able to ameliorate hypertension and yet do not improve renal function in the rats with renal ablation (48). However, given the critical role of hypertension, oxidative stress, and inflammation in progression of renal disease, the observed preservation of the integrity of the remnant kidney with melatonin administration must be largely mediated by favorable modification of all of the above factors.

In summary, the present study showed that melatonin administration attenuated oxidative stress, inflammation, and hypertension and retarded deterioration of the remnant kidney function and structure in rats with renal ablation. Clinical studies are needed to explore the efficacy of melatonin in humans with CKD, particularly since the results with other antioxidant therapies are more compelling in animal models than in human disease (19). If proven effective, melatonin would be an attractive adjunctive therapy, since it is a natural, inexpensive, widely available (3), orally administered (8), and relatively safe product (4).

ACKNOWLEDGMENTS

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GRANTS

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