Melatonin reduces renal interstitial inflammation and improves hypertension in spontaneously hypertensive rats

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Nava, Mayerly, Yasmir Quiroz, Nosratola Vaziri, and Bernardo Rodríguez-Iturbe. Melatonin reduces renal interstitial inflammation and improves hypertension in spontaneously hypertensive rats. Am J Physiol Renal Physiol 284: F447–F454, 2003. First published November 19, 2002; 10.1152/ajprenal.00264.2002.—Several studies have demonstrated that treatment with antioxidants improves hypertension in spontaneously hypertensive rats (SHR). Because our laboratory has shown that renal infiltration of immune cells plays a role in the development of hypertension (Rodriguez-Iturbe B, Quiroz Y, Nava M, Bonet L, Chavez M, Herrera-Acosta J, Johnson RJ, and Pons HA. Am J Physiol Renal Physiol 282: F191–F201, 2002), we did the present studies to define whether the antihypertensive effect of antioxidants was associated with an improvement in renal inflammation. Melatonin was administered as an antioxidant. For wk, melatonin was added to the drinking water (10 mg/100 ml) given to a group of SHR (SHR-Mel; n = 10), and we compared them with groups of untreated SHR (n = 10) and Wistar-Kyoto (WKY) control rats (n = 10). Hypertension became increasingly severe in the SHR group [195 ± 14.3 (SD) mmHg at the end of the experiment] and improved in the SHR-Mel group (149 ± 20.4 mmHg, P < 0.001) in association with a 40–60% reduction in the renal infiltration of lymphocytes, macrophages, and angiotensin II-positive cells. Intracellular superoxide and renal malondialdehyde content were reduced by melatonin treatment as was the immunohistological expression of the 65-kDA DNA-binding subunit of NF-κB. We conclude that melatonin treatment ameliorates hypertension in SHR in association with a reduction in interstitial renal inflammation. Decreased activation of NF-κB, likely resulting from a reduction in local oxidative stress, may play a role in the suppression of renal immune infiltration and, thereby, in the antihypertensive effects of melatonin.

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WE HAVE POSTULATED THAT the renal infiltration of immunocompetent cells plays a role in the pathogenesis of salt-sensitive hypertension (20, 44). The most compelling evidence in favor of our postulate comes from studies demonstrating that the salt-driven hypertension that follows angiotensin II infusion (43), nitric oxide synthesis inhibition (36), and protein overload proteinuria (2) is prevented or markedly ameliorated when the renal immune infiltrate is reduced by the administration of mycophenolate mofetil (MMF), an immunosuppressive anti-inflammatory drug. Furthermore, blood pressure in the Okamoto strain of spontaneously hypertensive rats (SHR) decreased to nearly normal levels when lymphocytes and macrophages infiltrating tubulointerstitial areas were reduced with MMF treatment (45). These recent studies from our group follow the pioneering observations by Svendsen (52), who showed that athymic nude mice were resistant to the late, salt-dependent phase of the DOCA-salt model of hypertension.

The role of the immune cells infiltrating the kidney would depend on the close interrelationship among inflammatory infiltration, generation of free oxygen radicals, and local vasoconstrictor activity (42). In fact, in the experimental models listed above (2, 36, 43, 44), the correction of hypertension is associated with a concomitant reduction of the renal immune infiltrate, interstitial angiotensin II-positive cells, and oxidative stress.

The importance of reactive oxygen species (ROS) in the development and maintenance of hypertension has been recognized for some time (4, 11, 23, 47, 57). Unchecked production of ROS results in the impairment of vasodilatory systems, particularly nitric oxide, that is degraded by oxygen free radicals (9, 39, 58). In addition to shifting the balance toward systemic vasoconstriction, the additional consequence of chronic oxidative stress can stimulate salt retention. Unchecked angiotensin II activity, which in the renal interstitium is unresponsive to systemic volume expansion (31), favors sodium retention by the combined effects of glomerular vasoconstriction, which reduces filtered sodium load, increased sodium reabsorption, and impairment of the pressure-natriuresis mechanism.

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While oxidative stress causes hypertension (27, 53, 54, 59) and a variety of antioxidant strategies improve hypertension in genetically hypertensive rats (reviewed in Refs. 40 and 56), it is not clear whether the blood pressure reduction induced by antioxidant therapy is associated with a decrease in renal interstitial inflammation. The confirmation of this prediction is central to our theory of the pathogenesis of salt-sensitive hypertension; therefore, the present studies were designed to answer this question.

Melatonin is a very potent antioxidant, due chiefly to its capacity to act as an electron donor (34, 40). Therefore, to test our hypothesis, we administered melatonin in the drinking water to SHR for a period of 6 wk. We found that hypertension was improved in association with suppression of oxidative stress, diminished activation of transcriptional NF-κB, and reduction in the lymphocyte and macrophage infiltration of the kidney.

**MATERIALS AND METHODS**

**Experimental design.** Male genetically hypertensive rats from the Okamoto strain (SHR) and control normotensive Wistar-Kyoto rats (WKY) 23–25 wk of age at the beginning of the experiment were obtained from the Instituto Venezolano de Investigaciones Científicas (Los Teques, Venezuela). Rats received regular rodent chow with 4% NaCl (Protinal, Purina, Maracay, Venezuela), had free access to food and water, and were handled according to the institutional guidelines of animal care. Animals were maintained on 12:12-h (7 AM-7 PM) light-dark cycles throughout the experiments.

Three animal groups were studied for 6 wk. The SHR-Mel group (n = 10) consisted of SHR that received melatonin (M-5250, Sigma). Because melatonin is water soluble at concentrations of 0.1 mg/ml (Sigma Technical Library) or more (48), we administered in the drinking water (10 mg/100 ml) using containers wrapped in aluminum foil because melatonin is light sensitive. The SHR group (n = 10) consisted of SHR that received no melatonin. Control WKY rats (n = 10) received no melatonin and were similarly studied.

Because the investigation demonstrated (see below) a reduction in the tubulointerstitial infiltrating cells after 6 wk of melatonin treatment, an additional group (n = 5) of SHR of 23–24 wk of age (SHR-23w) was studied to evaluate the spontaneous changes in renal cellular infiltration occurring in untreated SHR over 6 wk.

Rats are known to drink more water during the night than during the day (54), which was confirmed by measuring water consumption in 12-h cycles (7 AM-7 PM). Melatonin serum levels were determined (see below) at three different times during a 24-h period (10–11 AM, 5:30–6:30 PM, and 11:30 PM-12:30 AM) in the rats from the SHR-Mel and SHR groups.

Blood pressure was determined weekly. Weight, serum creatinine and proteinuria were determined before and at the end of the experiments. At the end of 6 wk, all rats were anesthetized with ether and euthanized by aortic desanguination, and the kidneys were harvested for studies. One kidney was divided into two parts and used for histological, histochemical, and immunohistochemical studies. As in previous investigations (2, 36, 43, 45), one part was used for histology and the contralateral kidney was used to determine malondialdehyde (MDA), GSH, and catalase content.

**Blood pressure determinations.** Blood pressure was determined in conscious restrained rats by tail-cuff plethysmography (ITTC, Life Scientific Instruments, Woodland Hills, CA) in a quiet, darkened room as previously reported (36, 43, 45). Before the beginning of the experiments, rats were conditioned three to four times to the procedure and, when blood pressure was determined, the value represented the mean of three to four separate determinations.

**Serum melatonin levels.** Serum melatonin levels were determined by ELISA with commercially available kits (ICN Biomedicals Research Products, Costa Mesa, CA). Melatonin extraction from the samples was done with reverse-phase extraction columns provided with the kit. The dried extracts were stored at −20°C and processed within 24 h. The samples of the SHR-Mel group were diluted 20 times for the assay, which has a sensitivity of 3 pg/ml. Intra-assay variability was 7.3 ± 4.2%.

**Histological studies.** Light microscopy was evaluated with hematoxylin and eosin and periodic acid-Schiff stains. Histological damage was graded using a glomerular injury score described initially by Raji et al. (38) and a tubulointerstitial injury score described initially by Pichler et al. (33), which depends on the percent extension of the damaged area, as detailed in several previous communications from our group (36, 43, 45): 0 = no changes present; 1+ = <10%; 2+ = 10–25%; 3+ = 25–50%; 4+ = 50–75%; and 5+ = 75–100% of the renal tubulointerstitial area showing injury. The entire cortical and juxtamedullary areas were evaluated. All studies were done in a blinded fashion.

**Infiltrating cells and antiserum.** Avidin-biotin-peroxidase methodology was used to identify infiltrating cells and angiotensin II-positive cells. Intraglomerular cells (positive cells/glomerular cross section (GCS)) and tubulointerstitial cells (positive cells/mm²) were analyzed separately.

Double-staining methodology was used to determine the proportion of angiotensin II-positive cells that were lymphocytes and macrophages. The technique has been described before in detail (41) and includes an initial incubation with monoclonal antibody anti-CD5 or anti-ED1; a second incubation with biotin-conjugated, affinity-purified F(ab’2) anti-mouse IgG; a third incubation with rabbit anti-angiotensin II antibody; and, finally, incubation with fluorescein-conjugated affinity-purified donkey anti-rabbit IgG antibody.

Monoclonal antibodies were used to identify lymphocytes (anti-CD5, clone MRCOX19, Biosource, Camarillo, CA) and macrophages (anti-ED1, Harlan Bioproducts, Indianapolis, IN). Angiotensin II-positive cells were identified with rabbit anti-angiotensin II human IgG (Peninsula Laboratories, Belmont, CA). The secondary antibody for CD5 and ED1 stainings was a biotin-conjugated, affinity-purified F(ab’2) fragment of rat anti-mouse IgG with minimal cross-reactivity to rat serum proteins (dilution 1:30). The secondary antibody for angiotensin II and NF-κB stainings was a biotin-conjugated F(ab’2) fragment of donkey anti-rabbit IgG with minimal cross-reactivity to rat serum proteins (dilution 1:40). Secondary antibodies were purchased from Accurate Chemical and Scientific (Westbury, NY).

**Oxidative stress.** Oxidative stress was studied by determination of the renal content of MDA, GSH, and catalase in one of the kidneys harvested at the end of the experiment and by determination of intracellular superoxide in frozen kidney sections of the contralateral kidney.

Renal homogenates were prepared, and the content of MDA, GSH, and catalase were measured by the methods of Okhawa et al. (32), Beutler et al. (5), and Aebi (1), respectively. MDA and GSH results are given as nanomoles per milligram of homogenate protein. Catalase results are given.
as the rate constant of a first-order reaction ($\kappa$) per milligram homogenate protein. Details of these methods in our laboratories have been published previously (30).

The cytochemical method of Briggs et al. (7) with minor modifications published previously (30) was applied to 8- to 10-$\mu$m cryostat sections of the kidneys to determine intracellular superoxide. Sections were fixed in formalin and counterstained with 1% methyl green. Results are given as positive cells per square millimeter.

**NF-κB.** Activated NF-κB was identified using polyclonal antibody specific for the 65-kDa DNA-binding subunit (rabbit anti NF-κB, Zymed, San Francisco, CA). Cryostat sections (4 $\mu$m) in citrate buffer, pH 6.0, were subject to microwave irradiation for 30 s to enhance epitope retrieval, fixed with 10% formalin, and stained using the avidin-biotin-peroxidase technique. The secondary antibody was a donkey anti-rabbit biotin-conjugated, affinity-purified F(ab’)2 IgG fragment with minimal cross-reactivity to rat serum proteins (Accurate Chemical and Scientific). Hematoxylin and eosin were used as counterstains.

**Statistical analysis.** Comparisons among groups were done with ANOVA and Tukey-Kramer posttests. Repeated-measures ANOVA was used to evaluate changes found in serial determinations. Results are expressed as means ± SD throughout the paper, and two-tailed P values < 0.05 were considered significant. A commercial statistical package (Instat, GraphPad, San Diego, CA) was used for statistical calculations.

**RESULTS**

**Weight, renal function, and serum melatonin levels.** Melatonin was administered in the drinking water. As expected (54), water consumption was higher during the night (7 PM-7 AM) when the animals consumed 70.9 ± 8.6% of the daily total. There were no apparent ill effects from melatonin, and SHR-Mel and SHR rats gained weight similarly. At the beginning of the experiments, the weight of the SHR-Mel group was 331.4 ± 18.6 g, and the weight of the SHR group was 338.5 ± 26 g. At the end of the experiments 6 wk later, the corresponding weights were 380.7 ± 23.4 and 373.0 ± 24.5 g (P not significant (NS)). Serum creatinine was similar in the SHR (0.4 ± 0.06 mg/dl) and SHR-Mel (0.4 ± 0.07 mg/dl) groups and remained unchanged at the end of the experiment. Proteinuria was <10 mg/dl before and at the end of the experiments.

Melatonin serum levels are shown in Fig. 1. Endogenous melatonin levels in the SHR rats were 77 ± 13 at 10–11 AM, 56 ± 12 at 5:30–6:30, and 202 ± 31 pg/ml at midnight. The melatonin levels in the SHR-Mel group were more than seven times higher at 10–11 AM and 5:30–6:30 PM and reached peak levels of 3,510 ± 319 pg/ml at midnight (Fig. 1).

**Blood pressure.** The serial systolic blood pressure (SBP) measurements are shown in Fig. 2. Rats from the SHR group exhibited a progressive rise in SBP during the 6 wk of experiments. The SBP in the melatonin-treated rats (SHR-Mel group) decreased slowly during the experiment, and at every interval it was significantly below the values in the SHR group.

**Histology and infiltrating cells.** Light microscopy did not demonstrate significant glomerular sclerosis. Mesangial expansion was observed in some glomeruli of both experimental groups. Tubulointerstitial damage score was 2.3 ± 0.8 in the untreated group and 1.9 ± 0.8 in the melatonin-treated group, and these differences were not statistically significant. Interstitial findings consisted of focal areas of widening with mononuclear cell infiltration.

**Glomerular infiltration of lymphocytes was scarce and similar in the experimental groups.** Mean values of intraglomerular CD5-positive cells were 0.4 positive cells/GCS. Intraglomerular macrophages were increased in the SHR group (1.94 ± 0.92 ED1-positive cells/GCS) and reduced by melatonin treatment (SHR-Mel group = 0.30 ± 0.07, P < 0.001) to values similar to those found in control WKY rats (0.20 ± 0.10). The SHR-23w group had 1.3 ± 0.90 ED1-positive cells/GCS (P NS vs. SHR experimental group and P < 0.01 vs. SHR-Mel group).

**Tubulointerstitial infiltration of both lymphocytes and macrophages was drastically reduced in the melatonin-treated rats,** as demonstrated in Figs. 3 and 4, respectively. SHR-23w rats had 61.8 ± 20.3 CD5-positive cells/mm² and 32.6 ± 9.9 ED1-positive cells/mm². These values are lower, but not statistically different from, the corresponding values found in the SHR group.
(corresponding to 6-wk-older SHR) and significantly higher ($P < 0.05$ for CD5-positive cells and $P < 0.01$ for ED1-positive cells) than the corresponding values found in the SHR-Mel group (Figs. 3 and 4).

Angiotensin II-positive cells in tubulointerstitium were increased in SHR, and treatment with melatonin resulted in an $\sim 50\%$ reduction in the number of angiotensin II-positive cells in the SHR-Mel group (Fig. 5). Double-staining studies (Fig. 6A) revealed that 18–25% of the angiotensin II-positive cells were lymphocytes and 12–18% were macrophages. Nearly 6 of 10 angiotensin II-positive cells were tubular epithelial cells (Fig. 6B).

Oxidative stress. Intracellular superoxide was found in tubular epithelial cells as well as in infiltrating mononuclear cells. Increased numbers of superoxide-positive cells were found in SHR and were reduced by melatonin treatment to values similar to those found in WKY rats (Fig. 7).

Table 1 shows the results of the MDA, GSH, and catalase content of the kidney in SHR and SHR-Mel groups and in WKY rats. The SHR group had increased MDA content with respect to WKY, and melatonin treatment resulted in a highly significant reduction of renal MDA.

NF-κB. Kidney sections of the SHR group demonstrated increased expression of the 65-kDA DNA-bind-
SHR-Mel group without reaching the low levels present in the WKY rats (Fig. 8). Representative microphotographs are shown in Fig. 9, A and B.

DISCUSSION

Melatonin is a hormone that functions as a biological modulator of sleep, reproduction and sexual behavior, immunological functions, and circadian rhythms. There are high-affinity melatonin receptors located primarily in the kidney, blood vessels, eye, brain, and gastrointestinal tract (6). The administration of melatonin has been reported to decrease arterial blood pressure when administered intravenously (24) as well as by intraperitoneal minipumps (21) as a consequence of various mechanisms, including a direct hypothalamic effect, a decrease in catecholamine levels, relaxation of the aortic smooth muscle wall, and, most importantly, as a result of its antioxidant properties (49). Antioxidant activity of melatonin results mainly from electron donation and its easy access to intracellular compartments (40). The antioxidant properties of melatonin are likely to be the most critical in the results obtained in our experiments because they involve the chronic oral administration of melatonin, and accumulating evidence gives support to the role of oxidative stress in the pathogenesis of hypertension (4, 11, 47, 56).

To our knowledge, the oral administration of melatonin for the treatment of hypertension has not been tried previously. One of the reasons may be that when melatonin is given by mouth, plasma melatonin levels increase rapidly but return to baseline levels after only 10.2 hours.

Table 1. Renal content of malondialdehyde, glutathione, and catalase

<table>
<thead>
<tr>
<th>Group</th>
<th>MDA, nmol/mg protein</th>
<th>GSH, nmol/mg protein</th>
<th>CAT, μmol/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHR</td>
<td>1.93 ± 0.23</td>
<td>10.7 ± 1.9</td>
<td>0.60 ± 0.21</td>
</tr>
<tr>
<td>SHR + melatonin</td>
<td>0.72 ± 0.07*†</td>
<td>13.4 ± 5.3</td>
<td>0.39 ± 0.14</td>
</tr>
<tr>
<td>WKY rats</td>
<td>0.44 ± 0.07</td>
<td>9.43 ± 2.68</td>
<td>0.68 ± 0.22</td>
</tr>
</tbody>
</table>

Values are means ± SD. MDA, malondialdehyde; CAT, catalase; SHR, spontaneously hypertensive rats; WKY, Wistar-Kyoto. *P < 0.001 vs. SHR. †P < 0.01 vs. WKY.
2–3 h. Such was the case in previous studies from our group in which melatonin was given orally to humans (18) as well as by gastric gavage to rats (31) in the treatment of conditions characterized by an acute increase in oxidative stress. In fact, in preliminary pilot studies, we found that a single daily dose of melatonin given by gastric gavage had no effect on the hypertension of SHR. Therefore, we gave the drug in the drinking water in containers protected from light, and this method resulted in drug concentrations in serum over 24 h that were 7 (in the morning) to 17 times (at midnight) higher than the endogenous melatonin concentration (Fig. 1). The endogenous melatonin levels in the SHR group were similar to those reported by others (19) as well as ourselves (30), and there were no apparent ill effects related to the pharmacologically high circulating levels of melatonin in the SHR-Mel group, which is not surprising because no ill effects have been reported when the plasma levels are increased 10,000 times (8). The rats gained weight normally, and they appeared to be as active as the untreated rats. No attempt was made to compare endogenous melatonin levels of the SHR group with the WKY controls because other investigations have shown that young SHR have higher, whereas adult SHR have lower midnight levels of melatonin than the WKY rats (22).

As expected, melatonin treatment for 6 wk reduced oxidative stress. Both intracellular superoxide production (Fig. 6) and renal MDA content (Table 1) were drastically reduced in rats from the SHR-Mel group. Previous studies from our group have also found reduction of these parameters of oxidative stress in SHR treated with an immnosuppressor drug, mycophenolate mofetil (45).

Rats from the SHR group developed an increasingly severe hypertension when given a regular diet with 4% NaCl. In contrast, the melatonin-treated rats exhibited a significant reduction of SBP without reaching normal levels (Fig. 2). These findings were associated with a significant reduction of the renal infiltration of lymphocytes (Fig. 3), macrophages (Fig. 4), and angiotensin II-positive cells (Fig. 5). Double-staining studies revealed that about one-fifth of the angiotensin II-positive cells were lymphocytes and an almost similar proportion were macrophages. The remaining 60% were tubular epithelial cells; these findings are similar to those reported in previous studies (43, 45).

The reduction of interstitial inflammation resulting from the administration of a potent antioxidant such as melatonin is the mirror image of the reduction of renal oxidative stress resulting from the administration of a drug with anti-inflammatory properties, such as mycophenolate mofetil (36, 43, 45).

The administration of melatonin induced an 80% normalization in blood pressure in association with a 50% normalization of lymphocyte and angiotensin II infiltration. A possible explanation could be that inflammatory infiltration is an epiphenomenon, but this would appear unlikely in view of the consistent improvement in hypertension obtained with MMF-induced reduction of interstitial inflammation in several models of acquired (2, 36, 43) and genetic (45) models of hypertension and the correlations found between the severity of hypertension and the intensity of the infiltration of lymphocytes and angiotensin II-positive cells in previous studies in the SHR (45). Furthermore, the suppression of the interstitial infiltration obtained by melatonin treatment reduced the number of interstitial lymphocytes, macrophages, and angiotensin II-positive cells below the levels present in the SHR-23w group, and, importantly, blood pressure in the SHR-Mel group actually improved with respect to baseline values. A more likely explanation for the finding of an 80% reduction in blood pressure in association with only a 50% reduction in the interstitial infiltrate is that a critical amount of lymphocyte infiltration can be tolerated by the kidney before it results in salt-sensitive hypertension.

Melatonin is widely available and free of side effects and has been used with moderate success in humans for the treatment of insomnia (8) and to reduce oxidative stress and inflammatory response (15, 18). Relevant to the present work, melatonin has been reported to modify characteristics of the immune system and of interstitial inflammation (16), being capable of inducing Th1 and Th2 cytokine responses (13, 25, 37) and to have proinflammatory (10, 28), as well as anti-inflammatory effects (12, 26). These conflicting results may be due, at least in part, to differences in dose, species, sex, age, and immune maturation, all of which are critical factors in the response to melatonin (51). However, there seems to be general agreement on the fact that melatonin inhibits the activation of NF-κB, an effect that may indirectly contribute to its role as an antioxidant (14, 29, 35). The participation of NF-κB in chronic inflammatory diseases (3) and, specifically, in renal disease (17), is well recognized, and we theorized that NF-κB activation was probably participating in the renal interstitial inflammation present in SHR because of overproduction of oxygen-free radicals in SHR with severe hypertension (44), which act as second messengers in NF-κB activation (48). Angiotensin II, which was found previously (43) as well as in the present studies (Fig. 6) to be present in cells in the renal tubulointerstitium, could also contribute to activation of NF-κB because several studies have shown this effect in vivo and in vitro (46).

In fact, we found increased expression of activated NF-κB in the kidney of severely hypertensive SHR that was reduced with melatonin treatment. NF-κB expression was observed in infiltrating mononuclear cells as well as in tubular epithelial cells (Fig. 9). Because NF-κB expression was not investigated in cells of the peripheral blood, it is not possible to say whether NF-κB activation of immune cells infiltrating the renal tubulointerstitium took place inside or outside the kidney. Nevertheless, activation of this transcriptional factor in the tubular epithelial cells suggests the participation of genes activated within these cells in the phenomena of immune cell recruitment and inflammation present in the kidney of SHR. It is also logical to assume that the reduction of NF-κB in the rats of the
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SHR-Mel group (Figs. 8 and 9B) likely played a significant role in the observed reduction of the renal inflammatory infiltrate.

It is of note that nearly complete correction of oxidative stress parameters (MDA and superoxide-positive cells) was accompanied by significant but incomplete (50%) reduction of inflammatory infiltrate and angiotensin II-positive cells. This phenomenon points to the dual action of melatonin in alleviating oxidative stress. First, through mechanisms discussed above, melatonin treatment results in reduction of the inflammatory infiltrate, which partially reduces but does not normalize ROS production. Second, there is the direct scavenging of the residual ROS by melatonin. Nearly complete inactivation of residual ROS can restore lipid peroxidation and MDA levels to normal.

In summary, we demonstrated that the progressive hypertension in SHR is significantly improved with the administration of melatonin in the drinking water, which resulted in pharmacological elevations in the hormone-circulating levels throughout the 24-h period. Melatonin administration resulted in reduction of oxidative stress, reduction of NF-κB activation, and reduction of interstitial renal inflammation. These results support the role of immune cells infiltrating the kidney in the pathogenesis of salt-sensitive hypertension and indicate that melatonin is a potential antihypertensive treatment that merits further investigation.

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