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ORIGINAL ARTICLE

Effect of L-NAME-induced hypertension on melatonin receptors and melatonin levels in the pineal gland and the peripheral organs of rats

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Melatonin plays a role in blood pressure (BP) control. The aim of this study was to determine whether melatonin concentrations and melatonin receptor levels are altered in L-NAME-treated, NO-deficient hypertensive rats. Two groups of male adult Wistar rats were investigated: rats (n=36) treated with NO-synthase inhibitor L-NAME (40 mg kg^{-1}) and age-matched controls (n=36). BP was measured weekly by tail-cuff plethysmography. After 4 weeks, L-NAME administration increased BP (178 ± 1 *vs.* control 118 ± 1 mm Hg). At the end of treatment, rats were killed in regular 4 h intervals over a 24-h period. Melatonin concentrations in the plasma, pineal gland, heart and kidney and melatonin receptor (MT_1) density in the aorta were determined. A significant daily rhythm of melatonin concentrations was found in the blood, pineal gland, kidney and heart of both control and hypertensive rats. Peak nighttime pineal melatonin concentrations were higher in L-NAME-treated rats than in controls (3.38 ± 0.48 *vs.* 1.75 ± 0.33 ng per pineal gland). No differences between both groups were found in melatonin concentrations in blood, kidney and heart or in the MT_1 receptor density in the aorta. Our results suggest that L-NAME treatment enhances melatonin production in the pineal gland, potentially by decreasing an inhibitory effect of NO on melatonin production in the pineal gland. However, the enhanced pineal melatonin formation was insufficient to increase melatonin concentrations in circulation, heart and kidney of L-NAME-treated rats, indicating an increased use of melatonin in hypertensive animals.
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INTRODUCTION

Melatonin is a hormone produced by the pineal gland in a circadian manner and exhibits a broad spectrum of effects, including those in the cardiovascular system. Melatonin has been suggested to play a role in blood pressure (BP) control. Earlier reports have shown that pinealectomized rats developed hypertension,[1] and the administration of melatonin in this model normalized the increased BP.[2] Melatonin decreased BP in hypertensive patients,[3] and the addition of melatonin to antihypertensive medication in patients with nocturnal hypertension reduced nighttime systolic BP.[4] However, the mechanisms behind melatonin's effect on BP are still not completely understood.

The hypotensive effect of melatonin could be mediated either by a direct effect on vessels or by decreasing brain serotonin release, resulting in sympathetic inhibition or parasympathetic stimulation.[5] There are rather limited data about changes of melatonin concentrations related to essential hypertension in the literature. Plasma melatonin and urinary 6-sulfatoxymelatonin levels have been reported to decrease in hypertensive patients with non-dipping BP profile,[6,7] but comparisons between normotensive and hypertensive patients are rare.[8] Similarly, in experimental hypertension, there are limited data on circulating melatonin concentrations and melatonin content in peripheral tissues in hypertensive animals.

The physiological effects of melatonin are mediated predominantly through membrane receptors. Melatonin receptors mRNA (MT_1 and MT_2) were localized in various organs, and can control different organs antagonistically by activating different postreceptor cascades.[9] High-affinity melatonin receptors, MT_1 and MT_2, are transmembrane proteins, and their mechanism of action is related to G-coupled proteins.[10] Expression of MT_1 and MT_2 receptor mRNA was found in different parts of the cardiovascular system, such as the heart,[11] coronary artery, aorta[12] and cerebral[13] and caudal arteries.[14] In the cardiovascular system, MT_1 and MT_2 receptor proteins were found in the heart[11] and coronary artery.[15] Activation of the MT_1 receptor causes vasoconstriction in the rat caudal artery, which is believed to be

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involved in the thermoregulation,[16] and activation of MT_2 results in vasodilatation.[17] Currently, the exact mechanism determining the activation of melatonin receptor subtypes by melatonin is obscure. Another important question is whether the density or activity of melatonin receptors is altered during hypertension.

The aim of our study was to determine melatonin concentrations in the pineal gland, plasma and in target tissues (heart and kidney) in rats with N^{ω}-nitro-L-arginine methyl ester (L-NAME)-induced hypertension throughout a 24-h period. As melatonin can exert its action through specific membrane MT_1 receptors, we also investigated the MT_1 protein density in the aorta of control and L-NAME hypertensive rats. The presence of MT_1 mRNA in the aorta and heart was measured by real-time RT-PCR.

METHODS

Animals

Mature male Wistar rats (Institute of Experimental Pharmacology, Dobra Voda, Slovak Republic) were housed in cages (two in each cage) with free access to drinking water and commercial pelleted chow. The animals were kept in the animal care facility throughout the experiment at 22–24 °C with a light (L) and dark (D) regimen in the ratio of 12:12, lights on at 08:00 hours. All experiments were carried out in accordance with protocols approved by the Ethical Committee for the Care and Use of Laboratory Animals at the Comenius University Bratislava. Rats (n=72, initial weight 237 ± 10 g) were allocated to two groups. Half of the animals (36) were treated with an inhibitor of NO-synthase, L-NAME (40 mg kg^{-1} in drinking water). The remainder served as a control group. BP was measured once a week by non-invasive tail-cuff plethysmography (AD Instruments, Spechbach, Germany). Data were evaluated using software Chart 5 (AD Instruments).

Tissue sampling

After 4 weeks of L-NAME treatment, the animals were killed in 4-h intervals over 24 h (10:00, 14:00, 18:00, 22:00, 02:00 and 06:00 hours). Tissue sampling proceeded under red light during the dark phase. Blood samples were collected into heparinized tubes, and immediately centrifuged at 2500 RPM for 10 min, and plasma was kept frozen at −20 °C until use. The pineal gland, heart, kidney and aorta were frozen in liquid nitrogen and stored at −80 °C until analysis.

Radioimmunoassay

The melatonin content was measured directly in plasma or after methanol extraction of the pineal gland and chloroform extraction of the heart and kidney.[18] Melatonin antiserum (Stockgrand Ltd, University of Surrey, Guildford, UK) and radioactive tracer [O-methyl-^{3}H]-labeled melatonin (specific activity: 3.07 TBq mmol^{-1}, GE Healthcare, Amersham, UK) were used. Bound hormone was separated by dextran-coated charcoal. Radioactivity was measured by liquid scintillation counter (Packard Tri-Carb 2900 TR, Packard Instruments, Meriden, CT, USA).

Western blot analysis

Aorta samples were homogenized in 0.3 M sucrose, 20 mM Tris (pH 7) supplemented with a protease inhibitor cocktail (4-[2-aminoethyl]benzenesulfonyl fluoride, pepstatinA, E-64, bestatin, leupeptin, aprotinin; Sigma, Saint Louis, MO, USA), and afterward, the samples were centrifuged (twice for 10 min at 3000 RPM and once for 11 min at 11 000 RPM). Supernatants were collected and protein concentrations were measured.[19] The samples were denatured in 10 mM Tris-HCl, 4% SDS, 20% glycerol, 0.05% coomassie brilliant blue, 4% 2-mercaptoethanol at 95 °C for 5 min. Proteins (50 µg) were separated by 12% SDS-PAGE and transferred to supported nitrocellulose membranes (Hybond-ECL, Amersham). After blocking for nonspecific binding in Tris-buffered saline (TBS), 1.2% Tween-20 and 5% nonfat dry milk at 4 °C overnight, membrane sheets were incubated with a goat antibody against melatonin receptor, MT_1 (1:500; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). As a secondary antibody, horseradish peroxidase-conjugated donkey antigoat IgG (1:1000; Santa Cruz Biotechnology Inc.) was used. The secondary antibody was visualized by an enhanced chemiluminiscence detection system (ECL, Amersham). Individual bands were densitometrically evaluated using Quantity One software (Bio-Rad, Hercules, CA, USA) to assess the proportional differences in the MT_1 protein content between samples from L-NAME hypertensive and normotensive rats. Protein expression in the aorta was normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Real-time RT-PCR

Total RNA was isolated with the use of TRI reagent (MRC, Cincinnati, OH, USA). The first-strand cDNA synthesis was carried out with the use of the ImProm-II Reverse Transcription System (Promega, Madison, WI, USA) according to the manufacturer's instruction. Denatured total RNA (0.6 µg for the aorta and 1 µg for the heart) was used as a template in a 20-µl cDNA synthesis reaction with random hexamer primers (0.5 µg µl^{-1}).

Aliquots of the cDNA (0.8 µl of RT product) were analyzed for gene expression with the appropriate primers in 20-µl real-time PCR reactions. The primers for the PCR analysis were as follows: for *gapdh* (AF106860) forward primer 5′-CTGAGAATGGGAAGCTGGTC-3′, reverse primer 5′-GTGGTTCA CACCCATCACAA-3′; for *mt1* (AF130341) forward primer 5′-CTGGTGG TTTTCCATTTCGT-3′, reverse primer 5′-CGGATCTGAGGCCACAATAA-3′. The quantification of cDNA was performed with the QuantiTect SYBR Green PCR Kit (QIAGEN, Hilden, Germany) and the StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Real-time PCR conditions were 95 °C, 15 min followed by 50 cycles of 94 °C, 15 s; 49 °C, 30 s; and 72 °C, 30 s. The specificity and identity of the PCR product were validated by adding melting curve analysis at the end of the program. The fluorescence dye, ROX, served as an internal reference for normalization of the SYBR Green I fluorescent signal.

Statistics

The values are presented as the mean ± s.e.m. Rhythmical 24-h profile of melatonin concentrations was determined by Cosinor analysis.[20] The unpaired t-test was used to compare variables between L-NAME hypertensive and normotensive rats.

RESULTS

L-NAME treatment induced hypertension. After 4 weeks of L-NAME treatment, BP reached 178.0 ± 0.6 and 118.0 ± 0.5 mm Hg in hypertensive and normotensive rats, respectively. The increase represented 55.6 ± 0.9 mm Hg in L-NAME, and in the controls, BP even decreased by 5.1 ± 1 mm Hg. The final body weight was lower in the L-NAME group compared with that in the control group (326 ± 4 *vs.* 343 ± 5 g; $P<0.01$). We found a significant higher weight of left ventricle in L-NAME hypertensive rats, confirming an expected left ventricle hypertrophy (462 ± 9 *vs.* 394 ± 5 mg, $P<0.0001$).

Pineal gland

Melatonin content showed a pronounced daily rhythm in the pineal gland of both normotensive and L-NAME hypertensive rats (Figure 1a). Pineal melatonin content during the light period was similar in both groups (Figure 1a). As expected, pineal melatonin levels reached a peak in the middle of the dark period. In L-NAME hypertensive rats, the peak melatonin levels were significantly higher (3377 ± 478 pg per pineal gland) than in the control group (1752 ± 329; $P<0.05$). The amplitude of the melatonin rhythm was about 1.8-fold higher in hypertensive than in normotensive rats (Table 1). No difference in pineal melatonin content was observed in other analyzed time points. Acrophases of the rhythms did not differ between both groups.

Plasma

Plasma melatonin concentrations exhibited a distinct daily rhythm in both normotensive and L-NAME hypertensive rats (Figure 1b). In normotensive rats, plasma melatonin levels ranged from 28 ± 4 to 37 ± 3 pg ml^{-1} during the light period. During the nighttime, plasma

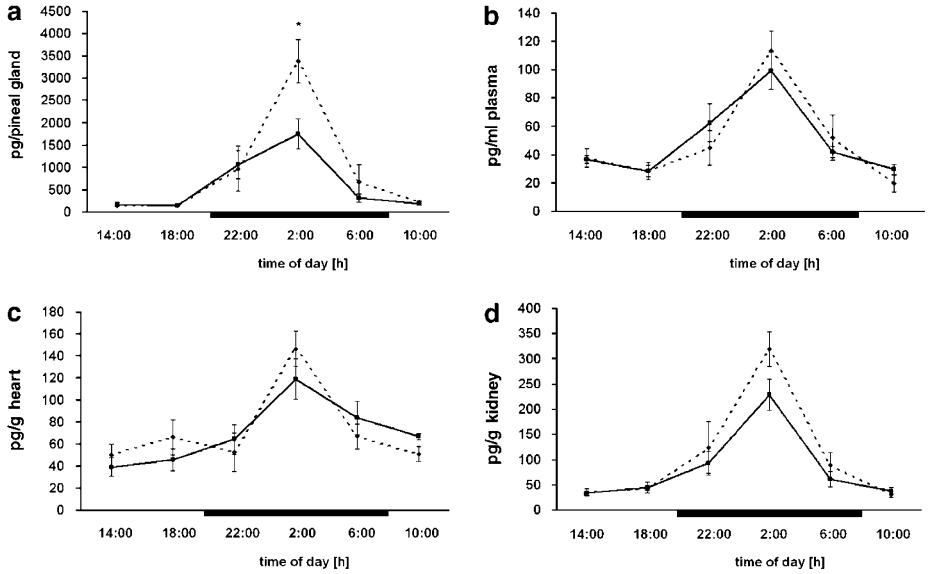

Figure 1 Circadian profile of melatonin concentrations in L-NAME-treated (dotted line) and control rats (solid line) in the pineal gland (**a**), plasma (**b**), heart (**c**) and kidney (**d**). Results are expressed as means ± s.e.m. (n=6; at 22:00 n=5), *$P<0.05$ in comparison with normotensive rats.

Table 1 Melatonin rhythm assessed by Cosinor analysis with 24-h period in the pineal gland, plasma, heart and kidney in normotensive and L-NAME hypertensive rats

Tissue (units)	*Group*	*Mesor*	*Amplitude*	*Acrophase (ZT—h:min)*	P-*value*
Pineal gland (pg per pineal gland)	L-NAME	940	1314	17:43	0.0001
	Control	614	735	17:22	0.0001
Plasma (pg ml^{-1})	L-NAME	50	34	17:49	0.0004
	Control	48	28	17:16	0.0001
Heart (pg g^{-1} tissue)	L-NAME	71	30	18:00	0.017
	Control	70	35	19:16	0.0002
Kidney (pg g^{-1} tissue)	L-NAME	106	119	17:35	0.0001
	Control	82	77	17:28	0.0001

ZT, Zeitgeber time; ZT0 corresponds to the begining of the light phase (08:00 hours).

melatonin concentrations peaked at 99 ± 13 pg ml^{-1}. In L-NAME hypertensive rats, plasma melatonin concentrations fluctuated from 19 ± 6 to 38 ± 7 pg ml^{-1} during the light period, whereas in the middle of the dark period, they reached 113 ± 14 pg ml^{-1} (Table 1). Daily profiles, mesor and amplitude of melatonin rhythm in plasma did not differ between both groups.

Heart

We observed a clear-cut daily rhythm in melatonin concentrations in both normotensive and L-NAME hypertensive rats (Figure 1c). In the heart of normotensive rats, melatonin levels fluctuated from 39 ± 9 to 67 ± 3 pg g^{-1} tissue during the light period, and maximal values were achieved in the middle of the dark period (119 ± 19 pg g^{-1} tissue). In L-NAME hypertensive rats, melatonin heart concentrations ranged from 50 ± 10 to 66 ± 16 pg g^{-1} tissue during the light period and peaked in the middle of the dark period (146 ± 16 pg g^{-1} tissue). Daily profiles and amplitude of melatonin concentrations rhythm in the heart did not differ between both groups (Table 1).

Kidney

A significant daily rhythm in melatonin levels of both normotensive and L-NAME hypertensive rats was found in the kidney (Figure 1d). Melatonin concentrations in the kidney in normotensive rats peaked in the middle of the nighttime (228 ± 31 pg g^{-1} tissue), whereas during the light period, they ranged from 33 ± 5 to 44 ± 10 pg g^{-1} tissue. In L-NAME hypertensive rats, melatonin concentrations fluctuated from 31 ± 6 to 41 ± 3 pg g^{-1} tissue during the light period. In the middle of the dark period, plasma melatonin levels increased up to 319 ± 35 pg g^{-1} tissue. The amplitude of the melatonin rhythm in the kidney reached about a 1.5-fold higher value in L-NAME hypertensive rats compared with that in normotensive rats (Table 1).

Melatonin receptors in the aorta

We identified MT_1 melatonin receptors in the aorta of both normotensive and L-NAME hypertensive rats. The major protein band represented a protein with molecular weight of approximately 60 kDa that corresponds to the glycosylated form of MT_1 receptors. We did not find a daily rhythm of protein expression in the aorta in any experimental group. Likewise, no significant difference between MT_1 receptors expression in normotensive and L-NAME hypertensive rats was observed (Figure 2).

In control animals, we examined a presence of mt_1 mRNA in the aorta and heart by real-time RT-PCR (Figure 3). We observed a significant expression of mt_1 in both tissues in all tested animals

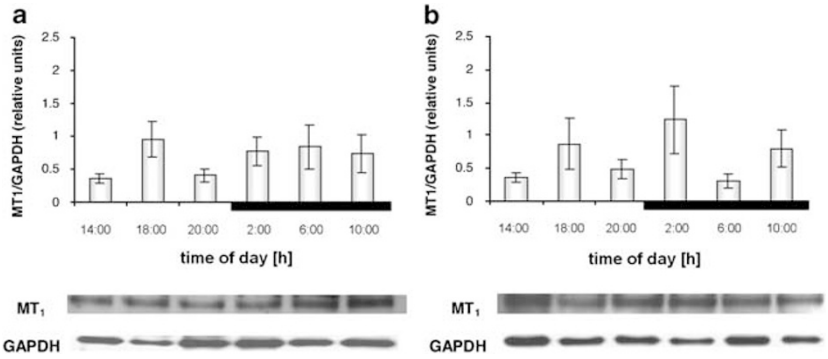

Figure 2 Daily profile of MT_1 receptor density within 24-h cycle in the aorta of control (**a**) and L-NAME-treated rats (**b**) with illustrating bands below the graphs. The expression of MT_1 is normalized to GAPDH, values are expressed as means ± s.e.m. from three to four independent measurements (n=3).

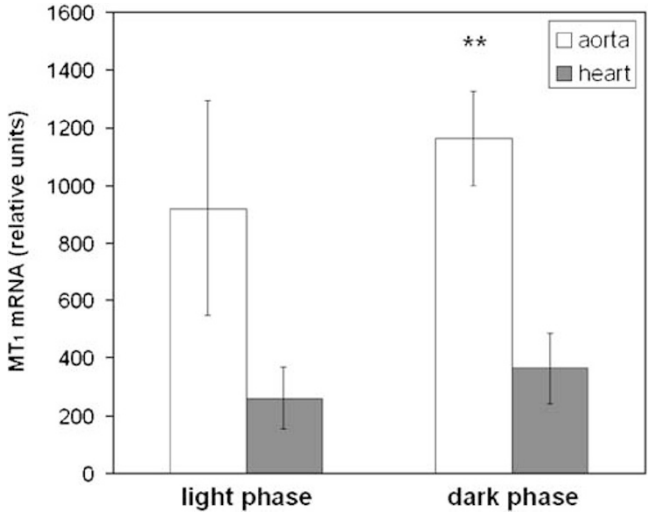

Figure 3 The expression of mRNA MT_1 receptor in the aorta and the heart during the light (aorta, n=12; heart, n=6) and dark phases (aorta, n=14; heart, n=8) of 24-h cycle. Values are presented as means ± s.e.m. $^{**}P<0.01$, t-test.

(n=26). The expression of mt_1 was significantly higher in the aorta compared with that in the heart ($P<0.05$, t-test).

DISCUSSION

Experimental hypertension induced by L-NAME is based on the inhibition of nitric oxide formation and impaired vascular relaxation. In our experiment, long-term administration of L-NAME induced hypertension and hypertrophy of the left ventricle in accordance with earlier findings from our and other laboratories.[21,22] Chronic depletion of NO and marked arterial hypertension may cause vasoconstriction and hypoperfusion of tissue with the consequent exacerbation of reactive oxygen species (ROS) formation.[23]

The administration of melatonin reduces BP and infarct size in rats with L-NAME induced hypertension in a regressive experimental setting,[24] as well as when melatonin administration started simultaneously with L-NAME treatment and before hypertension development.[25] The antihypertensive effect of melatonin was reduced compared with spontaneously hypertensive rats,[26] suggesting that the inhibited NO production by L-NAME reduces the hypotensive effect of melatonin. Melatonin can attenuate an increased BP by interfering with several control systems, such as the sympathetic nervous system, the NO–cGMP pathway[27] and the excessive production of ROS.[28] Modulation of different systems may have additional effects on BP; therefore, the hypotensive action of melatonin may differ among different experimental models of hypertension.

In this study, a distinct circadian rhythm of pineal melatonin concentrations with higher levels during the dark in comparison with the light period in both L-NAME hypertensive and normotensive rats was observed. Interestingly, after 4 weeks of L-NAME treatment, the nighttime melatonin concentrations in the pineal gland were significantly higher than in controls. The exact pathway upregulating melatonin biosynthesis in L-NAME hypertension is unknown and at least two possibilities exist. First, the formation of NO potentiates GABA-ergic inhibitory effects in the paraventricular nucleus (PVN) of the hypothalamus.[29] Therefore, reducing NO with L-NAME would reduce GABA-mediated inhibitory effects in the brain and enhance pineal melatonin biosynthesis. Moreover, an inhibitory action of NO on electrically active units in the rat pinealocytes was shown.[30] The suppression of NO generation by L-NAME treatment may further stimulate melatonin biosynthesis in the pineal gland. The second hypothesis is based on an increased sympathetic tone, which is related to hypertension development. As melatonin biosynthesis is stimulated by the adrenergic system through the suprachiasmatic nucleus, PVN and superior cervical ganglion during the nighttime,[31] upregulated melatonin biosynthesis may represent a protective feedback mechanism against excessive sympathetic excitation.[27]

In our study, the enhanced melatonin production in the hypertensive rats was not reflected in higher melatonin concentrations in the plasma and peripheral tissues. Melatonin is released into circulation immediately after biosynthesis, and melatonin concentrations in plasma should reflect melatonin concentrations in the pineal gland. Therefore, the absence of a close correlation between the pineal gland and plasma melatonin profile in our study is surprising. We suppose that lower melatonin concentrations in the plasma of hypertensive rats can reflect the higher production of ROS during hypertension.[32] In hypertensive rats with the metabolic syndrome, melatonin secretion is decreased during sleep hours, and supplementation with melatonin prevents the development of hypertension.[33] Melatonin as a potent antioxidant[34] participates in scavenging of ROS. The enhanced formation of free radicals in L-NAME hypertensive rats[35,36] could lead to a faster consumption of melatonin and thus diminish the differences in melatonin levels in plasma between L-NAME hypertensive and normotensive rats. In SHR, the melatonin-induced decline in BP was associated with a reduced intracellular content of superoxide and malondialdehyde in the kidney,[37] reduction of nuclear factor κB

expression[38] and depressed level of conjugated dienes.[26] Therefore, it is expected that the antioxidant activity of melatonin could contribute to its antihypertensive effects.[27]

The melatonin rhythm showed maximal levels during the night in the tissue of both the heart and the kidney. These data are congruent with the earlier reported circadian melatonin rhythm in the kidney of diabetic rats treated with streptozotocin.[39] Detection of a melatonin circadian profile in the heart is an original finding as earlier studies measured melatonin content in the heart only in one time point during the daytime.[11] The absence of differences in the melatonin content between L-NAME hypertensive and normotensive rats in the heart and kidney correlates with plasma melatonin profiles that are similar between both groups.

We detected MT_1 receptors in the rat aorta at the level of mRNA and also as a protein with a molecular weight of around 60 kDa, which corresponds to the glycosylated form of MT_1 melatonin receptors. To our knowledge, this is the first study that proved the presence of the MT_1 receptor protein in the aorta. The exact function of MT_1 receptors in the aorta is still not known. The activation of MT_1 in the rat caudal artery could lead to vasoconstriction,[17] but this action was not shown in other vessels, including the aorta. Receptor-mediated melatonin action in the aorta was suggested to enhance NO signaling[27] and upregulate antioxidant enzymes,[40] which may protect against increased ROS and consequent aorta stiffness, which is increased after the inhibition of NO synthase in rats.[41] The exact localization of receptors in different aorta layers, together with the determination of MT_2 density and changes in MT_1/MT_2 ratio are needed to clarify the physiological role of melatonin in the aorta.

In conclusion, melatonin concentrations in the pineal, blood, heart and kidney showed a distinct circadian profile in normotensive as well as in L-NAME-treated hypertensive rats. The nighttime peak levels of pineal melatonin were higher in L-NAME-treated animals compared with those in controls. Enhanced melatonin formation in the pineal gland of L-NAME-treated rats might be a consequence of reduced NO formation in the central nervous system. In this model, the elevated melatonin production by the pineal gland seems to be insufficient to alter plasma and tissue melatonin levels probably because of its increased consumption owing to oxidative load. The presence of mRNA and protein of MT_1 receptors in the aorta indicates that melatonin may be involved in the control of peripheral resistance and thus the functional relevance of melatonin receptors in the aorta should be further investigated.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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