Inhibitory Effect of Melatonin on $\alpha_1$-Adrenergic–Induced Vasoconstriction in Mesenteric Beds of Spontaneously Hypertensive Rats

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**Background:** The aim of this study was to assess the effects of melatonin on $\alpha_1$-adrenergic pathway in mesenteric arteries of spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats.

**Methods and Results:** The SHR are characterized by a higher vasoconstriction ($P < .001$) and inositol phosphate formation ($P < .001$) in response to phenylephrine (PHE) and an increased superoxide anion production ($P < .001$) in mesenteric arteries. Melatonin and 2-iodomelatonin produced a significant inhibition of the PHE-induced vasoconstriction in isolated and perfused mesenteric beds ($P < .001$) with the same magnitude in SHR and WKY rats. Melatonin significantly decreased the inositol phosphate (IPs) formation in isolated mesenteric arteries from SHR compared to WKY rats ($P < .001$). The inhibitory effect of melatonin was increased by the removal of endothelium ($P < .001$). No effects of superoxide dismutase (SOD), tempol, or catalase were observed on the PHE-induced vasoconstriction. Moreover, no superoxide anion scavenging effect of 2-iodomelatonin was observed in isolated mesenteric vascular muscle cells using lucigenin.

**Conclusions:** The present study showed that high melatonin concentrations inhibit the $\alpha_1$-adrenergic-induced vasoconstriction and inositol phosphate formation in mesenteric arteries from SHR and WKY rats. The vasorelaxant effect of the melatonin receptors agonist, 2-iodomelatonin, and the absence of any vasoactive effect of antioxidants such as SOD, tempol, and catalase suggest that melatonin exerts its inhibition on $\alpha_1$-adrenergic-induced vasoconstriction of mesenteric arteries through a low-affinity membrane receptor negatively coupled to the IPs formation and that this effect is independent of its antioxidant properties. Am J Hypertens 2004;17:339–346 © 2004 American Journal of Hypertension, Ltd.

**Key Words:** Melatonin, $\alpha_1$-adrenergic pathway, spontaneously hypertensive rats, mesenteric artery, superoxide anion.
effect to specific antioxidants such as superoxide dismutase (SOD), tempol (4-hydroxy-[2,2,4,4-tetramethylpiperidine-1-oxyl]), which is a mimic of SOD, and catalase.

Methods
Animals and Treatment
The SHR and normotensive WKY rats were obtained from Harlan Laboratories (Indianapolis, IN). The rats were treated and maintained in accordance with Canadian Council on Animal Care guidelines and the study was approved by the local Institutional Animal Ethics Committee. The rats were housed under conditions of constant temperature and humidity, exposed to a 12-h light/dark cycle with free access to standard laboratory rat chow (Basal Purified Diet 5755C; Purina Mills Inc., St. Louis, MO) and drinking water. Systolic blood pressure (BP) was measured the day before experiments, by tail cuff plethysmography on conscious restrained rats and was recorded on a system MacLab/8 (AD Instruments Ltd., CastleHill, Australia) fitted with a Harvard photocell as a pulse detector (Harvard Apparatus Ltd., South Natick, MA, USA). For each rat, at least three blood pressure (BP) readings were averaged to establish the blood pressure (BP) level.

Mesenteric Arterial Bed Preparation
Experiments were performed on 15-week-old rats as previously described.2 The superior mesenteric artery was cannulated near its origin in the aorta and was perfused at 4 mL/min using a peristaltic pump (Ismatec, Cole-Parmer Instrument Co., Chicago, IL) with an oxygenated (95%) Krebs–bicarbonate solution maintained at 37°C. To eliminate the contribution of prostaglandins in the vascular responses, all the experiments were realized in the presence of indomethacin (10μmol/L). Vasoconstriction was estimated from the increased perfusion pressure (in mm Hg) by using a strain gauge transducer (P322Dc; Gould Statham Instruments, Hatorey, Puerto Rico) connected to a side arm of the perfusion cannula. The isolated mesenteric bed was allowed to equilibrate for 60 to 90 min. Then, the arteries were repeatedly contracted with PHE (1 μmol/L) at 15-min intervals until the responses remained constant. In some experiments, the endothelium was removed by infusing 0.5% [3-(3-chloramidopropyl)dimethylammonio]-1-propanesulfate (CHAPS) for 45 sec.10 The presence of a functional endothelium was assessed by the observation of a vasodilator action of acetylcholine (10 μmol/L). Thereafter, the mesenteric bed was continuously perfused with concentrations (0.3 to 3μmol/L) of PHE that produced 70 mm Hg, which is about 50% of the maximal contraction. Melatonin or 2-iodomelatonin was perfused with increasing and cumulative concentrations in Krebs solution at 5-min intervals. The maximal effect of melatonin or 2-iodomelatonin at each concentration was reached after about 2 min. Responses to melatonin were also examined after the endothelium has been removed by the same procedure. The effect of scavenging free radicals on PHE-induced vascular responses was assessed by exposing the vascular beds with intact endothelium to superoxide dismutase (SOD) (a superoxide anion scavenger), catalase (a hydrogen peroxide scavenger), 4-hydroxy-[2,2,4,4-tetramethylpiperidine-1-oxyl] (tempol, a SOD mimic), mannitol (hydroxyl radical scavenger), and deferoxamine (iron chelator). Superoxide dismutase (120 U/mL), catalase (80 U/mL), Tempol (1 mmol/L), mannitol (10 mmol/L), and deferoxamine (0.1 mmol/L) were added to the perfusate for 20 min.

Measurement of Inositol Phosphate Formation
Mesenteric arteries were dissected, cleaned of adventitia, and incubated for 15 min in Erlenmeyer flasks containing 6 mL of physiologic oxygenated (95%) Krebs–bicarbonate solution. To evaluate the formation of phosphoinositol metabolites, the tissues were labeled with 0.3 μmol/L of d-myo-[3H]inositol (specific activity 24 Ci/mmol; DuPont Canada Inc. Diagnosis and Biotechnology Systems, Ontario, Canada) for 4 h in 4 mL of oxygenated (95%) Krebs–bicarbonate at 37°C. After this incubation, the arteries were washed three times in fresh Krebs. Each artery was then cut (4 to 8 mg/strip), transferred into individual vials, and incubated for 10 min at 37°C in 3.6 mL (controls) or 3.2 mL (activated tissues) of Krebs containing 500 mmol/L LiCl. Then, 0.4 mL of a solution containing PHE (1 μmol/L) along with melatonin (1 mmol/L) was added and tissues were incubated for 20 min. The reaction was terminated with 5 mL of cold Krebs. Thereafter, the arteries were removed from the medium, quickly frozen in liquid nitrogen, and homogenized with 0.9 mL of methanol–chloroform (40:20). After the addition of 0.4 mL of chloroform and 0.4 mL of distilled water, the samples were centrifuged (International Equipment, Needham Heights, MA) to separate lipid and aqueous phases. The inositol phosphates (IPs) were then extracted as previously described.11 The lipid phase was counted to measure the phosphatidylinositol (PIP) lipid pool. The accumulation of IPs was expressed as a relative value of (IPs / PIP) × 10³ (arbitrary units) to correct for the variation in the labeling of the lipid pool.

Cell Culture and Superoxide Anion Production
Smooth muscle cells (SMCs) from mesenteric arteries were isolated as described previously.11 Briefly, rat mesenteric arteries were dissected and the connective tissue was removed. The vessels were enzymatically digested with collagenase/dispase, elastase, and collagenase in a stepwise manner. Dispersed cells were plated in 75 cm² flasks in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum in a CO₂ incubator at 37°C. Isolated
cells between passages 2 to 8 were seeded into 132 mm six-well multidishes and used for superoxide anion assays. Lucigenin (0.25 mmol/L) was used to detect superoxide anion production by aortic SMCs (approximately 10^6 cells) placed in scintillation vials containing 2 mL of phosphate-buffered saline. The effects of melatonin (1 mmol/L) and 2-iodomelatonin (1 mmol/L) on the superoxide anion production were tested after an in vitro preincubation of 20 min. The chemiluminescence was measured using a scintillation counter (Wallac 1409, Turku, Finland) set to operate in the out-of-coincidence mode, with sampling at 30-sec intervals over a 10-min time period. The respective background was subtracted from total count. At the end of the chemiluminescence measurement, the total SMC protein content was determined with Lowry’s method.12

Statistical Analysis
The amplitude of the contraction immediately before the addition of melatonin or 2-iodomelatonin was considered to represent 100%. Values are given as the mean ± SE mean, and n indicates the number of observations. Statistical analysis was performed using analysis of variance (two-way ANOVA) in conjunction with a Bonferroni multiple comparison analysis when F values were significant for comparison of three or more groups and unpaired and paired Student t tests to compare two groups. For multiple comparisons to the same control group, significance was assessed by a one-way ANOVA followed by Dunnett’s test. Dose–response curves were fitted to the sigmoidal four parameter logistic equation by a curve-fitting analysis program (Graph Pad Prism for Windows version 2.01; GraphPad Inc., San Diego, CA) to evaluate ED50 and maximal relaxation response. This program was also used for all statistical analysis. The ED50 values were expressed as the negative logarithm of the drug concentration that produces 50% of the maximal response to each drug. Statistical significance was considered when P < .05.

Drugs
All drugs and chemical components of solutions were purchased from Sigma Chemical Company (St Louis, MO). The PHE and tempol were dissolved in water and indomethacin, melatonin, and 2-iodomelatonin were dissolved in ethanol to give a final concentration of 0.1%. The SOD and catalase were dissolved in polyethylene glycol (PEG) to give a final concentration of 0.1%. Ethanol (0.1% to 1%) or PEG concentrations in Krebs-Henseleit solution did not have any effect on PHE-induced vasoconstriction.

Results
BP and Body Weight
Body weights of SHR and WKY rats were similar (285 ± 6 compared with 290 ± 7 g, n = 8). Systolic arterial pressure was significantly higher in SHR than in WKY rats (191 ± 2 compared with 126 ± 4 mm Hg, n = 8, P < .001).

Effect of Melatonin on the Phenylephrine-Induced Vasoconstriction
Mesenteric arterial beds from hypertensive SHR were significantly more responsive to PHE than those obtained from age-matched normotensive rats, in the presence and in the absence of endothelium (P < .001; Fig. 1). However, mesenteric beds were more responsive to PHE in the absence of endothelium than in the presence of endothelium in both SHR and WKY rats (P < .05). Melatonin caused a concentration-dependent relaxation in mesenteric arterial beds precontracted with PHE. In the absence of endothelium, the ED50 of the concentration–response curve for melatonin-induced relaxation was lower than in the presence of endothelium both in SHR and WKY rats (Fig. 2 and Table 1). The concentration–response curves for melatonin in mesenteric beds with or without endothe-
Exogenous antioxidants were tested for their possible effect on the PHE-induced vasoconstriction. Mesenteric beds were perfused for 20 min for each dose of SOD and catalase. No effects of these antioxidant enzymes alone or combined were observed on the PHE-induced vasoconstriction in mesenteric beds from SHR and WKY rats (Fig. 3). To make sure that the absence of response of SOD is not attributed to its low membrane-permeant characteristic, its effect was compared with the effect of a stable membrane-permeant SOD mimetic, tempol (Fig. 3). No effects of tempol were observed in the PHE-induced vasoconstriction of mesenteric arteries from SHR and WKY rats with or without endothelium. Mannitol and deferoxamine also failed to inhibit the PHE-induced vasoconstriction (results not shown).
PHE after subtraction of basal values was still higher in SHR than in WKY rats (76.4 ± 1.7 compared with 56.9 ± 5.1, P < .01, n = 8). Melatonin (1 mmol/L) did not have any effects on basal IPs formation (results not shown), but it significantly inhibited the PHE-induced IPs production (Fig. 5). The inhibitory effect of melatonin on the PHE-induced formation of IPs was of the same magnitude in arteries from both SHR and WKY rats (84.9% ± 1.0% compared with 83.4% ± 0.5% of inhibition).

**Effects of Melatonin and 2-Iodomelatonin on Vascular Superoxide Anion Production**

To verify whether 2-iodomelatonin is a specific melatonin receptor agonist rather than an antioxidant, its capacity to scavenge superoxide anion was compared to the antioxidant potential of melatonin. In SHR, the lucigenin chemiluminescence was higher than that in WKY rats (1.87 ± 0.04 × 10³ count/min per milligram of dry tissue weight compared with 1.43 ± 0.20 × 10³ count/min per milligram of dry tissue weight; Fig. 6). Melatonin markedly decreased the lucigenin chemiluminescence in SMCs from SHR (0.50 ± 0.01 × 10³ count/min per milligram of dry tissue weight) and WKY rats (0.50 ± 0.01 × 10³ count/min per milligram of dry tissue weight), whereas 2-iodomelatonin did not significantly decrease the lucigenin chemiluminescence rats (1.70 ± 0.15 × 10³ count/min per milligram of dry tissue weight in SHR and 1.50 ± 0.14 × 10³ count/min per milligram of dry tissue weight in WKY rats; Fig. 6).

**Discussion**

The present study demonstrates for the first time that the pineal hormone melatonin inhibits the contractile responses induced by PHE in mesenteric beds from SHR and WKY rats. This vasorelaxant effect of melatonin has also been observed in aorta from normotensive rats, SHR, diabetic rats, and rabbits. In the present study, the vasorelaxant effect of melatonin did not differ between SHR and WKY rats. However, Wu et al demonstrated...
that the effect of melatonin on the norepinephrine-induced vasoconstriction is stronger in SHR than in WKY rats and it has been demonstrated that this difference relies on the antioxidant property of melatonin. In the presence of 2-iodomelatonin at saturated concentrations, melatonin further inhibited the norepinephrine-induced formation of inositol phosphate, suggesting that a receptor-independent pathway is implied in this effect. Moreover, other antioxidants, such as SOD, significantly inhibited the vasoconstrictive effect of norepinephrine in aorta from SHR. It has been demonstrated that the production of superoxide anion and the free radical-induced vasoconstriction are significantly enhanced in aorta from SHR. Results obtained in isolated SMCs from mesenteric arteries also showed an increased production of superoxide anion in SHR compared to their control WKY. However, SOD and tempol, which are superoxide anion scavengers did not modify the PHE-induced vasoconstriction. These results suggest that the superoxide anion production in mesenteric beds is not high enough to disturb the \( \alpha_1 \)-adrenergic pathway. The superoxide anion scavenging property of melatonin at a concentration that inhibits almost completely the PHE-induced vasoconstriction was very effective. It thus appears that although melatonin is a potent superoxide anion scavenger, its vasorelaxant effect in mesenteric arteries is not related to its superoxide anion scavenging capacity. The absence of an inhibition of the PHE-induced vasoconstriction by catalase, mannitol, and deferoxamine also strongly suggests that the effect of melatonin does not depend on its capacity to scavenge hydrogen peroxide and the hydroxyl radical. Moreover, \( \alpha_1 \)-adrenergic blockers, which possess broad antioxidant spectra and a low molecular weight, was found to also inhibit the PHE-induced vasoconstriction, but its effect depended entirely on endothelium and cyclo-oxygenase, which is not the case for melatonin.

Because the \( \alpha_1 \)-adrenergic pathway could be largely influenced by endothelium and that a chronic treatment with melatonin may improve the endothelium-dependent vasorelaxation in mesenteric arteries from SHR, we hypothesized that a part of the vasorelaxant effect of melatonin is endothelium dependent. Surprisingly, the vasorelaxant effect of melatonin was increased by the removal of the endothelium. This result could be explained by an acute inhibitory effect of melatonin on the endothelium-dependent vasorelaxant pathways. Melatonin may acutely inhibit nitric oxide synthase or scavenged various endothelium-derived relaxing factors such as nitric oxide, peroxynitrite, and hydroxyl radical.

Results obtained with 2-iodomelatonin suggest that the inhibitory effect of melatonin on the \( \alpha_1 \)-adrenergic-induced vasoconstriction is mediated by specific receptors. Furthermore, our experiments with lucigenin clearly demonstrated that 2-iodomelatonin is not an antioxidant as is melatonin. These results are consistent with those of Doolen et al., whereas the vasoconstrictive response of vessels was inhibited by high concentrations of melatonin (1 mmol/L to 10 mmol/L) through the MT\(_2\) melatonin receptor subtype activation. Moreover, melatonin-binding sites have been identified in arteries from SHR and WKY rats and MT\(_2\) receptors mediating vasorelaxation have been identified in rat caudal artery. Although this action of melatonin occurs at concentrations in excess of those found in the plasma (0.5 to 1 mmol/L), recent studies have demonstrated extrapineal sites for the production of melatonin by leukocytes and in hamster skin cell cultures. These studies suggest that a possible paracrine action of melatonin could reduce vascular smooth muscle tone. The vasorelaxant effect of 2-iodomelatonin was stronger that the effect of melatonin in the presence of endothelium and similar to the effect of melatonin in the absence of endothelium. These observations could be explained by the higher affinity of 2-iodomelatonin compared to melatonin for melatonin-binding sites and by the much lower efficiency of 2-iodomelatonin to scavenge molecules that are susceptible to produce a vasorelaxation such as hydroxyl radical.

In addition, the melatonin receptor seems to mediate the inhibition of the PHE-induced formation of IPs. These results are consistent with those of Wu et al. who showed that the inhibition of the norepinephrine-induced formation of IPs by melatonin is fully mediated by a membrane receptor-related mechanism in WKY rat aorta. However, no clear relationship with the inhibition of the phosphoinositide pathway by specific melatonin-binding sites has been demonstrated yet.

It has been demonstrated that acute and chronic administrations of melatonin decrease arterial pressure in SHR. Moreover, bolus injections of alloxan, allopurinol, oxypurinol, or Cu/Zn–SOD, which are superoxide anion scavengers, decreased arterial pressure in SHR.
and chronic treatments with antioxidant such as N-acetyl-cysteine or Tempol prevent the increase in arterial pressure in SHR. These results suggest that the antioxidant properties of melatonin could modulate arterial pressure. However, these antioxidant effects seem to be confined to the nitric oxide synthase response in mesenteric beds, whereas, in aorta, responses to muscarinic, α- and β-adrenergic agonists are modulated by antioxidants.

In conclusion, our results show that high melatonin concentrations inhibit the α1-adrenergic-induced vasoconstriction to a similar extend in mesenteric beds from SHR and WKY rats. This effect was not decreased and was even enhanced by the removal of the endothelium. Moreover, although the melatonin receptor agonist 2-iodomelatonin, which possesses no antioxidant properties, induced a vasorelaxant response similar to that of melatonin, and as no inhibitory effects of other antioxidants such as catalase, SOD, tempol, mannitol, and deferoxamine were observed on the PHE-induced vasoconstriction, it thus seems that melatonin exerts its inhibitory effect on α-adrenergic-induced vasoconstriction of mesenteric arteries through a low-affinity membrane receptor coupled to the inhibition of IPs formation and independent of its antioxidant scavenging properties.

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References