Melatonin mediates vasodilation through both direct and indirect activation of BK_{Ca} channels

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Abstract

Melatonin, synthesized primarily by the pineal gland, is a neuroendocrine hormone with high membrane permeability. The vascular effects of melatonin, including vasoconstriction and vasodilation, have been demonstrated in numerous studies. However, the mechanisms underlying these effects are not fully understood. Large-conductance Ca²⁺-activated K⁺ (BK_{Ca}) channels are expressed broadly on smooth muscle cells and play an important role in vascular tone regulation. This study explored the mechanisms of myocyte BK_{Ca} channels and endothelial factors underlying the action of melatonin on the mesenteric arteries (MAs). Vascular contractility and patch-clamp studies were performed on myocytes of MAs from Wistar rats. Melatonin induced significant vasodilation on MAs. In the presence of N^ω-nitro-L-arginine methyl ester (L-NAME), a potent endothelial oxide synthase (eNOS) inhibitor, melatonin elicited concentration-dependent relaxation, with lowered pIC₅₀. The effect of melatonin was significantly attenuated in the presence of BK_{Ca} channel blocker iberiotoxin or MT1/MT2 receptor antagonist luzindole in both (+) L-NAME and (–) L-NAME groups. In the (+) L-NAME group, iberiotoxin caused a parallel rightward shift of the melatonin concentration–relaxation curve, with pIC₅₀ lower than that of luzindole. Both inside-out and cell-attached patch-clamp recordings showed that melatonin significantly increased the open probability, mean open time and voltage sensitivity of BK_{Ca} channels. In a cell-attached patch-clamp configuration, the melatonin-induced enhancement of BK_{Ca} channel activity was significantly suppressed by luzindole. These findings indicate that in addition to the activation of eNOS, melatonin-induced vasorelaxation of MAs is partially attributable to its direct (passing through the cell membrane) and indirect (via MT1/MT2 receptors) activation of the BK_{Ca} channels on mesenteric arterial myocytes.

Key Words

- ▶ melatonin
- ▶ BK_{Ca} channels
- ▶ mesenteric artery
- ▶ vascular smooth muscle cell

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Introduction

Melatonin (N-acetyl-5-methoxytryptamine) is the primary hormone of the pineal gland, mainly secreted at night (Saarela & Reiter 1994). Increasing evidence indicates that melatonin plays an important role in

regulating cardiovascular homeostasis as well as in the formation and organization of biorhythms (Cook *et al.* 2011, Grossini *et al.* 2011, Pandi-Perumal *et al.* 2016, Sun *et al.* 2016, Tang *et al.* 2016, Tordjman *et al.* 2017).

By regulating blood vessel diameter, melatonin affects arterial blood pressure and local blood flow to organs and tissues (Cook *et al.* 2011, Pandi-Perumal *et al.* 2008).

Melatonin exerts many of its physiological actions through interaction with specific melatonin receptors that are present in mammalian tissues (Pandi-Perumal *et al.* 2008, Zlotos *et al.* 2014). Three high-affinity melatonin receptor subtypes – MT1, MT2, and MT3 receptors – have been identified and cloned (Nosjean *et al.* 2000, Dubocovich *et al.* 2003). Melatonin predominantly acts via two G-protein-coupled receptors: MT1 and MT2 (Pandi-Perumal *et al.* 2008). The activation of melatonin receptors on endothelial and vascular smooth muscle cells and antioxidant properties of melatonin could be responsible for the melatonin effects on vascular tone (Paulis & Simko 2007). The effects of melatonin on vascular function are complex; melatonin receptor activation causes vasoconstriction in some vasculatures and vasodilation in others. For instance, melatonin has been found to elicit vasoconstriction in certain arteries (Evans *et al.* 1992, Geary *et al.* 1997, Ting *et al.* 1997, Viswanathan *et al.* 1997), such as coronary vessels, the renal vascular bed (Yang *et al.* 2001, Cook *et al.* 2011, Tunstall *et al.* 2011) and cerebral arteries (Geary *et al.* 1997, 1998), whereas it induces vasodilation in others (Satake *et al.* 1991, Weekley 1991, Doolen *et al.* 1998), such as aorta, pulmonary and umbilical vascular bed, and mesenteric arteries (MAs) (Weekley *et al.* 1993, Girouard *et al.* 2001, Thakor *et al.* 2010). These opposite effects may be due to melatonin's binding to 2 different receptors, which are differently distributed on different vascular beds (Doolen *et al.* 1998). The activation of MT1 receptors has been widely associated with cAMP decrease and phosphatidylinositol-4,5-bisphosphate hydrolysis, which would lead to the inhibition of vasodilation or vasoconstriction (Paulis & Simko 2007). The activation of MT2 receptors on endothelial cells (ECs) may involve an increased nitric oxide (NO) production and an endothelium-dependent vasodilation (Anwar *et al.* 2001, Paulis & Simko 2007, Reiter *et al.* 2009).

Both endothelium-dependent and non-endothelium-dependent pathways are involved in melatonin's effects on blood vessels, which mainly occur following activation of MT1 and/or MT2 receptors (Paulis & Simko 2007, Hung *et al.* 2013). In addition to these well-established mechanisms, MT1 receptors have also been reported to couple to large-conductance Ca²⁺-activated K⁺ (BK_{Ca}) channels (13, 18) and to G protein-activated inward rectifier K⁺ channels (GIRK Kir 3) (Jiang *et al.* 1995), which indicates that K⁺ channels on vascular myocytes are

potential targets of melatonin. BK_{Ca} channels, activated by both membrane depolarization and intracellular calcium, are broadly expressed on smooth muscle cells (SMCs) and play a critical role in vascular tone regulation (Nelson & Quayle 1995, Ledoux *et al.* 2006, Ko *et al.* 2008). There is indirect evidence for the inhibition of BK_{Ca} channels by melatonin in rat cerebral arteries (Geary *et al.* 1997, 1998).

Previous studies have demonstrated that melatonin has high membrane permeability and displays high lipophilicity (Yu *et al.* 2016). Melatonin can enter and leave cells in seconds; the literature therefore suggests that melatonin is secreted by simple diffusion across the pinealocyte plasma membrane (Simonneaux & Ribelayga 2003, Yu *et al.* 2016). Given this background, we wondered whether these small molecules could pass through the cell membrane and act on BK_{Ca} channels directly. To date, no studies have investigated this hypothesis.

In the rat, the mesenteric circulation receives approximately one-fifth of the cardiac output; thus, regulation of this bed makes a significant contribution to the regulation of systemic blood pressure (Nichols *et al.* 1985). Previous studies have shown that melatonin induces vasodilation in the MAs (Girouard *et al.* 2001). Although endothelium-independent and cycle AMP-dependent mechanisms have been suggested, the precise mechanisms by which melatonin affects the vascular tone of the MAs have not been fully determined. Therefore, this study was designed to determine (1) whether melatonin activates BK_{Ca} channels on MA SMCs; (2) whether both direct and indirect activation of BK_{Ca} channels are involved in melatonin-induced vasorelaxation of MAs. The information obtained will expand the knowledge of melatonin's effect on the peripheral resistance arteries and enhance understanding of the potential effects of melatonin on blood pressure regulation in humans.

Materials and methods

Animals

Eight-week-old male Wistar rats weighing 180–220 g were used in these experiments. The animals were housed under controlled conditions with a 12 h light-darkness cycle and ambient temperature of 24°C, with food and water available *ad libitum*. All experimental procedures and protocols conformed to the recommended guidelines on the care and use of laboratory animals issued by the Chinese Council on Animal Research. The study was approved by the ethical committee of Beijing Sport University.

Immunofluorescence

For immunofluorescence staining, MA sections were fixed with 4% paraformaldehyde in PBS for an hour at room temperature and then permeabilized by 0.2% Triton X-100 for 15 min. After extensive washing with PBS, the tissue sections were blocked for non-specific antibody binding 60 min with 5% BSA, then incubated in a rabbit polyclonal antibody specific for Anti-Melatonin Receptor Type 1 (Alomone Labs, Jerusalem, Israel; 1:200) or Anti-Melatonin Receptor Type 2 overnight at 4°C. The second Alexa Fluor 488 Goat Anti-Rabbit IgG antibody (Molecular Probes, 1:1000) was used for 1 h next day after washing. The fluorescence images were captured using a TCS-SP5 confocal laser scanning microscope (Leica). Image analysis software was performed in ImageJ (NIH, version 1.46r).

Assessment of vascular function

The animals were euthanized with sodium pentobarbital (100 mg/kg, i.p.). The MA and its branches were removed and placed in cold Krebs solution with a composition of 131.5 mM NaCl, 5 mM KCl, 1.2 mM NaH_2PO_4 , 1.2 mM MgCl_2 , 2.5 mM CaCl_2 , 11.2 mM glucose, 13.5 mM NaHCO_3 , 0.025 mM EDTA (37°C, pH 7.4), and aerated with 95% O_2 and 5% CO_2 . Short segments of A3 were used for contractile studies performed with a multi myograph system (620M, DMT, Denmark) per the procedures in previous studies (Shi *et al.* 2015).

For all vessels, we evaluated the contractile response for tension by measuring the maximum peak height and expressing it as the percentage of maximal tension achieved to 60 mM K^+ ($K_{\max}$), and calculated pIC_{50} ($-\log\text{IC}_{50}$, the negative logarithm of the half-maximal inhibitory concentration, a measure of the effectiveness of a substance in inhibiting a specific biological or biochemical function). To examine the effect of melatonin on vascular tone, tissues were first contracted with 10^{-5} M norepinephrine (NE). Then, responses to the cumulative addition of melatonin (10^{-7} – 10^{-3} M) were tested. In some experiments, non-selective nitric oxide synthase (NOS) inhibitor N^{ω} -nitro-L-arginine methyl ester (L-NAME, 100 μM) was added after $K_{\max}$ measurement.

Cell isolation

MAs were removed, cleaned of fat and connective tissue, cut into tissue pieces (0.5–1 mm in both length and width) and placed in digestion buffer (Ca^{2+} -free ice-cold physiological salt solution I (PSS I)) containing 137 mM

NaCl, 5.6 mM KCl, 1 mM MgCl_2 , 10 mM HEPES and 10 mM glucose (adjusted to 7.4 with NaOH). Dissected segments were incubated for 30 min at 37°C in digestion buffer supplemented with papain (0.3 mg/mL, Worthington Biochemical, USA) and dithiothreitol (1 mg/mL, Sigma-Aldrich), followed by a second incubation (20 min at 37°C) in digestion buffer supplemented with collagenases Type F (1.5 mg/mL, Sigma) and Type 1-S (1 mg/mL, Sigma-Aldrich). Tissues were then washed 3–4 times with PSS II containing 137 mM NaCl, 5.6 mM KCl, 1 mM MgCl_2 , 10 mM HEPES, 10 mM glucose, 0.42 mM Na_2HPO_4 , and 0.44 mM NaH_2PO_4 (99.9% O_2 for 40 min, adjusted to 7.3–7.4 with NaOH), and triturated gently using a fire-polished pipette to create a cell suspension.

Electrophysiological recording techniques

Standard patch-clamp recording techniques were used to measure currents in the cell-attached or inside-out patch configuration (Hamill *et al.* 1981). Currents were amplified using an Axon700B amplifier, sampled at 10 KHz and filtered at 2 KHz with an 8-pole Bessel filter.

Conventional whole-cell recording Whole-cell K^+ currents were measured with conventional voltage-clamp configuration. The cell bath solution comprised the following (mM): 134 NaCl, 6 KCl, 1 MgCl_2 , 1.8 CaCl_2 , 10 glucose and 10 HEPES (pH 7.4 with KOH). The pipette solution contained the following (mM): 110 K-Asp, 30 KCl, 1 EGTA, 3 Na_2ATP , 0.85 CaCl_2 , 10 glucose and 10 HEPES (pH 7.2 with KOH). Outward K^+ currents were elicited by a series of 400 ms depolarizing voltage steps. To measure current (I)–voltage (V) relationships, cells were held at -60 mV and then stepped to test potentials from -60 to $+80$ mV in 10 mV increments for 400 ms at each potential. To assess BK_{Ca} and voltage-dependent potassium channel (K_v) current amplitudes, outward K^+ currents were elicited in the absence and presence of 100 nM iberiotoxin (IbTX) or 3 mM 4-aminopyridine (4-AP).

Single-channel recording The pipette resistance ranged from 10 to 15 M Ω in the cell-attached and inside-out patch experiments. Recording pipette electrodes were fabricated from disposable micro-pipettes (BF150-86-10, Sutter Instrument, USA; ID: 0.86 mm, OD: 1.50 mm) in 2 stages on a vertical electrode puller (PC10, Narishige, Japan) to give resistances of 10–15 M Ω when filled with solution. The bath solution (PSS) and the pipette solution were identical and contained 45 mM KCl, 100 mM K-Asp,

1 mM EGTA, 10 mM HEPES and 5 mM glucose, adjusted to pH 7.4 with KOH. Pipettes were filled with 100 mM KCl, 45 mM K-Asp, 1 mM EGTA, 10 mM HEPES and 5 mM glucose, adjusted to pH 7.4 with KOH. As previously described (Shi *et al.* 2013), Ca²⁺ (CaCl₂) was added to achieve the desired level of free Ca²⁺ (determined using WinMAXC software; Chris Patton, Stanford University).

For single BK_{Ca} channel recording, as an index of channel steady-state activity, we used the product of the number of channels in the patch (*N*) and the channel open probability (*P*_o). The number of BK_{Ca} was estimated from the maximum observed current level at relatively high voltage and/or bath Ca²⁺ concentration. The *P*_o, amplitude and kinetic characteristics of the channels were analyzed with pCLAMP software (Clampfit 10.2); only recordings with stable *P*_o values for a minimum of 2 min were analyzed.

Voltage-dependent behavior of the channel *P*_o was modeled with the Boltzmann function:

$$P_o = 1 / \left\{ 1 + \exp \left[-ZF / RT (V - V_{1/2}) \right] \right\} \quad (1)$$

where *V*_{1/2} is the membrane potential for the half-maximal channel activation.

The Ca²⁺-dependent activation was fitted with the Hill equation:

$$P_o = [Ca^{2+}]_i^{\eta^H} / (K_d^{\eta^H} + [Ca^{2+}]_i^{\eta^H}) \quad (2)$$

where η^H is the Hill coefficient, and *K*_d is the dissociation constant.

Chemicals and statistics

All chemicals were purchased from Sigma-Aldrich unless otherwise stated. Statistical analysis was conducted using either one-way analysis of variance (ANOVA) followed by a Tukey's *post hoc* test for multiple comparisons or a paired Student's *t*-test; values of *P* < 0.05 were considered significant. Data are expressed as mean ± S.D.; *n* refers to the number of animals studied. Concentration–response curves were analyzed by computer-assisted nonlinear regression to fit the data using GraphPad Prism (GraphPad Software) to obtain –logIC₅₀ (pIC₅₀). Melatonin is first dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO was less than 0.1% that had no effects on currents.

Results

MT1 and MT2 receptor expression in MAs

Using immunofluorescence and confocal imaging, we examined the distribution of MT1 and MT2 receptors in

MAs. As shown in Fig. 1A, MT1 and MT2 receptors were detected in both endothelium and VSMCs.

Melatonin-induced vasodilation of MAs

In each MA ring, a high dose of potassium (60 mM KCl) was first applied to induce the maximal contraction (100% *K*_{max}). Then, 2 sets of experiments with or without L-NAME (100 μM) treatment were conducted in separate artery rings. In the absence of L-NAME (Fig. 1B), norepinephrine (NE, 10^{–5} M) induced a significant increase of vascular tone, 160.5 ± 7.6% *K*_{max} (*n* = 8). At the plateau of NE-induced contraction, melatonin (10^{–7}–10^{–3} M) was administered in half-log increments. As shown in Fig. 1B and E, melatonin induced dose-dependent vascular relaxation in MAs. Here, 10^{–5} M NE-induced maximal tension increase was treated as 100%. The pIC₅₀ value (negative logarithm of the molar concentration required to block the NE-induced contraction by 50%) in the melatonin-treated group was 4.75 ± 0.11 (*n* = 8, Fig. 1E). In the presence of L-NAME (Fig. 1C), the maximal NE-induced force was 182.8 ± 9.2% *K*_{max} (*n* = 8), which was higher than that in the absence of L-NAME (160.5 ± 7.6% *K*_{max}, *P* < 0.01). Notably, L-NAME by itself had no effect on basal tone, but it did significantly increase contractile responses to NE. In the presence of L-NAME (Fig. 1F), melatonin also elicited a concentration-dependent relaxation with pIC₅₀ = 3.93 ± 0.12 (*n* = 8, *P* < 0.01, vs (–) L-NAME). These results suggest that melatonin-induced vasorelaxation is partially due to the activation of eNOS.

In order to examine the role of the BK_{Ca} channel in melatonin-induced vascular relaxation, iberiotoxin (IbTX, a specific BK_{Ca} channel blocker, 10^{–8} M) was added to the bath to incubate the MA rings. In both L-NAME-treated and untreated groups, 10^{–8} M IbTX did not change the resting tension (Fig. 1C). However, after 2-min incubation with IbTX, a parallel rightward shift of the melatonin concentration–relaxation curve was detected in both L-NAME-treated and untreated groups, compared to those without IbTX incubation (Fig. 1B, C, E and F). The pIC₅₀ was 4.11 ± 0.09 in the (–) L-NAME+IbTX group, which was lower than that in the group without IbTX treatment ((–) L-NAME: pIC₅₀ = 4.75 ± 0.11, both *n* = 8, *P* < 0.01). The pIC₅₀ was 2.74 ± 0.13 in the (+) L-NAME+IbTX group, which was also lower than that in the group without IbTX treatment ((+) L-NAME: pIC₅₀ = 3.93 ± 0.12, *P* < 0.01, *n* = 8 in each group). It should be noted that following IbTX incubation, the melatonin concentration–relaxation curve was markedly rightward-shifted in the (+) L-NAME group (pIC₅₀ = 2.74 ± 0.13) compared to that of the

Figure 1

Expression of MT1/MT2 receptors and melatonin-induced vasodilation on MAs. (A) Immunofluorescence images of MT1 (red) and MT2 (green) receptors in MAs. The black and white pictures are the negative control (NC) images of MAs with secondary antibody only, no corresponding MT1 and MT2 receptor primary antibody. Scale bar = 20 μ m. (B, C and D) Representative tracings of the effect of melatonin on NE (10^{-5} M)-induced vasoconstriction in the absence (B) or presence (C) of L-NAME (100μ M), or in the denuded vessels (D). (a) Control; (b) IbTX-treated group; (c) Luz-treated group; (d) Luz + IbTX-treated group. L-NAME, non-selective nitric oxide synthase inhibitor N^G -nitro-L-arginine methyl ester; IbTX, ibuprofen (10^{-8} M), BK_{Ca} channel blocker; Luz, Luzindole (2×10^{-6} M), MT1/MT2 receptor blocker. Black triangles mark the time of addition of melatonin (10^{-7} , 10^{-6} , 3×10^{-6} , 10^{-5} , 3×10^{-5} , 10^{-4} , 3×10^{-4} , 10^{-3} M). (E, F and G) Cumulative concentration-response curve for effects of melatonin on NE-induced contraction. $n=8$ in each group. Results are expressed as change in NE-induced vascular tone increase (% NE).

non-L-NAME-treated group ($pIC_{50}=4.11\pm0.09$). These results indicate that in addition to the activation of eNOS, melatonin-induced vasorelaxation is partially attributable to its activation of the BK_{Ca} channels on vascular smooth muscle cells (VSMCs).

To determine whether the vasorelaxation induced by melatonin in MAs occurred through MT receptors, luzindole (MT1 and MT2 receptor blocker) was applied to the preparations before NE. As shown in Fig. 1B and C, in both the L-NAME-treated and untreated groups, 2×10^{-6} M luzindole did not change the resting tension. However, after 2-min incubation with luzindole, a parallel rightward shift of the melatonin concentration–relaxation curve was detected in both the L-NAME-treated and untreated groups, compared to those without luzindole incubation (Fig. 1E and F). The pIC_{50} was 3.55 ± 0.10 in the (–) L-NAME+Luz group, which was lower than that in the group without luzindole treatment ((–) L-NAME: $pIC_{50}=4.75\pm0.11$, both $n=8$, $P<0.01$). The pIC_{50} was 3.36 ± 0.12 in the (+) L-NAME+Luz group, which was also lower than that in the group without luzindole treatment ((+) L-NAME: $pIC_{50}=3.93\pm0.12$, $P<0.01$, $n=8$ in each group). These results indicate that melatonin-induced vasorelaxation may operate partially through the binding of melatonin to MT receptors. Unlike with IbTX, following luzindole incubation, the melatonin concentration–relaxation curve was not significantly shifted in the (+) L-NAME group ($pIC_{50}=3.36\pm0.12$) compared to that of the non-L-NAME-treated group ($pIC_{50}=3.55\pm0.10$). These results indicate that in melatonin-induced vasorelaxation, the eNOS-dependent part occurs through MT receptors. Therefore, if the MT receptors are blocked by luzindole, there is no significant difference between the (+) L-NAME and (–) L-NAME groups. Iberitoxin when combined with luzindole provided an additional inhibition of relaxation as compared to luzindole alone in either (–) L-NAME or (+) L-NAME group (Fig. 1B, C, E and F). Another implication of the results is that melatonin-induced partial vasorelaxation, which depends on myocyte BK_{Ca} channel activation, involves both MT receptor-mediated and non-MT receptor-mediated mechanisms.

Since endothelium-dependent component in the vascular bed also includes NO-independent endothelium-derived hyperpolarizing factor (EDHF) mechanism, the vasorelaxation effect of melatonin was also investigated on de-endothelized MAs. As shown in Fig. 1D and G, the

vasodilation induced by melatonin was not significantly different from that in (+) L-NAME group, which indicates that EDHF mechanisms may not be involved in melatonin-induced vasorelaxation of MAs.

Melatonin increases whole-cell BK_{Ca} currents in MA SMCs

To compensate for differences in cell size, membrane I_K is expressed relative to cell capacitance (pA/pF). To assess the effects of melatonin on BK_{Ca} or K_V components, the selective BK_{Ca} channel inhibitor IbTX (100 nM) or selective K_V channel inhibitor 4-AP (3 mM) was applied first to the cells, and then melatonin (10 and 100 μ M) was applied in the presence of IbTX or 4-AP (Fig. 2). As shown in Fig. 2A, B, C and D, either 4-AP or IbTX suppressed the currents. At the holding potential (HP) of +80 mV, the currents were inhibited by 4-AP to $72.05\pm5.60\%$ of control and by IbTX to $27.60\pm2.90\%$ of control, respectively ($n=12$ cells/6 rats), indicating that the outward K^+ currents consisted of at least two components: 4-AP-sensitive (K_V) and IbTX-sensitive ones (BK_{Ca}). After 4-AP incubation, the outward K^+ current density was significantly inhibited, and the following melatonin increased the left parts (mainly BK_{Ca} currents) significantly in a dose-dependent manner. At an HP of +80 mV, the peak BK_{Ca} current density was increased from 20.99 ± 2.20 pA/pF to 32.08 ± 3.10 pA/pF and 46.17 ± 3.80 pA/pF after 10 μ M and 100 μ M melatonin treatment (each $n=12$ cells/6 rats, both $P<0.01$), respectively (Fig. 2A and B). As shown in Fig. 2C and D, after IbTX pretreatment, the whole-cell I_K was markedly inhibited. The inhibited parts are IbTX-sensitive currents (BK_{Ca}), and the left parts are mainly K_V currents. However, the melatonin applied after IbTX pretreatment had no significant change on K_V currents. These data indicate that melatonin activates BK_{Ca} channels but not K_V channels.

To examine whether the activation of BK_{Ca} channels is through MT1/MT2 receptors, luzindole (2×10^{-6} M) was applied before melatonin. As shown in Fig. 2E and F, luzindole had no significant change on BK_{Ca} currents. At HP=+80 mV, after luzindole treatment, the peak BK_{Ca} current density was increased from 20.64 ± 2.13 pA/pF to 36.37 ± 3.51 pA/pF by 100 μ M melatonin. The increase by melatonin was significantly inhibited by luzindole compared to that of control (from 22.65 ± 2.40 pA/pF to 44.64 ± 3.50 pA/pF by 100 μ M melatonin, each $n=6$, $P<0.01$), suggesting that the activation of BK_{Ca} currents by melatonin was mediated by MT1/MT2 receptors.

Figure 2

Melatonin increases the whole-cell BK_{Ca} currents in MA SMCs. (A and C) Whole-cell I_K recorded in the presence of 4-aminopyridine (4-AP, 3 mM) or iberiotoxin (IbTX, 100 nM). Representative currents recorded in response to 400 ms voltage clamp steps from -60 to $+80$ mV in 10 mV steps. (B and D) Current-voltage relationships showing the effect of melatonin (10 μ M and 100 μ M) on the whole-cell I_K density in the presence of 4-AP or IbTX (each $n=12$ cells/6 rats). (E and F) The effects of luzindole on the melatonin-induced increase of the whole-cell BK_{Ca} currents in MA SMCs. All the whole-cell I_K was recorded in the presence of 4-AP (3 mM) together with vehicle (DMSO, 0.1%), luzindole (2×10^{-6} M), MT1/MT2 receptor blocker. See text for further details.

Figure 3

Melatonin increased the activity of BK_{Ca} channels from mesenteric arterial myocytes in cell-attached patch-clamp configuration. $[Ca^{2+}]_{free}$ in bath was 0.1μ M. The upward deflection indicates outward currents. (A) A schematic model showing the cell-attached patch-clamp configuration. (B) Representative recordings of single-channel currents at a membrane voltage of $+40$ mV in the presence of melatonin (left panel) or luzindole plus melatonin (right panel). Mel: melatonin, 0, 100, 300μ M; Luz: Luzindole, 2×10^{-6} M. Traces shown are 50 s (a) or 2 s (b) recordings. (C) Summary of the conductance, P_o , dwell time of open state and closed state ($n=8$). P_o : open probability. * $P < 0.01$ vs control (0μ M melatonin). # $P < 0.01$ vs 100μ M melatonin. \$ $P < 0.01$, Luz+melatonin treatment vs melatonin-only treatment with same concentration.

Activation of BK_{Ca} channel in MA SMC by melatonin in cell-attached patches

Using cell-attached patch-clamp recording (Fig. 3A), we examined the effect of melatonin on BK_{Ca} channel activity and gating properties. Figure 3B shows representative records of single BK_{Ca} channels under cell-attached configuration with symmetrical 145 mM K⁺ at a HP of +40 mV ([Ca²⁺]_{free} = 0.1 μM). Po increased along with membrane potential (data not shown). A linear fit revealed an average single-channel conductance (G) of 270.8 ± 24.5 pS (*n* = 8). Melatonin (100 and 300 μM) had no significant impact on the channel conductance (Fig. 3C).

Melatonin significantly increased the Po of BK_{Ca} channels in a concentration-dependent manner. For example, with 100 μM melatonin treatment, the Po (0.025 ± 0.003, *n* = 8) was significantly increased compared with that of the control (0.002 ± 0.002, *n* = 8, *P* < 0.01), whereas 300 μM melatonin increased the Po to 0.040 ± 0.005 (*n* = 8). To examine whether the activation of BK_{Ca} channels occurred through MT1/MT2 receptors, luzindole (2 × 10⁻⁶ M) was applied before melatonin. Luzindole significantly inhibited the increase of Po of BK_{Ca} channels induced by melatonin (100 μM melatonin: Po = 0.009 ± 0.001; 300 μM melatonin: Po = 0.030 ± 0.001; both *n* = 8, *P* < 0.01 vs control). Luzindole itself had no significant impact on the activity of BK_{Ca} channels; however, it could markedly inhibit the Po increase induced by melatonin. The conductance of BK_{Ca} was unaffected by luzindole.

We further examined the effects of melatonin on the gating properties of the BK_{Ca} channels in cell-attached patch configuration (*n* = 8 in each group). At the testing potential of +40 mV, the Po was significantly increased in the melatonin treatment group (Fig. 3B and C). The mean open time (To) of a single BK_{Ca} channel was increased in the 100 μM melatonin treatment group (10.80 ± 1.20 ms) compared with the control group (7.54 ± 0.85 ms), but not significantly changed in the 300 μM melatonin treatment group (9.70 ± 1.90 ms). The mean close time (Tc) was significantly decreased in both the 100 and 300 μM melatonin treatment groups (411.60 ± 34.50 ms and 337.90 ± 21.30 ms, respectively) compared with the control group (3531.95 ± 350.60 ms, *n* = 8 in each group). These data show that luzindole inhibits Po increase mainly by prolonging the closed state of BK_{Ca} channels.

Activation of BK_{Ca} channel by melatonin in inside-out patches

We also examined the effect of melatonin on BK_{Ca} channel activity and gating properties in an inside-out patch-clamp

configuration (Fig. 4). Since the BK_{Ca} activation induced by melatonin is much stronger in an inside-out patch-clamp configuration than in a cell-attached configuration, lower concentrations of melatonin (10, 30, 100 μM) were used here. Figure 4A shows representative records of single BK_{Ca} channels in an inside-out configuration at different voltages with symmetrical 145 mM K⁺ in the presence of 0.1 μM and 1 μM Ca²⁺ with or without melatonin (0, 10, 30, 100 μM). A linear fit revealed an average single-channel conductance (G) of 279.3 ± 45.0 pS ([Ca²⁺]_{free} = 0.1 μM, *n* = 8) and 305.5 ± 48.0 pS ([Ca²⁺]_{free} = 1 μM, *n* = 8), respectively. Melatonin (10, 30, 100 μM) had no significant effect on the channel conductance (Fig. 4B).

We further examined the effects of melatonin at different doses on the gating properties of the BK_{Ca} channels (Fig. 4B, *n* = 8 in each group). At the testing potential of +40 mV, the Po was significantly increased by melatonin in a concentration-dependent manner. When the [Ca²⁺]_{free} = 0.1 μM, the mean open time (To) of a single BK_{Ca} channel was longer in the melatonin group than in the control group (7.77 ± 0.80 ms (10 μM melatonin), 9.92 ± 1.20 ms (30 μM), 16.44 ± 1.82 ms (100 μM) vs 4.96 ± 0.40 ms (control); all *P* < 0.01). The Tc was shorter in the melatonin group than in the control group (208.10 ± 21.20 ms (10 μM melatonin), 155.80 ± 25.60 ms (30 μM), 51.46 ± 6.70 ms (100 μM) vs 251.20 ± 19.60 ms (control), all *P* < 0.01). In summary, melatonin treatment decreases Tc and increases To in a dose-dependent manner. When the [Ca²⁺]_{free} = 1 μM, melatonin treatment also decreases Tc and increases To, which is most prominent in 100 μM melatonin.

The influence of melatonin on the voltage and calcium dependence of the BK_{Ca} channels was also examined (Fig. 5). Notably, Po increased along with membrane potential (Fig. 5A). The NPo was normalized to the max probability. The Po–voltage relationships were fitted with the Boltzmann distribution to determine the voltage for half activation of the channel, *V*_{1/2} (equation described in ‘Materials and methods’ section). As can be seen from Fig. 5B, *V*_{1/2} was shifted from 54.78 ± 0.56 mV (control) to 44.47 ± 0.52 mV by 10 μM melatonin, and to 30.44 ± 0.48 mV by 100 μM melatonin (0.1 μM [Ca²⁺]_{free}). However, melatonin had no significant effect on the slope.

A similar leftward shift in Ca²⁺-dependent activation was observed with melatonin treatment. Figure 5C plots the relations of NPo normalized to their maximum value against [Ca²⁺]_{free}. The Po–Ca²⁺ curve fitted (using least squares) by Hill equation with a *K*_d value of 1.90 ± 0.10 (control), 1.35 ± 0.11 (10 μM Melatonin) and 0.32 ± 0.02 (100 μM Melatonin, *n* = 6 in each group), respectively.

Figure 4

Melatonin increased the activity of BK_{Ca} channels from mesenteric arterial myocytes in inside-out patch-clamp configuration. The upward deflection indicates outward currents. (A) Representative recordings of single-channel currents at a membrane voltage of +40 mV in the presence or absence of melatonin (0, 10, 30 and 100 μM). $[Ca^{2+}]_{free}$ in bath was 0.1 μM (left) or 1 μM (right). (B) Summary of the Conductance, Po, Dwell time of open state and closed state ($n=8$). Po: open probability. * $P < 0.01$ vs control (0 μM melatonin). # $P < 0.01$ vs 10 μM melatonin. \$ $P < 0.01$ vs 30 μM melatonin.

Figure 5

Effect of melatonin on voltage and calcium dependence of BK_{Ca} channels in mesenteric arterial myocytes in inside-out patch-clamp configuration.

(A) Representative recordings of single-channel currents in the presence of melatonin (100 μ M) at different membrane voltages (-10 to 50 mV). $[Ca^{2+}]_{free}$ in bath was 0.1 μ M. (B) Effect of melatonin (0, 10, 100 μ M) on voltage dependence of BK_{Ca} channels. The line was drawn according to the best fit with the Boltzmann equation (Eq. 1, $n=8$). (C) Effect of melatonin (0, 10 and 100 μ M) on calcium dependence of BK_{Ca} channels. HP = +40 mV. The data points were fitted with the Hill equation (Eq. 2) to obtain the calcium concentration necessary to open half of the channels (K_d) and the Hill coefficient (n_H).

(D) Luzindole has no significant effect on melatonin-induced increase of Po in inside-out patch-clamp configuration. HP = +40 mV, $[Ca^{2+}]_{free} = 0.1$ μ M. Vehicle: DMSO (0.1%); Luz: 2×10^{-6} M. (E) Bar plot summarizes luzindole on melatonin-induced increase of Po in inside-out patch-clamp configuration.

* $P < 0.01$ vs control; # $P < 0.01$, vs vehicle (a) or Luz (b). $n=6$ in each group.

The Hill coefficient (H) was 1.67 ± 0.12 (control), 1.57 ± 0.17 ($10 \mu\text{M}$ Melatonin) and 1.25 ± 0.11 ($100 \mu\text{M}$ Melatonin).

To rule out the direct effect of luzindole on single BK_{Ca} channels, we examined the effects of luzindole on single BK_{Ca} channels in inside-out configuration (Fig. 5D and E). Luzindole ($2 \times 10^{-6} \text{M}$) itself had no effect on single BK_{Ca} activity in inside-out configuration. After luzindole pretreatment, the Po increase induced by melatonin ($100 \mu\text{M}$) was not significantly changed.

Discussion

The results of this study demonstrate that (1) melatonin induces concentration-dependent vasodilation in MAs; (2) in addition to activating eNOS, melatonin significantly activates BK_{Ca} channels on the mesenteric arterial SMCs; (3) this enhancement of BK_{Ca} channel activity involves both direct and indirect (MT1/MT2 receptor-mediated) pathways. Thus, in MAs, vasodilation by melatonin depends on the activity of both endothelial factors and BK_{Ca} channels. These findings suggest a novel but important pathway of melatonin in regulating vascular tone: passing through the cell membrane and activating the BK_{Ca} channel directly.

Increasing evidence shows that melatonin exerts many of its physiological actions through interaction with MT1 and MT2 receptors, which belong to the G-protein-coupled family of membrane receptors (Pandipati *et al.* 2008, Tunstall *et al.* 2011). The different vascular effects observed with melatonin are attributed to the relative distribution of MT1 and MT2 receptors, since MT1 receptor activation causes vasoconstriction and MT2 receptor activation causes vasodilation (Doolen *et al.* 1998).

eNOS is primarily responsible for the generation of NO in the vascular endothelium (Fish *et al.* 2006). NO produced by eNOS in the vascular endothelium plays crucial roles in regulating vascular tone (Förstermann & Münzel 2006). Hence, a functional eNOS is essential for a healthy cardiovascular system. Once produced in ECs, NO diffuses across the VSMC membranes and activates the enzyme-soluble guanylate cyclase (sGC), which catalyzes the conversion of guanosine triphosphate into cyclic guanosine monophosphate (cGMP) (Denninger & Marletta 1999). cGMP, in turn, activates protein kinase G (PKG), which promotes multiple phosphorylation of cellular targets, lowering cellular Ca²⁺ concentrations and promoting vascular relaxation

(Cornwell *et al.* 1994). In this study, melatonin-induced vasodilation was significantly inhibited in the presence of L-NAME (eNOS inhibitor). Thus, vasodilation depends on the presence of NO released by the endothelium. This finding suggests that the endothelium (endothelial-derived NO) is a target for melatonin action. Melatonin increases NO availability (Paulis & Simko 2007) to induce vasodilation, which is consistent with previous studies (Girouard *et al.* 2001).

In another experiment, we found that pretreatment of the MAs with luzindole (MT1/MT2 receptor inhibitor) also significantly inhibited melatonin-induced vasorelaxation. This finding suggests that melatonin may act partially through MT receptors. However, the melatonin concentration–relaxation curve was not significantly shifted in the (+) L-NAME group ($pIC_{50} = 3.36 \pm 0.12$) compared with the non-L-NAME-treated group ($pIC_{50} = 3.55 \pm 0.10$). These results suggest that in melatonin-induced vasorelaxation, the eNOS-dependent part occurs mainly through MT receptors. Therefore, if the MT receptors are blocked by luzindole, there is no significant difference in pIC_{50} between the (+) L-NAME and (–) L-NAME groups.

In addition to the endothelium, BK_{Ca} channels on VSMCs also play an important role in vessel tone regulation (Bukiya *et al.* 2007). BK_{Ca} channels are mainly activated and hyperpolarize the membrane, which, in turn, suppresses the activity of the voltage-gated Ca²⁺ channels and thereby relaxes the artery (Nelson & Quayle 1995, Davis & Hill 1999). In the present study, IbTX caused a parallel rightward shift of the melatonin concentration–relaxation curve in both L-NAME-treated and untreated groups, which indicates that in addition to the activation of eNOS, melatonin-induced vasorelaxation is partially attributable to its activation of the BK_{Ca} channels on VSMCs. Another interesting thing to be noted is that the NO-dependent component of relaxation appears sensitive to iberiotoxin too (Fig. 1). As reported, BK_{Ca} channels are also detected in ECs in rat MAs, and the activation of BK_{Ca} channels on ECs also induces vasodilation (Jackson-Weaver *et al.* 2013). This may partially explain the sensitivity of NO-dependent component to iberiotoxin in the present study. Then, whether melatonin's action on BK_{Ca} channels in VSMCs was receptor mediated or non-receptor mediated was still unclear. We found that in the presence of L-NAME, luzindole ($2 \times 10^{-6} \text{M}$) had no significant effect on MAs, but suppressed the vasodilation induced by melatonin. If melatonin-induced BK_{Ca} activation were all through MT1/MT2 receptors, then

Figure 6

A model for a mechanistic explanation of vasodilation of melatonin on MAs. Both endothelium-dependent and non-endothelium-dependent pathways are involved in melatonin-induced vasodilation of MAs. In the endothelium-dependent pathway, by binding to MT1/MT2 receptors, melatonin enhances eNOS to reduce NO production, which induces partial vasodilation. In the non-endothelium-dependent pathway, melatonin causes vasodilation by activating BK_{Ca} channels on VSMCs either directly or indirectly (via MT1/MT2 receptors).

luzindole should abolish the vasodilation of melatonin in the presence of L-NAME. However, this was not the case. The melatonin concentration–relaxation curve was only right-shifted in the luzindole-treated group ($pIC_{50}=3.36\pm0.12$) compared with the control group ($pIC_{50}=3.93\pm0.12$). This finding indicates that melatonin-induced vasorelaxation dependent on myocyte BK_{Ca} channel activation involves not only MT receptor-mediated mechanisms, but also non-MT receptor-mediated mechanisms.

Previous studies have demonstrated that melatonin constricts cerebral arteries (Geary *et al.* 1997) *in vitro*, following G protein-dependent inhibition of Ca^{2+} -activated BK_{Ca} channels. By using an open-skull preparation in anesthetized rats, Régrigny also showed that melatonin directly constricts small-diameter cerebral arterioles in rats (Régrigny *et al.* 1999). This vasoconstriction effect is mediated by the inhibition of BK_{Ca} channels following activation of MT1 and/or MT2 receptors. Our results were not consistent with those findings. If melatonin inhibits BK_{Ca} channels on mesenteric arterial myocytes, then melatonin should induce vasoconstriction. However, we found just the opposite effect: melatonin induced vasodilation in MAs. IbTX pretreatment significantly inhibited the melatonin-induced relaxation. These data suggest that melatonin activates, rather than inhibits, BK_{Ca} channels on mesenteric arterial myocytes.

The mechanisms underlying melatonin-induced activation of BK_{Ca} channels in MA myocytes were further studied using electrophysiological studies. Whole-cell recording showed that melatonin significantly increased BK_{Ca} currents but not K_v currents. In previous studies, both activation and inactivation of potassium channels by melatonin have been reported. For example, it was found that 2-indomelatonin (a melatonin receptor agonist) increased BK_{Ca} channel activity in nonpregnant and pregnant rat uterine myocytes in the cell-attached patch-clamp configuration (Steffens *et al.* 2003). However, the effect on NPo was transient; 2 min after the addition

of 2-iodomelatonin, the open probability had returned to the basal value. However, in this study, we found that melatonin had a sustained, concentration-dependent activating effect on BK_{Ca} channels. A cell-attached patch clamp showed that melatonin significantly activated BK_{Ca} channels. Blocking MT1/MT2 receptors by luzindole significantly inhibited the NPo increase, which suggests that melatonin acts at least partially by binding to MT receptors. Our vascular contraction experiments supported this theory: MT receptor blocker inhibited melatonin-induced vasorelaxation.

Melatonin is a highly permeant neutral molecule that can pass through the cell membrane rapidly. Due to its highly lipophilic nature, melatonin passes through cell membranes with ease (Yu *et al.* 2016). We speculated that melatonin could act on BK_{Ca} channels directly. To evaluate this hypothesis, an inside-out patch clamp was conducted. The results clearly showed that melatonin could activate BK_{Ca} channels directly. Our vessel contractility experiments with luzindole also lent strong support to this hypothesis. In the presence of L-NAME, both luzindole and IbTX pretreatment caused a parallel rightward shift of the melatonin concentration–relaxation curve (Luzindole: $pIC_{50}=3.36\pm0.12$; IbTX: $pIC_{50}=2.74\pm0.13$) compared with the control group ($pIC_{50}=3.93\pm0.12$). IbTX shifted the curve more than luzindole, which suggests that melatonin-induced activation of BK_{Ca} channels occurs not only through binding to MT receptors but also through other means (non-receptor-mediated pathway). One possibility is that melatonin activates the BK_{Ca} channel directly, just as our inside-out patch-clamp study demonstrated. In both the cell-attached and inside-out patch-clamp configuration, melatonin displayed an alteration of the gating properties on BK_{Ca} channels by prolonging the time in the open state (T_o) and shortening the time in the closed state (T_c). In inside-out patch-clamp configuration, luzindole itself had no effect on single BK_{Ca} activity. Moreover, luzindole pretreatment did not change

the Po increase induced by melatonin, which can rule out the possibility that MT1/2 receptors are physically associated with BK_{Ca} channel similar to thromboxane receptor and other channels (Li *et al.* 2010). However, it is still difficult to determine whether such a compound acts inside or outside of the cell. Usually, a highly permeant compound is observed on both sides of the membrane at almost equal concentrations, whether it is applied inside the cell or outside the cell (Yu *et al.* 2016).

Based on these findings, we propose a model for a mechanistic explanation of the vasodilation of melatonin on MAs (Fig. 6). First, in addition to the enhancement of eNOS on the endothelium, melatonin activates BK_{Ca} channels on mesenteric arterial SMCs to induce vasodilation. Second, the activation of BK_{Ca} channels by melatonin involves direct (non-receptor-mediated) and indirect (receptor-mediated) mechanisms. Melatonin can pass through the cell membrane with ease and provides on-site activation of BK_{Ca} channels. As an inexpensive and well-tolerated drug, melatonin may be a new therapeutic option for cardiovascular diseases such as hypertension.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

L S and Y W participated in research design; T Z, H Z, C J and F Q conducted experiments; T Z, C J and F Q performed data analysis; T Z and L S wrote or contributed to the writing of the manuscript.

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