Melatonin potentiates contractile responses to serotonin in isolated porcine coronary arteries

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The rings were suspended in water-jacketed organ chambers filled with 25 ml of physiological salt solution. The solution was aerated with a mixture of 95% O2-5% CO2, and the temperature was maintained at 37°C throughout the experiment. Each ring was suspended by two fine stainless steel wire clips passed through the lumen; one clip was anchored inside the organ bath and the other was connected to a force transducer (model FT03, Grass Instruments, Quincy, MA). Isometric tension was measured and recorded on a Grass polygraph. The tissues were stretched progressively to the optimal point of their length-tension relationship by using KCl (20 mM) to generate a standard contractile response (10). After this procedure, the preparations were allowed to equilibrate at their optimal length for at least 30 min before being exposed to other vasoactive substances.

**Experimental protocols.** For contractile responses, concentration-response curves to melatonin (10⁻¹⁰⁻¹⁰⁻⁵ M), serotonin (10⁻²⁻¹⁰⁻⁷ M), and 9,11-dideoxy-11α,9α-epoxymethanol-PGF₂α (U-46619, 10⁻⁵⁻¹⁰⁻⁷ M) were obtained in resting coronary arterial rings with and without endothelium. The concentration-response curves to serotonin and U-46619 were obtained in the absence and presence of melatonin (10⁻⁷ M), which was added to the organ chamber immediately before the addition of the contractile agonist. This melatonin was selected because in preliminary experiments it produced the greatest potentiating effect on contractile responses in isolated pig coronary arteries, which is in agreement with previous studies in other blood vessels (29, 49). Potentiation was also observed with lower [melatonin] in this preparation, but the results were inconsistent. [Melatonin] > 10⁻⁷ M had no further effect, which is in agreement with previous findings (29, 49).

In some experiments the preparations were incubated with the melatonin-receptor antagonist N-[2-naphth-1-yl-ethyl]-cyclobutyl carboxamide (S-20928, 10⁻⁶ M) (58) for 30 min before exposure to melatonin. In a separate series of experiments, concentration-response curves to serotonin and U-46619 were obtained in the absence and presence of melatonin (10⁻⁷ M), which was added to the organ chamber immediately before the addition of the contractile agonist. This melatonin was selected because in preliminary experiments it produced the greatest potentiating effect on contractile responses in isolated pig coronary arteries, which is in agreement with previous findings (29, 49). Potentiation was also observed with lower [melatonin] in this preparation, but the results were inconsistent. [Melatonin] > 10⁻⁷ M had no further effect, which is in agreement with previous findings (29, 49).

In some experiments the preparations were incubated with the melatonin-receptor antagonist N-[2-naphth-1-yl-ethyl]-cyclobutyl carboxamide (S-20928, 10⁻⁶ M) (58) for 30 min before exposure to melatonin. In a separate series of experiments, concentration-response curves to serotonin in the absence and presence of melatonin were obtained in the presence of the soluble guanylyl cyclase inhibitors methylene blue (10⁻⁵ M) (32) and 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 10⁻⁵ M) (18) or the inhibitor of nitric oxide (NO) synthases (NOS), N⁵-nitro-L-arginine (L-NNA, 3 × 10⁻⁵ M) (36). In these experiments, the inhibitors were added to the organ chamber 20 min before exposure to serotonin and remained in contact with the tissues throughout the remainder of the experiment.

Relaxation of coronary arteries was studied in rings contracted with U-46619 (1–3 × 10⁻⁹ M). After the U-46619-induced contraction had reached a stable plateau, increasing concentrations of the following drugs were added to the bath solution: melatonin (10⁻¹⁰⁻¹⁰⁻⁵ M), sodium nitroprusside (10⁻⁵⁻¹⁰⁻⁷ M), isoprotrenal (10⁻⁵⁻¹⁰⁻⁷ M), or serotonin (10⁻⁶⁻¹⁰⁻⁵ M). The experiments with serotonin were performed in the presence of the 5-HT₁ receptor antagonist ketanserin (10⁻⁶ M) to inhibit the direct contractile effect of serotonin on coronary smooth muscle (43). Concentration-response curves to sodium nitroprusside, serotonin, and isoproterenol were obtained in the absence and presence of melatonin (10⁻⁷ M), which was added to the organ bath immediately before exposure to the vasodilator. After completion of each concentration-response curve, papaverine (10⁻⁴ M) was added to the preparations to ensure maximal relaxation of the tissues.

In all experiments concentration-response curves were obtained by increasing the drug concentration in the organ chamber in a cumulative manner by approximately threefold, after the response to the previous concentration had been allowed to reach its maximum (52). Control and treated rings from the same animal were studied in parallel. Only one concentration-response curve was obtained in each blood vessel ring.

**Data analysis.** Contractile responses were normalized by expressing them as a percentage of the contraction evoked by KCl (60 mM), which was added to the organ chamber at the conclusion of the experiment. Relaxations were expressed as a percentage of the initial increase in isometric tension induced by U-46619. The data were quantified by determining both the maximal effect (E₅₀ max) and the concentration of the agonist necessary to produce 50% of its own maximal response (EC₅₀). The EC₅₀ values were converted to negative logarithms and expressed as −log molar EC₅₀. Results are expressed as means ± SE, and n refers to the number of animals from which blood vessels were taken. Values were compared by Student’s t-test for paired or unpaired observations and were considered to be significantly different when P < 0.05.

**Drugs and solutions.** The following drugs used in this study were obtained from Sigma Chemical (St. Louis, MO): bradykinin, dl-isoproterenol hydrochloride, melatonin, methylene blue, L-NNA, papaverine hydrochloride, serotonin creatinine sulfate, and sodium nitroprusside. Ketanserin tartrate was obtained from Janssen Pharmaceuticals (Beersse, Belgium); ODQ was from Tocris Cookson (Ballwin, MO); S-20928 was from Institut de Recherches Internationales Servier (Courbevoie, France); and U-46619 was from Upjohn (Kalamazoo, MI). Drug solutions were prepared daily, kept on ice, and protected from light until used. All drugs were dissolved initially in distilled water with the exception of melatonin, which was dissolved in ethanol, and ODQ and S-20928, which were dissolved in DMSO before further dilution in distilled water. Drugs were added to the organ chambers in volumes not greater than 0.2 ml. Drug concentrations are reported as final molar concentrations in the organ chamber. The composition of the physiological salt solution was as follows (in mM): 118.3 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, 25.0 NaHCO₃, and 11.1 glucose.

**RESULTS**

**Direct effect of melatonin.** Melatonin (10⁻¹⁰⁻¹⁰⁻⁵ M) itself had no direct contractile effect on quiescent coronary arteries, nor did the hormone cause relaxation of tissues contracted with the thromboxane A₂ analog U-46619 (data not shown). The results were similar in coronary arteries with and without endothelium.

**Effect of melatonin on coronary vasoconstrictors.** Concentration-response curves to the coronary vasoconstrictors serotonin and U-46619 were obtained in the absence and presence of melatonin (10⁻⁷ M). In rings with endothelium, the maximal contractile response to serotonin was increased in the presence of melatonin (10⁻⁷ M) (Fig. 1A), whereas the concentration-response curve for U-46619 was unaffected under these same conditions (Fig. 2). Removal of the endothelium shifted the concentration-response curve for serotonin to the left and abolished the potentiating effect of melatonin on serotonin-induced contractions (Fig. 1B). In the presence of the melatonin-receptor antagonist S-20928 (10⁻⁷ M), melatonin had no potentiating effect on the concentration-response curve for serotonin in coronary arteries with intact endothelium (Fig. 3). The
antagonist itself did not affect the concentration-response curve for serotonin (data not shown).

Concentration-response curves to serotonin in the absence and presence of melatonin (10^{-7} M) were also obtained in coronary arteries with endothelium treated with L-NNA (3 \times 10^{-5} M) or ODQ (10^{-5} M). Similar to the results obtained in rings in which the endothelium had been removed, the concentration-response curve for serotonin was shifted to the left in the presence of either L-NNA or ODQ compared with the responses obtained under control conditions (i.e., Fig. 1A), and the potentiating effect of melatonin on serotonin-induced contractions was abolished (Fig. 4). Likewise, melatonin had no potentiating effect on the concentration-response curve for serotonin in arteries with endothelium treated with methylene blue (10^{-5} M) (data not shown).

Effect of melatonin on coronary vasodilators. In the presence of ketanserin (10^{-6} M), serotonin caused concentration-dependent relaxations of coronary arteries with endothelium contracted with U-46619 (Fig. 5).
The endothelium-dependent relaxations to serotonin were reduced significantly in the presence of melatonin (10^{-7} M) (Fig. 5). In coronary arteries without endothelium contracted with U-46619 (10^{-9} M), sodium nitroprusside caused concentration-dependent relaxations that were also inhibited by melatonin (10^{-7} M) (Fig. 6). The log molar EC_{50} values for sodium nitroprusside were 7.42 ± 0.19 versus 6.70 ± 0.27 (P < 0.05) in the absence and presence of melatonin (10^{-7} M), respectively.

Isoproterenol caused concentration-dependent relaxations of coronary arterial rings contracted with U-46619 (−log molar EC_{50} = 7.62 ± 0.20; E_{max} = 99.5 ± 0.5). The addition of melatonin (10^{-7} M) to the organ chamber had no significant effect on the concentration-response curve for isoproterenol (−log molar EC_{50} = 7.53 ± 0.10; E_{max} = 99.3 ± 0.7; n = 4).

**DISCUSSION**

Although considerable progress has been made regarding the neurobiology of melatonin (50), its role in the cardiovascular system is poorly understood. The discovery of receptors for melatonin in mammalian blood vessels suggests that the hormone may be involved in controlling vasomotor tone (44, 45, 55). Indeed, melatonin either causes or potentiates vasocon-
striction in certain vascular beds. For example, in rat cerebral arteries, melatonin causes vasoconstriction and reduces cerebral blood flow (9, 19, 56). In the caudal artery of the same species, melatonin does not directly cause contraction of the smooth muscle but it potentiates vasoconstrictor responses to norepinephrine and adrenergic nerve stimulation (29, 55). The results of the present study demonstrate that the coronary circulation is also a site of action for the vasoconstrictor-potentiating effect of melatonin, an effect that is mediated by receptors sensitive to inhibition by the melatonin-receptor antagonist S-20928 (58).

A novel finding of this study is that the potentiating effect of melatonin in coronary arteries is selective for contractions elicited by serotonin, inasmuch as the contractile response to U-46619, a thromboxane A₂ analog, was unaffected by the hormone. Moreover, the effect of melatonin on contractions evoked by serotonin was observed only in coronary arteries with an intact endothelium. These results most likely relate to the role of the endothelium in modulating serotonin-induced constriction of coronary arteries (10). Both U-46619 and serotonin act directly on vascular smooth muscle cells to cause contraction, but serotonin, unlike U-46619 (12), also causes endothelium-dependent relaxation via the release of NO (41, 54). In porcine coronary arteries, these opposing effects of serotonin are mediated by different serotonin receptor subtypes. The direct contractile effect of serotonin is mediated by 5-HT₁D receptors located on the smooth muscle cells (13), whereas NO release is mediated by 5-HT₂A-like receptors found on the coronary endothelial cells (42). Thus, in coronary arteries with endothelium, the direct contractile response to serotonin is suppressed by the indirect inhibitory effect of NO on the smooth muscle (10, 12). Indeed, the ability of the endothelium to modify serotonin-induced contractions in this preparation is evident from the increased responsiveness of rings without endothelium to serotonin observed in this study and others (12). That the synergistic effect of melatonin on serotonin-induced contractions occurred only in coronary arteries with endothelium suggests that the potentiating effect of the hormone may involve impairment of the NO pathway (i.e., decreased synthesis or release of NO or inhibition of its action on vascular smooth muscle). Such a mechanism would also explain the lack of potentiating effect of melatonin on the response to U-46619, because the thromboxane A₂ analog does not release endothelium-derived NO in coronary arteries (12).

The hypothesis that melatonin potentiates serotonin-induced contractions by inhibiting the NO pathway is supported by those experiments in which the synergistic effect of melatonin was abolished in the presence of either ODQ or l-NNA. By inhibiting the soluble form of guanylyl cyclase (18), ODQ inhibits the action of NO at the level of the vascular smooth muscle, whereas the NOS inhibitor l-NNA (36) prevents the formation of NO in endothelial cells. Thus, in tissues treated with ODQ or l-NNA, the inhibitory effect of endothelium-derived NO on the contractile response to serotonin is negated. If the synergistic effect of melatonin on serotonin-induced contractions is indeed due to decreased vascular relaxation by NO, then, consistent with the observations reported in this study, melatonin would not be expected to have an effect in the presence of either of these inhibitors. Further support for this hypothesis is provided by the experiments in which melatonin inhibited endothelium-dependent relaxations to serotonin, which are mediated solely by NO in porcine coronary arteries (41, 54). This effect of melatonin was not due to a nonspecific inhibitory action of the hormone, because the response to isoproterenol, which acts independently of NO, was unaffected by exposure to melatonin.

There are several potential mechanisms by which melatonin could interfere with the NO pathway and thus potentiate serotonin-induced contractions in coronary arteries. One possibility is that melatonin may inhibit NOS (3, 31, 40). Although an effect of melatonin on NOS in coronary arteries cannot be ruled out under the present experimental conditions, the inhibitory effect of melatonin on relaxations to sodium nitroprusside, which serves as an exogenous NO donor and is not dependent on NOS (28), suggests a site of action other than or in addition to inhibition of NOS. Alternatively, melatonin could act by attenuating the action of NO at the level of the vascular smooth muscle. NO relaxes vascular smooth muscle by increasing intracellular cGMP levels (23, 38) and by activating potassium channels in the cell membrane (2, 4). Recent evidence suggests that melatonin prevents increased cGMP accumulation in several cell types (24, 31, 39, 51) and that the hormone has potassium channel-blocking properties (19, 20). Thus it is likely that melatonin may potentiate serotonin-induced vasoconstriction by inhibiting the action of NO on coronary vascular smooth muscle cells rather than by inhibiting the release of NO from endothelial cells (20).

The results of the present study suggest that melatonin may play a role in regulating coronary vasomotor tone. The [melatonin] in blood rises during the night and falls to nearly undetectable levels during the day. Circulating peak plasma [melatonin] generally ranges from 0.5–1 nM (27), concentrations that are lower than those used in this study. However, because of its high lipophilicity, tissue [melatonin] may exceed plasma [melatonin]. Moreover, several extrapineal sources of melatonin have now been identified, including mast cells, leukocytes, platelets, and, particularly relevant to the present study, endothelial cells (16, 30). This further increases the potential for elevations in the local tissue [melatonin]. Indeed, [melatonin] two to three orders of magnitude higher than those typically found in blood have been reported in bile and bone marrow (47, 48). Elevated levels of melatonin may increase the sensitivity of the coronary arteries to serotonin, a putative mediator in coronary vasospasm and unstable angina (21, 34), and thus contribute to episodes of myocardial ischemia. Given the considerable interindividual variation in the pattern of melatonin release, further studies will be needed to deter-
mine the significance of this effect of melatonin in relation to the circadian variations in melatonin levels and acute cardiac events.

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REFERENCES


18. Garthwaite J, Southam E, Boulton CL, Nielsen EB, Schmidt K, and Mayer B. Potent and selective inhibition of nitric oxide-sensitive guanylyl cyclase by 1H-[1,2,4]oxadiazo-


