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Melatonin inhibits nitrate tolerance in isolated coronary arteries

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1 The present study was designed to test the hypothesis that melatonin inhibits nitrate tolerance in coronary arteries.

2 Rings of porcine coronary arteries were suspended in organ chambers for isometric tension recording. Nitrate tolerance was induced by incubating the tissues with nitroglycerin (10^{-4} M) for 90 min, followed by repeated rinsing for 1 h. Control rings that had not been exposed previously to nitroglycerin, but were otherwise treated identically, were studied simultaneously. The rings were contracted with U46619 ($1-3\times10^{-9}$ M) and concentration–response curves to nitroglycerin (10^{-9}–10^{-4} M) were obtained.

3 Nitrate tolerance was evident by a 15- to 20-fold rightward shift in the concentration–response curve to nitroglycerin in rings with and without endothelium exposed previously to the drug for 90 min. Addition of melatonin (10^{-9}–10^{-7} M) to the organ chamber during the 90-min incubation period with nitroglycerin partially inhibited nitrate tolerance in coronary arteries with intact endothelium; however, melatonin had no effect on nitrate tolerance in coronary arteries without endothelium.

4 The effect of melatonin on nitrate tolerance in coronary arteries with endothelium was abolished by the melatonin receptor antagonist, S20928 (10^{-6} M). In contrast to melatonin, the selective MT_3-melatonin receptor agonist, 5-MCA-NAT (10^{-8}–10^{-7} M), had no effect on nitrate tolerance in coronary arteries.

5 The results demonstrate that melatonin, acting *via* specific melatonin receptors, inhibits nitrate tolerance in coronary arteries and that this effect is dependent on the presence of the vascular endothelium.

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Abbreviations: EC_{50}, concentration necessary to produce 50% of its own maximal response; E_{max}, maximal effect; 5-MCA-NAT, 5-methoxycarbonylamino-*N*-acetyltryptamine; MT-receptor, melatonin receptor; NO, nitric oxide; S20928, *N*-[2-napth-1-yl-ethyl]-cyclobutyl carboxamide; U46619, 9,11-dideoxy-11α,9α-epoxymethano-prostaglandin $F_{2\alpha}$

Introduction

Nitroglycerin is widely used in the treatment of ischemic heart disease, but its long-term clinical usefulness is limited by the development of tolerance (Parker & Parker, 1998). Although the mechanisms underlying nitrate tolerance are not yet fully understood, diminished blood vessel responsiveness to the vasodilator effect of nitroglycerin is a characteristic feature of this phenomenon (Molina *et al*., 1987; Kukovetz & Holzmann, 1990; Munzel *et al*., 1995). Nitroglycerin and other nitrovasodilators exert their inhibitory effect on vascular smooth muscle cells *via* the formation of nitric oxide (NO), which activates soluble guanylyl cyclase and increases intracellular levels of cyclic GMP (Ignarro *et al*., 1981; Murad, 1986). Although considerable evidence indicates that vascular tolerance to nitroglycerin is ultimately associated with an impairment of one or more steps in the NO/guanylyl cyclase/cyclic GMP cascade (Molina *et al*., 1987; Kukovetz & Holzmann, 1990), it is not yet clear to what extent activation of these same pathways plays a causal role in the development of nitrate tolerance. Thus, efforts have been made to prevent nitrate tolerance with inhibitors of the NO/guanylyl cyclase/cyclic GMP pathway (De la Lande *et al*., 1999), while potentiators of this pathway have been used to reverse nitrate tolerance (Kim *et al*., 2001). Increased oxidative stress may also contribute to the development of nitrate tolerance and several antioxidants have demonstrated at least partial efficacy as inhibitors of this phenomenon (Munzel *et al*., 1995; 1996; Bassenge *et al*., 1998; Fink *et al*., 1999).

Melatonin, the primary hormone secreted by the pineal gland, alters vasomotor tone in a variety of blood vessels (Viswanathan *et al*., 1990; Evans *et al*., 1992; Geary *et al*., 1997; Mahle *et al*., 1997). The vascular effects of melatonin are mediated, in part, by inhibition of NO signaling pathways and by protection against oxidative stress (Geary *et al*., 1998; Okatani *et al*., 1999; Wakatsuki & Okatani, 2000; Yang *et al*., 2001), properties that may be beneficial in preventing nitrate tolerance. Thus, the present study was designed to test the hypothesis that melatonin prevents tolerance to the vasorelaxant effects of nitroglycerin.

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Methods

Tissue preparation

Fresh porcine hearts were obtained from a local abbatoir and were immediately immersed in cold physiological salt solution. After transfer to the laboratory, the left anterior descending coronary artery was dissected free from surrounding myocardium, cleaned of adherent fat and connective tissue, and cut into rings 4–5 mm in length. Four to eight coronary arterial rings were prepared from each heart. In some rings, the endothelium was removed by gently rubbing the intimal surface with a fine forceps. The absence or presence of endothelial cells was confirmed in each preparation by the absence or presence of relaxation to the endothelium-dependent vasodilator, bradykinin (10^{-7} M).

Coronary arterial rings were suspended in water-jacketed organ chambers filled with 25 ml of physiological salt solution. The organ chamber solution was aerated with a mixture of 95% O_2/5% CO_2 and the temperature was maintained at 37°C throughout the experiment. Each ring was suspended by means of two fine stainless-steel wire clips passed through the lumen; one clip was anchored inside the organ chamber, the other connected to a force transducer (Model FT03, Grass Instrument Company, Quincy, MA, U.S.A.). Isometric tension was measured and recorded on a Grass polygraph. The tissues were stretched progressively to the optimal point of their length–tension relationship (approximately 10 g), using KCL (20 mM) to generate a standard contractile response. After this procedure, the preparations were allowed to equilibrate at their optimal length for at least 30 min prior to further exposure to any vasoactive substances.

Induction of nitrate tolerance

At the end of the equilibration period, the preparations were rendered tolerant to nitroglycerin by a method established previously (O'Rourke, 1996). Briefly, adjacent rings prepared from the same coronary artery were incubated with nitroglycerin (10^{-4} M) for 90 min, followed by repeated rinsing with physiological salt solution every 10 min for 1 h. In each experiment, control rings that were not exposed previously to nitroglycerin, but were otherwise treated identically, were studied simultaneously with those exposed to nitroglycerin.

Relaxation studies

After the 1 h washout period, the tissues were contracted with the thromboxane A_2-mimetic, U46619 ($1-3\times10^{-9}$ M). When the contractions had reached a stable plateau, relaxation responses to nitroglycerin (10^{-9}–10^{-4} M) were obtained. Cumulative concentration–response curves were obtained in a stepwise manner by increasing the concentration of the nitrovasodilator in the organ chambers by approximately three-fold. At the end of each experiment, papaverine (10^{-4} M) was added to the organ chamber in order to produce maximal relaxation of the tissues. Only one concentration–response curve to nitroglycerin was obtained from each arterial ring.

In some experiments either melatonin itself (10^{-10}–10^{-6} M) or the selective MT_3-receptor agonist (Molinari *et al*., 1996; Dubocovich *et al*., 2000), 5-MCA-NAT (10^{-8}–10^{-7} M) was added to the organ chamber during the 90-min incubation period with nitroglycerin. In addition, some rings were treated with the melatonin receptor antagonist (Ying *et al*., 1996), S20928 (10^{-6} M), which was added to the organ chamber 20 min prior to the addition of melatonin, and remained in contact with the tissues throughout the 90-min coincubation period with nitroglycerin plus melatonin. At the conclusion of the coincubation period with nitroglycerin, all tissues treated with melatonin or melatonin-receptor ligands were washed for 1 h as described above.

Data analysis

Relaxation responses are expressed as a percentage of the initial tension induced by U46619. For each vasodilator, both the maximal decrease in tension (E_{max}) and the concentration necessary to produce 50% of its own maximal response (EC_{50}) were determined. The EC_{50} values were converted to the negative logarithms and expressed as $-\log$ molar EC_{50}. Results are expressed as means $\pm$ s.e.m. and n refers to the number of animals from which blood vessels were taken. Agonist potencies and maximal effects were compared by Student's t-test or analysis of variance. Values were considered to be significantly different when $P<0.05$.

Drugs and solutions

The following drugs were used: bradykinin, isoproterenol, melatonin, papaverine hydrochloride (Sigma Chemical Co., St Louis, MO, U.S.A.); cromakalim, 5-MCA-NAT (5-methoxycarbonylamino-*N*-acetyltryptamine; Tocris, Ellisville, MO, U.S.A.); nitroglycerin (Parke-Davis, Morris Plains, NJ, U.S.A.); S20928 (*N*-[2-napth-1-yl-ethyl]-cyclobutyl carboxamide; Institut de Recherches Internationales Servier, Courbevoie, France) and U46619 (9,11-dideoxy-11α,9α-epoxymethano-prostaglandin $F_{2\alpha}$; Pharmacia & Upjohn, Kalamazoo, MI, U.S.A.). 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 cromakalim, melatonin and 5-MCA-NAT, which were dissolved in ethanol, and S20928, which was dissolved in dimethylsulfoxide, 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 concentration in the organ chamber. The composition of the physiological salt solution was as follows (in mM): NaCl 118.3, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.0, and glucose 11.1.

Results

Effect of melatonin on nitrate tolerance in coronary arteries with endothelium

Nitroglycerin caused concentration-dependent relaxation of isolated porcine coronary arteries with endothelium contracted by U46619 (Figure 1). In control preparations not previously exposed to nitroglycerin, the $-\log$ (M) EC_{50} for nitroglycerin was 7.46 ± 0.14. Prior exposure of coronary rings to nitroglycerin (10^{-4} M) for 90 min caused a significant rightward shift in the concentration–response curve to nitroglycerin ($-\log$ (M) $EC_{50}=6.11\pm0.12$; $P<0.05$) (Figure 1). The magnitude of the

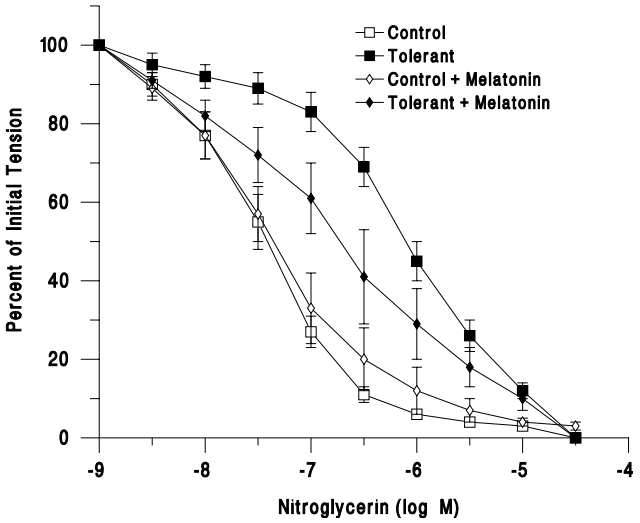

Figure 1 Effect of melatonin on nitrate tolerance in isolated porcine coronary arteries with endothelium. The tissues were incubated without (control) or with (tolerant) nitroglycerin (10^{-4} M) for 90 min, in the absence or presence of melatonin (10^{-9} M). Following the incubation period, the rings were rinsed for 1 h and then contracted with U46619 ($1-3\times10^{-9}$ M) before cumulative addition of nitroglycerin. The data are expressed as a percentage of the U46619-induced increase in tension, which averaged 11.5 ± 1.0 g in control rings and did not differ significantly in rings incubated with nitroglycerin or melatonin. Each point represents the mean$\pm$s.e.m.; $n=6$. As compared with control rings, there was a significant rightward shift in the nitroglycerin concentration–response curve in nitrate-tolerant rings ($P<0.05$). The effect of nitrate tolerance on the concentration–response curve to nitroglycerin was significantly reduced in tolerant rings exposed previously to melatonin ($P<0.05$).

shift in the concentration–response curve to nitroglycerin under these conditions was approximately 22-fold, indicating the development of nitrate tolerance in isolated coronary arteries.

Treatment of the tissues with melatonin (10^{-9} M) during the nitrate tolerance induction phase (i.e. coincubation with melatonin and nitroglycerin for 90 min, followed by a 1-h washout period) had no effect on the response to nitroglycerin in control rings (−log (M) $EC_{50}=7.31\pm0.25$; $P>0.05$ vs control without melatonin) but partially reduced the inhibitory effect of prior exposure to nitroglycerin on the concentration–response curve to nitroglycerin (Figure 1). Under these conditions, the −log (M) EC_{50} for nitroglycerin was 6.69 ± 0.27 ($P<0.05$ vs nitrate tolerant without melatonin), resulting in only a six-fold shift in the nitroglycerin concentration–response curve. Similar results were also obtained when the tissues were incubated with either melatonin (10^{-8} M) or (10^{-7} M) during the nitrate tolerance induction phase, whereas melatonin (10^{-10} M) failed to inhibit nitrate tolerance (data not shown). Treatment of the tissues with concentrations of melatonin greater than 10^{-7} M had no further inhibitory effect on the development of nitrate tolerance in isolated coronary arteries with endothelium. Melatonin ($10^{-10}-10^{-6}$ M) itself had no direct contractile effect on quiescent coronary arteries, nor did the hormone cause relaxation of tissues contracted with the thromboxane A_2 analog, U46619 (Yang *et al*., 2001). These results were similar in coronary arteries with and without endothelium.

When the melatonin receptor antagonist, S20928 (10^{-6} M), was added to the organ chamber prior to the 90-min coincubation period with melatonin and nitroglycerin, melatonin had no significant effect on the development of nitrate tolerance in coronary arteries with endothelium (Figure 2). Prior exposure to S20928 itself had no effect on the response to nitroglycerin in control or nitrate-tolerant tissues.

In a similar series of experiments, the selective MT_3-receptor agonist, 5-MCA-NAT ($10^{-8}-10^{-7}$ M) was added in place of melatonin to the organ chamber during the incubation period with nitroglycerin (10^{-4} M). Unlike melatonin, treatment of the tissues with 5-MCA-NAT during the nitrate tolerance induction phase failed to reduce the inhibitory effect of prior exposure to nitroglycerin on the subsequent concentration–response curve to nitroglycerin in coronary arteries with endothelium (Figure 3).

Effect of melatonin on nitrate tolerance in coronary arteries without endothelium

Nitroglycerin also caused concentration-dependent relaxation of isolated porcine coronary arteries without endothelium contracted with U46619 (Figure 4). In preparations not previously exposed to nitroglycerin, the −log (M) EC_{50} for nitroglycerin was 7.67 ± 0.24. As was observed in coronary arteries with intact endothelium, nitroglycerin-induced relaxation was significantly impaired in coronary rings without endothelium previously exposed to nitroglycerin (10^{-4} M) for 90 min (Figure 4). Under these conditions, the nitroglycerin

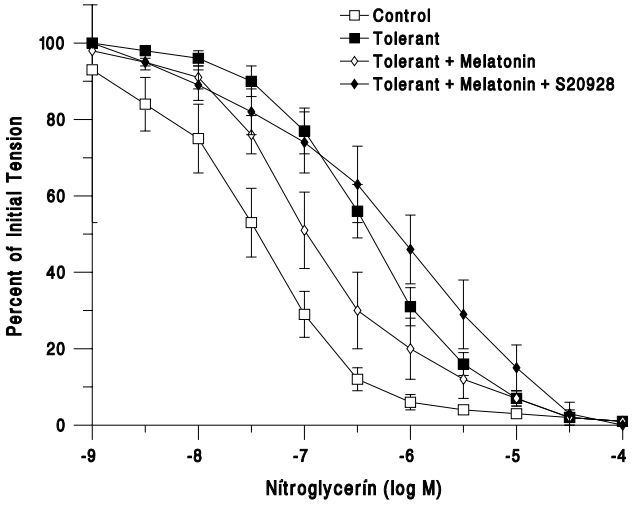

Figure 2 Effect of melatonin, in the absence and presence of S20928, on nitrate tolerance in isolated porcine coronary arteries with endothelium. The tissues were incubated without (control) or with (tolerant) nitroglycerin (10^{-4} M) for 90 min, in the absence or presence of melatonin (10^{-7} M). S20928 (10^{-6} M) was added to the organ chambers 20 min prior to the addition of melatonin. Following the incubation period, the rings were rinsed for 1 h and then contracted with U46619 ($1-3\times10^{-9}$ M) before cumulative addition of nitroglycerin. The data are expressed as a percentage of the U46619-induced increase in tension, which averaged 11.6 ± 1.1 g in control rings and did not differ significantly in rings incubated with nitroglycerin or melatonin. Each point represents the mean$\pm$s.e.m.; $n=5$. As compared with control rings, there was a significant rightward shift in the nitroglycerin concentration–response curve in nitrate-tolerant rings ($P<0.05$). The effect of nitrate tolerance on the concentration–response curve to nitroglycerin was significantly reduced in tolerant rings exposed previously to melatonin ($P<0.05$), but not in tolerant rings exposed previously to melatonin plus S20928.

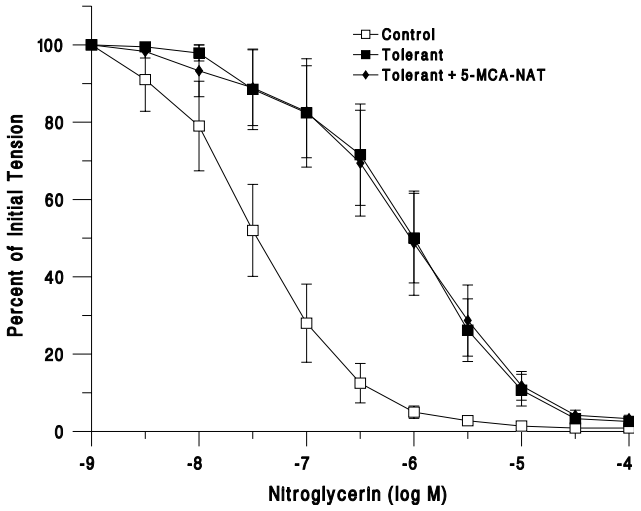

Figure 3 Effect of 5-MCA-NAT on nitrate tolerance in isolated porcine coronary arteries with endothelium. The tissues were incubated without (control) or with (tolerant) nitroglycerin (10^{-4} M) for 90 min, in the absence or presence of 5-MCA-NAT (10^{-7} M). Following the incubation period, the rings were rinsed for 1 h and then contracted with U46619 ($1-3\times10^{-9}$ M) before cumulative addition of nitroglycerin. The data are expressed as a percentage of the U46619-induced increase in tension, which averaged 12.1 ± 2.4 g in control rings and did not differ significantly in rings incubated with nitroglycerin or 5-MCA-NAT. Each point represents the mean $\pm$ s.e.m.; $n=6$. As compared with control rings, there was a significant rightward shift in the nitroglycerin concentration–response curve in nitrate-tolerant rings incubated with or without 5-MCA-NAT ($P<0.05$). Similar results were obtained with 5-MCA-NAT (10^{-8} M) (not shown for the sake of clarity).

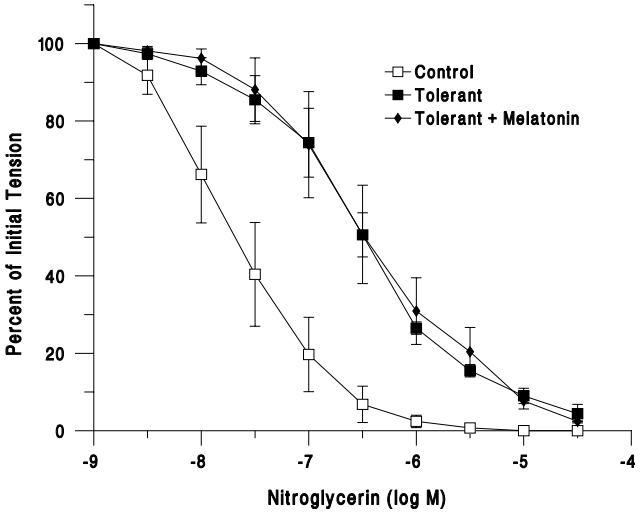

Figure 4 Effect of melatonin on nitrate tolerance in isolated porcine coronary arteries without endothelium. The tissues were incubated without (control) or with (tolerant) nitroglycerin (10^{-4} M) for 90 min, in the absence or presence of melatonin (10^{-9} M). Following the incubation period, the rings were rinsed for 1 h and then contracted with U46619 ($1-3\times10^{-9}$ M) before cumulative addition of nitroglycerin. The data are expressed as a percentage of the U46619-induced increase in tension, which averaged 15.9 ± 1.6 g in control rings and did not differ significantly in rings incubated with nitroglycerin or melatonin. Each point represents the mean $\pm$ s.e.m.; $n=6$. As compared with control rings, there was a significant rightward shift in the nitroglycerin concentration–response curve in nitrate-tolerant rings incubated with or without melatonin ($P<0.05$).

concentration–response curve was shifted to the right (−log (M) $EC_{50}=6.53\pm0.13$; $P<0.05$ vs control) by approximately 14-fold (Figure 4), indicating the development of nitrate tolerance in isolated coronary arterial rings without endothelium.

In contrast to the results obtained in rings with endothelium, treatment of endothelium-denuded coronary arteries with melatonin during the nitrate tolerance induction phase had no significant effect on the development of tolerance to nitroglycerin (Figure 4). Following the 90-min coincubation period with melatonin (10^{-9} M) and nitroglycerin (10^{-4} M), the −log (M) EC_{50} for nitroglycerin was 6.48 ± 0.26 ($P>0.05$ vs nitrate tolerant without melatonin), which was not significantly different from the potency of nitroglycerin observed in nitrate-tolerant rings not treated with melatonin. Similar results were also obtained with melatonin (10^{-8} M) and (10^{-7} M) in coronary arteries without endothelium (data not shown). Moreover, melatonin had no significant effect on the development of nitrate tolerance in coronary arteries without endothelium even after the time of exposure to the hormone was increased by up to 30 additional minutes prior to the nitrate tolerance induction phase.

Effect of nitrate tolerance on responses to nonnitrovasodilators

Isoproterenol, a cyclic AMP-dependent vasodilator, and cromakalim, a potassium channel opener, caused concentration-dependent relaxations in isolated porcine coronary arteries contracted with U46619. In control preparations not previously exposed to nitroglycerin, the −log (M) EC_{50} for isoproterenol was 7.77 ± 0.15 and the E_{max} was $99\pm1\%$ ($n=5$). Prior exposure of coronary rings to nitroglycerin (10^{-4} M) for 90 min had no significant effect on the concentration–response curve to isoproterenol (−log (M) $EC_{50}=7.96\pm0.18$; $E_{max}=99\pm1\%$; $n=5$; $P>0.05$). The concentration–response curve for cromakalim was not significantly different in control and nitrate-tolerant coronary arteries. The −log (M) EC_{50} values for cromakalim were 6.84 ± 0.12 and 7.03 ± 0.11 and the E_{max} values were $95\pm2\%$ and $95\pm2\%$ in control and nitrate-tolerant coronary arteries, respectively ($n=5$; $P>0.05$).

Discussion

Recent studies indicate that the pineal hormone, melatonin, may play a role in regulating vascular function by altering vasomotor tone, regional blood flow and systemic blood pressure (Krause *et al*., 2000). The present study demonstrates a novel effect of melatonin on the vasculature; the inhibition of nitrate tolerance. Indeed, at concentrations of the hormone that had no direct effect on vascular tone, melatonin reduced the desensitizing effect of exposure to nitroglycerin on subsequent responses to the nitrovasodilator in coronary arteries. That melatonin had no effect on the response to nitroglycerin under control conditions further demonstrates that this action of melatonin is specific to inhibiting nitrate tolerance and not due to an overall enhancement in the ability of nitroglycerin to cause relaxation of vascular smooth muscle. Moreover, the procedure used to induce nitrate tolerance in the present study selectively impairs responses to nitrovasodi-

lators (O'Rourke, 1996), as evidenced by the lack of effect on responses to the cyclic AMP-dependent vasodilator, isoproterenol, and the potassium channel opener, cromakalim.

Interest in the biological activity of melatonin within the cardiovascular system has increased greatly since the discovery of specific binding sites for melatonin in mammalian blood vessels (Viswanathan *et al*., 1990; Stankov & Fraschini, 1993). At present, two distinct melatonin receptor subtypes, designated MT_1 and MT_2, have been cloned (Dubocovich *et al*., 2000). That the effect of melatonin on nitrate tolerance is inhibited by S20928, a potent and selective melatonin receptor antagonist (Ying *et al*., 1996; Conway *et al*., 2000), strongly suggests that the response to melatonin is mediated by specific melatonin receptors in the blood vessel wall. These results are in agreement with previous studies demonstrating the presence of melatonin receptors in porcine and human coronary arteries (Ekmekcioglu *et al*., 2001; Yang *et al*., 2001). Another melatonin binding site, MT_3, has been recently purified and identified as the quinine reductase 2 (Nosjean *et al*., 2000). Although S20928 does not discriminate among the two receptor subtypes and the MT_3-binding site, the lack of effect of 5-MCA-NAT, at concentrations selective for the MT_3-binding site (Molinari *et al*., 1996; Pintor *et al*., 2001), provides preliminary evidence for a role for either MT_1 or MT_2 receptors, or both, in the inhibitory effect of melatonin on nitrate tolerance.

Despite considerable progress made during the past decade, the mechanisms underlying nitrate tolerance remain incompletely understood. Much of the evidence garnered to date indicates that tolerance is likely to be multifactorial. Upon exposure to nitroglycerin, tolerance develops rapidly and is likely the result of both counter-regulatory neurohumoral reflex mechanisms ('pseudotolerance') and decreased vascular responsiveness to the nitrovasodilator (true 'pharmacologic tolerance'). In regard to the latter phenomenon, considerable attention has focused on disruption of the nitric oxide/guanylyl cyclase/cyclic GMP signaling pathway as a potential target for nitrate tolerance and several mechanisms have been postulated. These include: (1) impaired bioactivation of nitrovasodilators (Chung & Fung, 1990; Sage *et al*., 2000), resulting in decreased nitric oxide formation; (2) decreased guanylyl cyclase activity and/or increased phosphodiesterase activity (Waldman *et al*., 1986; Kim *et al*., 2001), resulting in decreased cyclic GMP accumulation; and (3) increased generation of free radicals such as superoxide anions (Munzel *et al*., 1995; 1996), resulting in chemical inactivation of nitric oxide.

The cellular mechanism by which melatonin inhibits nitrate tolerance in coronary arteries remains to be determined. Since melatonin reportedly acts as a scavenger of oxygen-derived free radicals (Tan *et al*., 1993; Reiter *et al*., 1999), it is tempting to speculate that this mechanism contributes to the effect of melatonin on nitrate tolerance in the present study; however, several lines of evidence argue against this explanation, including: (1) the free radical scavenging properties of melatonin are typically observed only at concentrations of the hormone that are at least three orders of magnitude higher than those shown to be effective in the present study (Okatani *et al*., 1999; Wakatsuki & Okatani, 2000); and (2) the observation that the effect of melatonin on nitrate tolerance is inhibited by a specific melatonin receptor antagonist, S20928, makes it unlikely that a direct physicochemical interaction accounts for the effect of melatonin in the present study.

An alternative site of action is suggested by the finding that nitrate tolerance occurred in coronary arteries with and without endothelium, but inhibition of nitrate tolerance by melatonin was observed only in preparations in which the endothelium was intact. These observations suggest that the vascular endothelium, where the presence of melatonin receptors has been documented (Lotufo *et al*., 2001; Masana *et al*., 2002), is the primary site of action with regard to the effect of melatonin on nitrate tolerance. One potential explanation of these results is that melatonin activates receptors on endothelial cells to produce a factor that diffuses to the smooth muscle and inhibits the mechanism underlying nitrate tolerance. Although this possibility cannot be ruled out under the present experimental conditions, it is not clear what diffusible factor would be responsible for this effect. Since nitrate tolerance may be mediated, in part, by increased generation of superoxide anions by endothelial cells exposed to nitroglycerin (Munzel *et al*., 1995; 1996; Kaesemeyer *et al*., 2000), a more plausible hypothesis is that melatonin could exert its inhibitory effect on nitrate tolerance by activating endothelial melatonin receptors and increasing the activity of antioxidant enzymes (Barlow-Walden *et al*., 1995; Pablos *et al*., 1997; Okatani *et al*., 2000), such as superoxide dismutase, within the endothelium. Moreover, since the actions of nitroglycerin on endothelial cells are likely due to its bioconversion to nitric oxide and subsequent activation of the guanylyl cyclase/cyclic GMP cascade (Feelisch *et al*., 1995), it can be postulated that the ability of melatonin to impair signaling *via* this pathway in endothelial cells might also contribute to the inhibitory effect of melatonin on nitrate tolerance (Jockers *et al*., 1997; Petit *et al*., 1999).

Evidence is now beginning to emerge that indicates the mechanisms involved in nitrate tolerance development may differ between blood vessels with and without endothelium. In a rat model of nitrate tolerance, both superoxide dismutase and losartan prevented tolerance to nitroglycerin only in blood vessels in which the endothelium was intact, even though the degree of tolerance was similar in blood vessels with and without endothelium (Gruhn *et al*., 2002). These findings are similar to those obtained in the present study, inasmuch as nitrate tolerance occurred in coronary arteries with and without endothelium, while the inhibitory effect of melatonin on nitrate tolerance was observed only in preparations with intact endothelium. Taken together, these results suggest that different mechanisms of nitrate tolerance may be operative in endothelial cells and vascular smooth muscle cells. Thus, melatonin may selectively interfere with a nitrate tolerance mechanism(s) that requires the presence of endothelial cells.

In summary, the present study demonstrates that nanomolar concentrations of melatonin inhibit the development of nitrate tolerance in coronary arteries. This effect of melatonin appears to be mediated *via* MT_1- or MT_2-melatonin receptors and requires the presence of endothelial cells. Melatonin may prove to be a useful pharmacologic tool in unraveling mechanisms of nitrate tolerance in blood vessels with intact endothelium vs those in which the endothelium is absent or dysfunctional.

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References

BARLOW-WALDEN, L.R., REITER, R.J., ABE, M., PABLOS, M., MENENDEZ-PELAEZ, A., CHEN, L.D. & POEGGELER, B. (1995). Melatonin stimulates brain glutathione peroxidase activity. *Neurochem. Int.,* **26,** 497–502.

BASSENGE, E., FINK, N., SKATCHKOV, M. & FINK, B. (1998). Dietary supplement with vitamin C prevents nitrate tolerance. *J. Clin. Invest.,* **102,** 67–71.

CHUNG, S.H. & FUNG, H.L. (1990). Identification of a subcellular site for nitroglycerin metabolism to nitric oxide in bovine coronary smooth muscle cells. *J. Pharmacol. Exp. Ther.,* **253,** 614–619.

CONWAY, S., CANNING, S.J., HOWELL, H.E., MOWAT, E.S., BARRETT, P., DREW, J.E., DELAGRANGE, P., LESIEUR, D. & MORGAN, P.J. (2000). Characterisation of human melatonin mt_1 and MT_2 receptors by CRE-luciferase reporter assay. *Eur. J. Pharmacol.,* **390,** 15–24.

DE LA LANDE, I.S., STAFFORD, I. & HOROWITZ, J.D. (1999). Effects of guanylyl cyclase and protein kinase G inhibitors on vasodilatation in non-tolerant and tolerant bovine coronary arteries. *Eur. J. Pharmacol.,* **370,** 39–46.

DUBOCOVICH, M.L., CARDINALI, D.P., DELAGRANGE, P., KRAUSE, D.N., STROSBERG, D., SUGDEN, B. & YOCCA, F.D. (2000). Melatonin receptors. In: *The IUPHAR Compendium of Receptor Characterization and Classification* ed. Girdlestone D., 2nd edn, pp. 270–277. London, U.K: International Union of Pharmacology Media.

EKMEKCIOGLU, C., HASLMAYER, P., PHILIPP, C., MEHRABI, M.R., GLOGAR, H.D., GRIMM, M., LEIBETSEDER, V.J., THALHAMMER, T. & MARKTL, W. (2001). Expression of the MT1 melatonin receptor subtype in human coronary arteries. *J. Recept. Signal Transduct. Res.,* **21,** 85–91.

EVANS, B.K., MASON, R. & WILSON, V.G. (1992). Evidence for direct vasoconstrictor activity of melatonin in "pressurized" segments of isolated caudal artery from juvenile rats. *Nauyn-Schmiedeberg Arch. Pharmacol.,* **346,** 362–365.

FEELISCH, M., BRANDS, F. & KELM, M. (1995). Human endothelial cells bioactivate organic nitrates to nitric oxide: implications for the reinforcement of endothelial defence mechanisms. *Eur. J. Clin. Invest.,* **25,** 737–745.

FINK, B., SCHWEMMER, M., FINK, N. & BASSENGE, E. (1999). Tolerance to nitrates with enhanced radical formation suppressed by carvedilol. *J. Cardiovasc. Pharmacol.,* **34,** 800–805.

GEARY, G., KRAUSE, D.N. & DUCKLES, S.P. (1997). Melatonin directly constricts rat cerebral arteries through modulation of potassium channels. *Am. J. Physiol. Heart Circ. Physiol.,* **273,** H1530–H1536.

GEARY, G.G., DUCKLES, S.P. & KRAUSE, D.N. (1998). Effect of melatonin in rat tail artery: role of K^+ channels and endothelial factors. *Br. J. Pharmacol.,* **123,** 1533–1540.

GRUHN, N., BOESGAARD, S., ANDERSEN, C. & ALDERSHVILE, J. (2002). Nitroglycerin tolerance: different mechanisms in vascular segments with or without intact endothelial function. *J. Cardiovasc. Pharmacol.,* **40,** 201–209.

IGNARRO, L.J., LIPPTON, H., EDWARDS, J.C., BARICOS, W.H., HYMAN, A.L., KADOWITZ, P.J. & GRUETTER, C.A. (1981). Mechanism of vascular smooth muscle relaxation by organic nitrates, nitrites, nitroprusside and nitric oxide: evidence for the involvement of S-nitrosothiols as active intermediates. *J. Pharmacol. Exp. Ther.,* **218,** 739–749.

JOCKERS, R., PETIT, L., LACROIX, I., DE COPPET, P., BARRETT, P., MORGAN, P.J., GUARDIOLA, B., DELAGRANGE, P., MARULLO, S. & STROSBERG, A.D. (1997). Novel isoforms of Mel1c melatonin receptors modulating intracellular cyclic guanosine 3′,5′-monophosphate levels. *Mol. Endocrinol.,* **11,** 1070–1081.

KAESEMEYER, W.H., OGONOWSKI, A.A., JIN, L., CALDWELL, R.B. & CALDWELL, R.W. (2000). Endothelial nitric oxide synthase is a site of superoxide synthesis in endothelial cells treated with glyceryl trinitrate. *Br. J. Pharmacol.,* **131,** 1019–1023.

KIM, D., RYBALKIN, S.D., PI, X., WANG, Y., ZHANG, C., MUNZEL, T., BEAVO, J.A., BERK, B.C. & YAN, C. (2001). Upregulation of phosphodiesterase 1A1 expression is associated with the development of nitrate tolerance. *Circulation,* **104,** 2338–2343.

KRAUSE, D.N., GEARY, G.G., DOOLEN, S. & DUCKLES, S.P. (2000). Melatonin and cardiovascular function. In: *Melatonin after Four Decades* ed. Olcese, J. pp. 299–310. New York: Kluwer Academic/Plenum Publishers.

KUKOVETZ, W.R. & HOLZMANN, S. (1990). Mechanisms of nitrate-induced vasodilatation and tolerance. *Eur. J. Clin. Pharmacol.,* **38,** S9–S14.

LOTUFO, C.M.C., LOPES, C., DUBOCOVICH, M.L., FARSKY, S.H.P. & MARKUS, R.P. (2001). Melatonin and *N*-acetylserotonin inhibit leukocyte rolling and adhesion to rat microcirculation. *Eur. J. Pharmacol.,* **430,** 351–357.

MAHLE, C.D., GOGGINS, G.D., AGARWAL, P., RYAN, E. & WATSON, A.J. (1997). Melatonin modulates vascular smooth muscle tone. *J. Biol. Rhythms,* **12,** 690–696.

MASANA, M.I., DOOLEN, S., ERSAHIN, C., AL-GHOUL, W.M., DUCKLES, S.P., DUBOCOVICH, M.L. & KRAUSE, D.N. (2002). MT(2) melatonin receptors are present and functional in rat caudal artery. *Pharmacol. Exp. Ther.,* **302,** 1295–1302.

MOLINA, C.R., ANDRESEN, J.W., RAPOPORT, R.M., WALDMAN, S. & MURAD, F. (1987). Effect of *in vivo* nitroglycerin therapy on endothelium-dependent and independent vascular relaxation and cyclic GMP accumulation in rat aorta. *J. Cardiovasc. Pharmacol.,* **10,** 371–378.

MOLINARI, E.J., NORTH, P.C. & DUBOCOVICH, M.L. (1996). 2-[^{125}I]Iodo-5-methoxycarbonylamino-*N*-acetyltryptamine: a selective radioligand for the characterization of melatonin ML_2 binding sites. *Eur. J. Pharmacol.,* **301,** 159–168.

MUNZEL, T., KURZ, S., RAJAGOPALAN, S., THOENES, M., BERRINGTON, W.R., THOMPSON, J.A., FREEMAN, B.A. & HARRISON, D.G. (1996). Hydralazine prevents nitroglycerin tolerance by inhibiting activation of a membrane-bound NADH oxidase. A new action for an old drug. *J. Clin. Invest.,* **98,** 1465–1470.

MUNZEL, T., SAYEGH, H., FREEMAN, B.A., TARPEY, M.M. & HARRISON, D.G. (1995). Evidence for enhanced vascular superoxide anion production in nitrate tolerance A novel mechanism underlying tolerance and cross-tolerance. *J. Clin. Invest.,* **95,** 187–194.

MURAD, F. (1986). Cyclic guanosine monophosphate as a mediator of vasodilation. *J. Clin. Invest.,* **78,** 1–5.

NOSJEAN, O., FERRO, M., COGÈ, F., BEAUVERGER, P., HENLIN, J.M., LEFOULON, F., FAUCHÈRE, J.L., DELAGRANGE, P., CANET, E. & BOUTIN, J.A. (2000). Identification of the melatonin binding site MT_3 as the quinone reductase 2. *J. Biol. Chem.,* **257,** 31311–31317.

OKATANI, Y., WAKATSUKI, A. & KANEDA, C. (2000). Melatonin increases activities of glutathione peroxidase and superoxide dismutase in fetal rat brain. *J. Pineal Res.,* **28,** 89–96.

OKATANI, Y., WAKATSUKI, A., MORIOKA, N. & WATANABE, K. (1999). Melatonin inhibits the vasorelaxant action of peroxynitrite in human umbilical artery. *J. Pineal Res.,* **27,** 111–115.

O'ROURKE, S.T. (1996). K_{ATP} channel activation mediates nicorandil-induced relaxation of nitrate tolerant coronary arteries. *J. Cardiovasc. Pharmacol.,* **27,** 831–837.

PABLOS, M.I., REITER, R.J., CHUANG, J.I., ORTIZ, G.G., GUERRERO, J.M., SEWERYNEK, E., AGAPITO, M.T., MELCHIORRI, D., LAWRENCE, R. & DENEKE, S.M. (1997). Acutely administered melatonin reduces oxidative damage in lung and brain induced by hyperbaric oxygen. *J. Appl. Physiol.,* **83,** 354–358.

PARKER, J.D. & PARKER, J.O. (1998). Nitrate therapy for stable angina pectoris. *N. Engl. J. Med.,* **338,** 520–531.

PETIT, L., LACROIX, I., DE COPPET, P., STROSBERG, A.D. & JOCKERS, R. (1999). Differential signaling of human Mel1a and Mel1b melatonin receptors through the cyclic guanosine 3′-5′-monophosphate pathway. *Biochem. Pharmacol.,* **58,** 633–639.

PINTOR, J., MARTIN, L., PELAEZ, T., HOYLE, C.H. & PERAL, A. (2001). Involvement of melatonin MT3 receptors in the regulation of intraocular pressure in rabbits. *Eur. J. Pharmacol.,* **416,** 251–254.

REITER, R.J., TAN, D.X., CABRERA, J. & D'ARPA, D. (1999). Melatonin and tryptophan derivatives as free radical scavengers and antioxidants. *Adv. Exp. Med. Biol.,* **467,** 379–387.

SAGE, P.R., DE LA LANDE, I.S., STAFFORD, I., BENNETT, C.L., PHILLIPOV, G., STUBBERFIELD, J. & HOROWITZ, J.D. (2000). Nitroglycerin tolerance in human vessels: evidence for impaired nitroglycerin bioconversion. *Circulation,* **102,** 2810–2815.

STANKOV, B. & FRASCHINI, F. (1993). High affinity melatonin binding sites in the vertebrate brain. *Neuroendocrinol. Lett.,* **15,** 149–164.

TAN, D.X., CHEN, L.D., POEGGELER, B., MANCHESTER, L.C. & REITER, R.J. (1993). Melatonin: a potent endogenous hydroxyl radical scavenger. *Endocrinol. J.,* **1,** 57–60.

VISWANATHAN, M., LAITINEN, J.T. & SAAVEDRA, J.M. (1990). Expression of melatonin receptors in arteries involved in thermoregulation. *Proc. Natl. Acad. Sci. U.S.A.,* **87,** 6200–6203.

WAKATSUKI, A. & OKATANI, Y. (2000). Melatonin protects against the free radical-induced impairment of nitric oxide production in the human umbilical artery. *J. Pineal Res.,* **28,** 172–178.

WALDMAN, S.A., RAPOPORT, R.M., GINSBURG, R. & MURAD, F. (1986). Desensitization to nitroglycerin in vascular smooth muscle from rat and human. *Biochem. Pharmacol.,* **35,** 3525–3531.

YANG, Q., SCALBERT, E., DELAGRANGE, P., VANHOUTTE, P.M. & O'ROURKE, S.T. (2001). Melatonin potentiates contractile responses to serotonin in isolated porcine coronary arteries. *Am. J. Physiol.,* **280,** H76–H82.

YING, S.W., RUSAK, B., DELAGRANGE, P., MOCAER, E., RENARD, P. & GUARDIOLA-LEMAITRE, B. (1996). Melatonin analogues as agonists and antagonists in the circadian system and other brain areas. *Eur. J. Pharmacol.,* **296,** 33–42.

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