Melatonin Modulates Mesenteric Blood Flow and TNFα Concentrations after Lipopolysaccharide Challenge

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ABSTRACT

Objective: To investigate the effect of various doses of melatonin on reduction in mesenteric blood flow (MBF) and increase in tumour necrosis factor alpha (TNFα) concentration caused by injection of lipopolysaccharide (LPS).
Design: University Hospital, Turkey.
Setting: Open experimental study.
Animals: 59 Swiss albino mice.
Interventions: Animals were injected with melatonin solvent or 1, 10, 100, or 500 mg/kg melatonin. Ten minutes later control animals were injected with saline, and the experimental group with LPS.
Main outcome measures: Mesenteric blood flow and serum TNFα concentration.
Results: In control animals, 100 and 500 mg/kg melatonin reduced MBF. LPS reduced MBF in solvent, 1, and 10 mg/kg melatonin groups. The concentration of TNFα was considerably increased in the mice given LPS. Melatonin reduced this response significantly.
Conclusion: In high doses melatonin directly reduces MBF. It has no protective effect on the LPS-induced decrease in MBF. In lower doses it blocks, but at higher doses reduces, LPS-induced TNFα production.

Key words: sepsis, cytokine, shock, blood flow, endotoxin.

INTRODUCTION

Endotoxaemia in rodents is associated with a cascade of release of proinflammatory cytokines as well as hypotension and a lack of responsiveness to vasoconstrictor (16, 19, 21, 30) and vasodilator (18) stimuli, which are thought to be largely mediated by nitric oxide (19, 21, 30). Endotoxaemia not only renders the tissues less responsive to vasoconstrictor stimuli (17) but also causes mesenteric ischaemia (7). Septic shock, which ensues as a result of these interactions, is intricately associated with mesenteric ischaemia, the remote complications of which are essentially those of sepsis, whether as a cause or as a consequence. Reduced mesenteric blood flow (MBF) in septic shock followed by reperfusion injury has been incriminated in the pathophysiology of multiple organ failure syndrome.

Melatonin is a pineal hormone with immunostimulating (23, 26) and antioxidant effects (25). Studies in mice have suggested a beneficial role for melatonin in experimental models of haemorrhagic shock (29, 33) and the sepsis that follows haemorrhagic shock (32). The vascular system has melatonin receptors, and functional studies have suggested that melatonin might regulate vascular tone (11, 22, 31). The pineal gland, possibly through melatonin, may modulate mesenteric vascular reactivity (9) or the vascular response to vasoconstrictor agents (10).

Considering these data, we planned to investigate the effect of various doses of melatonin with and without lipopolysaccharide (LPS) challenge on MBF and tumour necrosis factor alpha (TNFα) concentrations. TNFα is the first cytokine that is released in endotoxic shock.

MATERIAL AND METHODS

We used inbred female Swiss albino mice weighing 30–35 g. The experiments were done in the laboratories of Hacettepe University School of Medicine, Departments of Pharmacology, and Surgical Research. Guidelines for the care and use of laboratory animals were adhered to throughout the study. Animals were given free access to standard rodent chow and tap water.

Melatonin

Synthetic melatonin (N-acetyl-5-methoxytryptamine, ICN Pharmaceuticals, CA) was dissolved in 95% ethanol and diluted with saline. Melatonin-treated animals were given supraphysiological doses (1, 10, 100 and 500 mg/kg) dissolved in about 0.1 ml 95% ethanol plus 0.4 ml saline. Animals in the solvent group

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were injected with 0.1 ml 95% ethanol plus 0.4 ml saline.

Experimental groups and procedures

Animals were randomly divided into two groups: one group was challenged with LPS and the other acted as controls. Each group was divided into five subgroups which were given melatonin at doses of 1, 10, 100, and 500 mg/kg. In the control group, each of these five subgroups contained four mice, making a total of 20. In the LPS group, the solvent alone subgroup contained 11 animals, whereas each of the melatonin injection subgroups contained 7 animals, making a total of 39. Melatonin solvent and 1, 10, 100, 500 mg/kg melatonin solutions were injected subcutaneously.

Ten minutes after injection of the solvent or the dose of melatonin, control animals were injected with saline, and LPS challenge mice were injected with LPS 20 mg/kg (Sigma Chemical Co., St. Louis, MO, E. coli serotype O55:B5) intraperitoneally. Ninety minutes after the injection of saline or LPS, all animals were anaesthetised with pentobarbital 60 mg/kg intraperitoneally and a midline laparotomy was done. The superior mesenteric artery (SMA) was dissected free from the surrounding tissues and a Doppler ultrasonic flowprobe was placed around the artery to measure MBF. Blood samples were then withdrawn for estimation of the TNFα concentration. The time from the start of the laparotomy to completion of withdrawal of blood was about 5 minutes in each animal.

Measurement of mesenteric blood flow

Perivascular ultrasonic Doppler flowprobes (Transonic Systems Inc, USA) were used to measure the MBF and it was monitored by displaying the beat-to-beat oscillations on a Harvard Universal Oscillograph (Harvard Apparatus Ltd, UK), a method we have previously described (1–3). After placement of a flowprobe around the SMA, the flow rate was monitored and the first value that remained stable for 10 seconds was recorded as the point value of MBF.

Collection of serum

Blood was collected by cardiac puncture and transferred to Eppendorf tubes. After about 120 minutes, the serum was separated by centrifugation at 1000 g for 20 minutes and stored at −70 °C until the assays were done.

TNFα assay

TNFα concentrations were measured using Cytoscreen™ Mouse solid phase sandwich enzyme linked immunosorbent assay (ELISA) kit (Biosource-International). Aliquots (50 µl) of standards, samples, and controls were added to microtitre wells that contained 50 µl of standard diluent. Biotinylated anti-TNFα antibody solution (50 µl) was added to each well. After the wells had been incubated for 1.5 hours at room temperature and excess antibody washed away, streptavidin-horse radish peroxidase working conjugate 100 µl was added to each well.

The wells were then incubated for 30 minutes at room temperature and washed, and stabilised chromogen 100 µl was added to each well. After a final incubation for 25 minutes in the dark at room temperature, stop solution 100 µl was added. Absorbencies were then read at 450 nm. Standard absorbencies were plotted against the standard concentrations and a graph was obtained. Sample concentrations were calculated by using this graph. Reading and computations were done by an automated microplate reader (Biotech, Elx 800). For out of range results, procedures were repeated after serial dilutions. Results were expressed as pg/ml.

Statistical analysis

Kruskal-Wallis analysis of variance was used followed by Mann-Whitney U test for two group comparisons as a post hoc test. After melatonin doses were transformed logarithmically, linear regression was done to establish the correlation patterns of different doses of melatonin with MBF and TNFα concentrations after saline and LPS challenges. Probabilities of less than 0.05 were considered significant.

RESULTS

Mesenteric blood flow

Results are shown in Figure 1. Comparison of saline groups showed that without LPS melatonin at 100 and 500 mg/kg doses significantly reduced MBF compared with solvent alone ($p = 0.03$ and 0.02, respectively).

Comparison of LPS groups with corresponding saline groups showed that LPS injection reduced MBF significantly in only solvent and 10 mg/kg melatonin groups ($p = 0.04$ and 0.01, respectively).

However, there was no significant difference in intragroup comparison of LPS groups ($p = 0.28$).

Comparison of MBF in solvent-saline animals with those given melatonin and LPS showed that the reduction in MBF at 10, 100, and 500 mg/kg doses was significant ($p = 0.02$, 0.02 and 0.01, respectively), whereas it was not significant at 1 mg/kg ($p = 0.15$).

TNFα concentrations

Results are shown in Figure 2. In the saline groups melatonin did not alter TNFα concentrations significantly ($p = 0.08$). In the LPS groups there were significant differences between the groups ($p =$

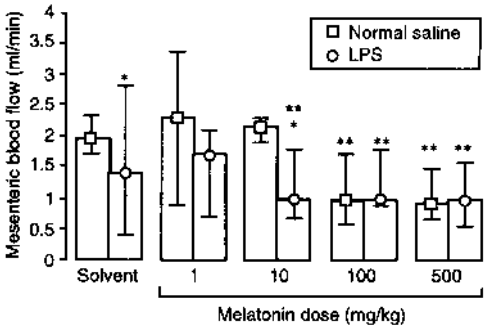

Fig. 1. Box plots showing median (range) mesenteric blood flow in each group ($n = 11$ in the lipopolysaccharide (LPS)/solvent group, $n = 4$ in each group treated with normal saline, and $n = 7$ in each of the four groups given LPS treated with melatonin). *$p = 0.04$ and 0.01 compared with corresponding saline groups; **$p < 0.05$ compared with solvent/saline group.

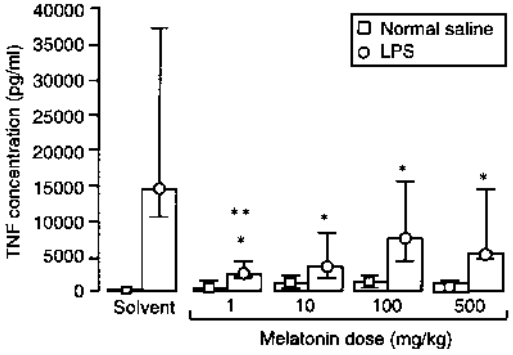

Fig. 2. Box plots showing median (range) tumour necrosis factor alpha (TNFα) concentrations in each group ($n = 11$ in the lipopolysaccharide (LPS)/solvent group, $n = 4$ in each group treated with normal saline, and $n = 7$ in each of the four groups given LPS treated with melatonin). *$p < 0.05$ compared with the LPS/solvent group, LPS/saline group, and the corresponding saline groups; **$p = 0.003$ and 0.002 compared with 100 and 500 mg/kg LPS groups.

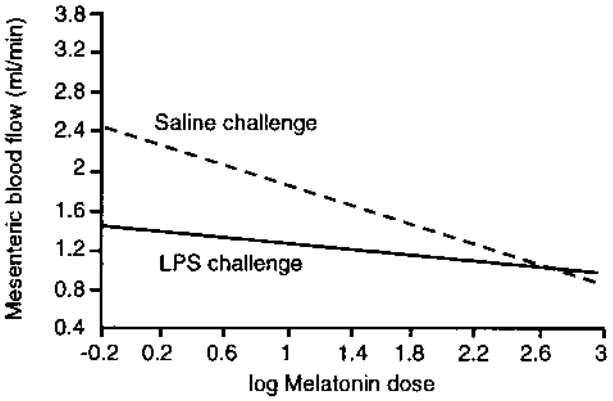

Fig. 3. Correlation of mesenteric blood flow and log doses of melatonin. Saline challenge: mesenteric blood flow = 2.347–0.516*log dose of melatonin + epsilon (e) (the random error associated with measurement); $r = -0.68$, $p = 0.004$. Lipopolysaccharide challenge: mesenteric blood flow = 1.425–0.171*log dose of melatonin + epsilon; $r = -0.4$, $p = 0.04$.

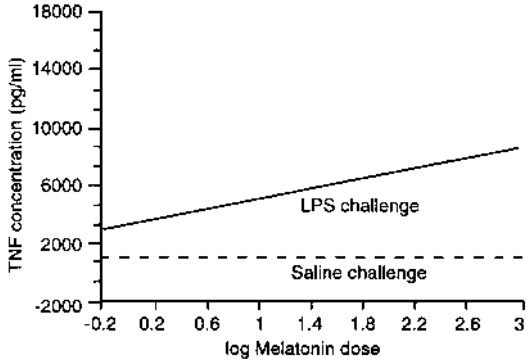

Fig. 4. Correlation of tumour necrosis factor alpha (TNFα) concentrations with log doses of melatonin. Saline challenge: TNFα = 1.048e3–80.257*log dose of melatonin + epsilon (e) (the random error associated with measurement); $r = 0.126$, $p = 0.66$. Lipopolysaccharide challenge: TNFα = 3.289e3 + 1.703e3*log dose of melatonin + epsilon; $r = 0.507$, $p = 0.006$.

0.002), and the median TNFα concentration in the LPS/solvent group was significantly higher than in any other group ($p < 0.05$ for any combination). The median TNFα concentration after LPS challenge was significantly lower after 1 mg/kg melatonin than after 100 mg/kg ($p = 0.003$) and 500 mg/kg ($p = 0.002$).

In the solvent-injected mice, LPS significantly increased the median TNFα concentration compared with saline injection ($p = 0.008$).

Comparison of melatonin-saline mice with their corresponding LPS groups showed that melatonin pre-treatment did not prevent the LPS-induced increase in TNFα with 1 ($p = 0.02$), 10, 100, and 500 mg/kg doses ($p = 0.01$ for all three groups).

There was significant difference in comparison of solvent-saline group with all of the melatonin-LPS groups ($p = 0.008$ for any combination).

Correlation of melatonin doses with MBF and TNFα levels

Linear regression showed that melatonin doses were linearly correlated with a negative slope of MBF after saline ($r = -0.68$, $p = 0.004$) and LPS challenges ($r = -0.4$, $p = 0.04$) (Figure 3). There was no linear correlation between melatonin doses and TNFα concentrations after saline challenge ($r = 0.126$, $p = 0.7$), whereas there was a linear correlation after LPS challenge ($r = 0.507$, $p = 0.006$) (Figure 4).

DISCUSSION

In experimental animals, the MBF is significantly reduced by endotoxaemia and is lowest around 120 minutes after LPS infusion (14). The TNFα concentration peaks around 60–120 minutes after injection of LPS (4, 35). As we aimed to investigate the interplay between melatonin, MBF, and TNFα, we chose 90 minutes after injection of LPS to evaluate the data. After intravenous (34) or subcutaneous (12) injection of melatonin, its half-life is around 20 minutes and the peak values are also reached around 20 minutes (12). The time of the day that melatonin is injected does not significantly influence its pharmacokinetic profile (12). After injection of LPS, the TNFα gene is expressed in 15–20 minutes, (8, 20) and reaches a maximum at 40 minutes (20), whereas calcium-independent inducible nitric oxide synthase in blood vessels is found within 30 minutes (13). These studies confirm the rapid accumulation and clearance of extracellular melatonin, as well as the rapid initiation of the mechanisms leading to raised TNFα values and reduced MBF after injection of LPS. As we aimed to block the effects of LPS-dependent mediator release as soon as possible to achieve the maximal inhibitory effect, we decided to give melatonin 10 minutes before LPS.

At 100 and 500 mg/kg doses, melatonin reduced MBF significantly in the absence of LPS. As this occurred in the absence of any other inciting factors and the vascular system is known to have melatonin receptors (11, 21, 31), the most plausible explanation is a direct effect of melatonin on vascular smooth muscle. LPS challenge was not able to reduce MBF further, which shows how powerful high concentrations of melatonin are in decreasing MBF.

It seems that melatonin might increase MBF at low doses but reduce it at high doses, probably by a direct effect. The fact that the melatonin doses had a negative linear correlation with MBF after both saline and LPS challenges supports this hypothesis. Recently two distinct melatonin receptors were identified that modulate the vascular smooth muscle, one of which caused relaxation, and the other vasoconstriction (11). It is possible that different receptors may be activated by different doses.

None of the melatonin doses prevented the LPS-induced increase in TNFα values compared with saline controls. Comparison of melatonin/LPS groups with the solvent/saline group showed that the TNFα values were significantly higher in each of the melatonin/LPS groups. However, comparison of the solvent/LPS group and melatonin/LPS groups showed that TNFα concentrations were significantly lower in each of the melatonin/LPS groups. These data suggest that although melatonin pretreatment does not prevent LPS-induced increase in TNFα concentrations, it does reduce the TNFα response. Small doses of melatonin seem more effective for this purpose as TNFα values were significantly lower after 1 mg/kg dose of melatonin than after 100 and 500 mg/kg doses.

At low concentrations melatonin inhibits nitric oxide synthase (5, 15, 27, 28). Nitric oxide inhibition significantly reduces serum TNFα concentrations in endotoxaemic rats (6) and our results showed that the suppressive effect of melatonin on the LPS-induced increase in TNFα concentrations was less at higher doses. One possibility is that the nitric oxide synthase inhibition by melatonin might be dose-related, with diminution of the inhibitory effect by increasing doses.

Melatonin given a short while after haemorrhagic shock reduced mortality from a subsequent septic challenge, whereas continuous melatonin treatment increased mortality (33). In another study, a high dose of 200 mg/kg melatonin was shown to have immunosuppressive effects, contrary to its usual immunostimulatory effects (24). In support of these data, we found that TNFα was more vigorously suppressed and MBF was less affected by LPS at lower doses of melatonin, whereas at higher doses there was a significant reduction in MBF even in the absence of an LPS challenge. All these data indicate that the effects of this substance are dose-dependent and that its beneficial effects are reversed at higher doses, and may even become detrimental.

In conclusion, the present results showed that melatonin suppresses LPS-induced increases in TNFα concentrations. This suppressive effect is more powerful at lower doses. High doses of melatonin reduce MBF in the absence of any challenge, whereas low doses may alleviate LPS-induced reduction in MBF. The doses of melatonin used in this study were all pharmacological. Further studies with lower doses might provide further support to our conclusions. However, it should also be taken into account that the present results apply to mice and one should be sceptical about applying them to humans.

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