Protective effect of melatonin on myocardial infarction

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Melatonin has been shown to scavenge hydroxyl radicals (OH), and hydrogen peroxide (H$_2$O$_2$) are generated intracellularly as by-products of oxidative metabolism. These reactive oxygen species can cause peroxidation of membrane lipids, denaturation of proteins, and modification of DNA, all of which ultimately lead to cell death. In mammals, cell damage induced by reduced oxygen species can also initiate local inflammatory responses,melatonin receptor; antioxidant

The pathogenesis of myocardial ischemia-reperfusion (I/R) injury is a multifactorial process involving the interaction of multiple mechanisms. Numerous studies indicate that the three pivotal factors in the pathogenesis of I/R injury are elevated oxidative damage, depressed energy metabolism, and altered calcium homoeostasis (12). Other evidence suggests that these three factors are intimately interrelated, forming a deleterious network.

Partially reduced species of oxygen, including superoxide anions (O$_2^-$), hydroxyl radicals (-OH), and hydrogen peroxide (H$_2$O$_2$), are generated intracellularly as by-products of oxidative metabolism. These reactive oxygen species can cause peroxidation of membrane lipids, denaturation of proteins, and modification of DNA, all of which ultimately lead to cell death. In mammals, cell damage induced by reduced oxygen species can also initiate local inflammatory responses, which then lead to further oxidant-mediated tissue injury.

The finding that reactive oxygen species cause myocardial I/R injury suggests that an increasing activity of cellular antioxidant enzymes should protect tissues from reperfusion damage. Indeed, the addition of SOD to the perfusion solution protects a number of isolated heart models against I/R injury (18, 21). However, the protective effect of exogenous SOD is highly variable and dose dependent. In addition, numerous studies showed no protective effect of SOD and/or catalase treatment on ischemic injury (20, 23). In all likelihood, these ambiguous results are due to the inability of the exogenous antioxidant enzymes to cross the cell membrane and reach the subcellular sites of free radical generation. To circumvent this problem, we have used transgenic mice overexpressing antioxidant enzymes to study the mechanism of I/R injury. Our results indicate that overexpression of mitochondrial MnSOD or cytosolic Cu/ZnSOD protects hearts against reperfusion injury (2, 3). In addition, transgenic mice overexpressing glutathione peroxidase have also shown protection against I/R injury (34). These studies clearly show that free radicals play an important role in I/R injury and that antioxidant enzymes can attenuate damage induced by free radicals.

Because there is strong evidence that free radicals contribute to postischemic injury, antioxidant enzyme therapy could potentially be extremely effective. In reality, however, the usefulness of this therapy is limited by a number of factors, in particular the ability of the antioxidants to penetrate the cell membrane and scavenge free radicals in situ. Fortunately, this limitation can in part be addressed with the use of melatonin, a potent antioxidant and free radical scavenger that can easily diffuse through the cell membrane to exert its antioxidant effects. Indeed, unlike the confined distribution of other antioxidants such as vitamin C or vitamin E, melatonin distributes readily in all subcellular compartments because of its solubility in water and in lipids.

Another advantage of melatonin over other antioxidants is the effectiveness of its scavenging ability. Melatonin has been shown to scavenge hydroxyl radicals, peroxyl radicals (ROO-), superoxide anions (10, 27), and H$_2$O$_2$ (31). Melatonin is 5 and 14 times more...

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effective at scavenging hydroxyl radicals than glutathione and mannitol, respectively (10), and it is twice as efficient as vitamin E in removing peroxyl radicals (25). Because of its permeability properties as well as its powerful antioxidant activity, a significant amount of research has been performed to explore melatonin’s potential as a cardioprotective agent against I/R injury. Previously, we reported (4) that melatonin indeed protects mouse hearts against I/R injury in vitro. Furthermore, several studies found that melatonin confers protection against I/R injury in a perfused rat heart model (13, 15) and in myocytes (29), and another study demonstrated that I/R-induced arrhythmia can be prevented by melatonin in perfused rat hearts (32).

Importantly, recent studies have suggested that melatonin’s receptors may play a role in a number of its physiological effects. Melatonin acts principally via high-affinity receptors coupled to heterotrimeric G proteins. Two high-affinity receptor subtypes have been cloned so far: Mel1a and Mel1b melatonin receptors (7). Mel1a melatonin receptors, which are believed to mediate major neurobiological functions of melatonin in mammals (16), are expressed in peripheral tissues such as quail heart (22). Mel1a melatonin receptors overexpressed in HEK293 cells were recently demonstrated to be coupled to Go/11 proteins (1), which are involved in several signal transduction pathways, including the regulation of ion channels, activation of MAPKs, and PLC-independent stimulation of phospholipase D (8). Some of these pathways lead to the activation of protein kinase C, which has been shown to play an important role in ischemic preconditioning (30). In contrast to Mel1a melatonin receptors, Mel1b melatonin receptors modulate cGMP production (24).

The experiments in our study expand on the current body of research on the cardioprotective effect of melatonin in four important ways. First, in contrast to the in vitro experiments performed by other researchers, our experiments explored the cardioprotective effects of melatonin in vivo. This is important because a number of agents that work well in vitro fail to protect the heart in vivo. Second, our experiments determined the effective dose and time frame of administration of melatonin, which is important information if melatonin is to have clinical applications. Third, our experiments explored the role of melatonin receptors in melatonin’s cardioprotective effect. Finally, our experiments compared the effects of melatonin in rats and mice.

METHODS

All protocols in this study were approved by the University Committee on Animal Care of East Tennessee State University.

Determination of dose-dependent effects of melatonin. Melatonin was administered intraperitoneally (ip) to male ICR mice weighing 25–30 g. Melatonin stock solution (10 mg/ml) was dissolved in 95% alcohol, and each mouse received 0.1 ml of saline containing 2 µl of alcohol with melatonin at the specified dose (33, 75, 150, or 300 µg/kg ip). After injection, mice were anesthetized with chloral hydrate (360 mg/kg ip). An endotracheal tube (PE-90) was inserted 5–8 mm from the larynx, and the mice were ventilated with room air (tidal volume of 0.5 ml) with a rodent respirator (Columbus Instruments International, Columbus, OH) set at 110–120 beats/min.

Thirty minutes after injection, mice were subjected to 60 min of left anterior descending artery (LAD) ligation and 4 h of subsequent reperfusion as described previously (3). After reperfusion mice were anesthetized with pentobarbital sodium (120 mg/kg ip), and hearts were perfused as Langendorff preparations for 5 min. The left coronary artery was reocluded, and 1% Evans blue was infused into the aorta and coronary arteries to determine the area at risk. Hearts were transversely cut into five sections, with one section made at the site of the ligature. Macroscopic staining with triphenyltetrazolium chloride (TTC) was used to quantitate the infarct sizes as described previously (3). The area at risk was expressed as the percentage of the left ventricle, and the area of infarct was expressed as the percentage of the area at risk as described previously (3). This method of measuring infarct was used for all subsequent experiments.

Determination of time-dependent effects of melatonin. To explore the time-dependent effects of melatonin, we first determined the optimal dosage of melatonin based on the results of the first set of experiments. A different set of mice from the first experiment was pretreated with melatonin at this dosage (150 µg/kg ip) 0.5, 2, 4, or 24 h before they were subjected to LAD ligation and 4-h reperfusion, as described previously (3). To determine the window of protection of melatonin, the previous experiment was repeated, except that mice were given melatonin (150 µg/kg ip) at the following time periods: 30 min before ligation, 45 min after ligation, or during reperfusion. Finally, an independent experiment was performed in which melatonin was administered into the ventricle immediately before reperfusion.

Determination of plasma levels of melatonin. Ten microliters of blood were collected at different times from the femoral vein in anesthetized mice and diluted with normal saline. Plasma levels of melatonin were determined by a melatonin RIA kit available from American Laboratory Products (Windham, NH).

Determination of role of melatonin receptor. Because of the scarcity of melatonin receptors in peripheral tissues, the presence or absence of Mel1a and Mel1b melatonin receptors in the heart was characterized by RT-PCR. Total RNA was extracted from mouse heart and brain by TRI Reagent LS (Molecular Research Center, Cincinnati, OH) and digested by RNase-free DNase I. RNA (2 µg) was primed with oligo(dT)16, reverse transcribed, and then amplified for 40 cycles with two mouse specific primers (each at 500 nM) with a RT-PCR kit (Applied Biosystems, Foster City, CA). The Mel1a melatonin receptor primers, which amplify a band of 117 bp, were 5’-GGAGGAGGAGGACCCAC-3’ and 5’-TGGCGGTACACAGACAGGATG-3’ (28). The Mel1b melatonin receptor primers, which amplify a band of 196 bp, were 5’-ATGATCAGCTGAGCCAGACC-3’ and 5’-CGAATATCCTCGGGCTCTC-3’ (16). After PCR, the reaction products were subjected to electrophoresis through 1.5% agarose gel.

Male C57BL/6J mice with targeted disruption of the Mel1a melatonin receptor (provided by Drs. Steven Reppert and David Weaver) and wild-type (Mel1a+/+) mice were given melatonin (150 µg/kg ip) 30 min before they were subjected to 50 min of LAD ligation, followed by 4 h of reperfusion. In addition, mice were given 8-methoxy-2-propionamidotetralin (8-M-PDOT; 150 µg/kg ip) 30 min before they were subjected to 60 min of LAD ligation; followed by 4 h of reperfusion. 8-M-PDOT is a potent Mel1a and Mel1b melatonin receptor agonist (7) that cannot scavenger free radicals because of a
lack of an indole structure, as demonstrated in our previous study (17).

Regional ischemia in vivo rat model. To determine the effect of melatonin on myocardial infarction in rats, Sprague-Dawley rats weighing 250 g were given melatonin (150 μg/kg ip) 30 min before ligation. The anterior descending branch of the left coronary artery was ligated near the middle of the heart with a curved T-6 needle and a 7-0 silk suture. After 45 min of artery occlusion, the exteriorized thread was gently removed to reperfuse the heart for 24 h.

Sources of chemicals. Melatonin was purchased from Sigma (St. Louis, MO). 8-M-PDOT was a product of Tocris Cookson (Ballwin, MO). All other reagents were of the highest grades commercially available.

Statistical analysis. All data are expressed as means ± SE and were analyzed with Instat software. Statistical difference was assessed by a one-way ANOVA test followed by the Student’s t-test for comparison between the control and melatonin- or 8-M-PDOT-treated groups. Statistical significance was set at \( P < 0.05 \).

RESULTS

Our first series of experiments were designed to explore the dose-dependent effects of melatonin. In these experiments, the risk area/left ventricle (LV) was comparable among all the groups. Pretreatment of mice with melatonin at a dose as low as 75 μg/kg for 30 min resulted in a significant protective effect. Melatonin treatment (150 μg/kg) was able to reduce the infarct size/risk area (59 ± 4% for control vs. 33 ± 5% for the melatonin group; \( P < 0.05 \)) as well as the infarct size/LV (19 ± 1% for control vs. 11 ± 2% for the melatonin group; \( P < 0.05 \)) (Fig. 1). Because administering higher doses conferred no additional protective effects, 150 μg/kg was chosen as the dosage for all subsequent experiments.

Figure 2 summarizes the results of the experiments exploring the time-dependent effects of melatonin. Melatonin’s protective effect was observed in mice 0.5–2 h after melatonin administration but not after 4 or 24 h. The results of the experiment designed to assess the window of protection of melatonin are summarized in Fig. 3. Administration of melatonin 45 min after ligation reduced the infarct size/risk area from 59 ± 4% (control) to 39 ± 5%, and it reduced the infarct size/LV from 19 ± 1% (control) to 11 ± 1%. Administration of melatonin 45 min after ligation conferred approximately the same magnitude of protection as administration of melatonin 30 min before ligation, as determined by infarct size/risk area. However, melatonin administered intraperitoneally 60 min after ligation was not protective. In the experiment in which melatonin was injected directly into the LV, melatonin was still able to protect the heart (Fig. 4).

To correlate the protective effect of melatonin with plasma levels of melatonin, mice were injected with melatonin (150 μg/kg ip) and plasma levels of melatonin...
Melatonin levels of melatonin were determined (Fig. 5). Plasma levels of melatonin rose rapidly to 40 ± 3 ng/ml or 170 nM within 5 min of injection, decreased 50% within 15 min, and returned to lower levels (1.8 ± 0.8 ng/ml or 7.7 nM and 0.8 ± 0.3 ng/ml or 3.4 nM, respectively) after 120 and 240 min.

We also investigated the role of melatonin receptor in melatonin’s protective effect. Expression of Mel1a and Mel1b melatonin receptors was examined in wild-type mice by RT-PCR of RNA isolated from the heart and the brain with specific primers for the receptors. As shown in Fig. 6A, a single band of 117 bp specific for the Mel1a melatonin receptor was present in the heart (lane 1) and the brain (lane 2). In addition, a single band of 196-bp Mel1b melatonin receptor was also detected in the heart and the brain (Fig. 6A, lanes 3 and 4). In mice deficient for the Mel1a melatonin receptor, melatonin was still able to protect the heart against myocardial infarction (Fig. 6B), suggesting that the Mel1a receptor is not involved in the protective effect. A confirmatory experiment was performed with 8-M-PDOT, a melatonin receptor agonist with no antioxidant activity. 8-M-PDOT was shown to have no protective effect (Fig. 7).

To verify that the protective effect of melatonin on myocardial infarction is not unique to mice, experiments were also performed with rats, the most commonly used experimental model for I/R injury. Melatonin treatment was effective in reducing the infarct size/risk area of rat hearts subjected to as long as 45 min of ligation (Fig. 8; 45 ± 5% for control vs. 28 ± 3.7%, P < 0.05). The values for the risk area as a percentage of LV were not different between control and melatonin-treated rats.

**DISCUSSION**

In this study, melatonin treatment was able to reduce the infarct size/risk area in an in vivo mouse heart...
model, which proves that melatonin’s effects are not just limited to in vitro settings. In terms of dose dependence of melatonin, we determined that the amount of plasma melatonin needed to confer cardiac protection is between 7.7 and 170 nM (Fig. 5) and that the optimal dosage of melatonin is 150 μg/kg. In terms of the time dependence of melatonin, we showed that melatonin’s protective effect lasts ~2 h. The lack of a protective effect after 24 h indicates that melatonin has no late preconditioning effect. Our results support a previous report that plasma melatonin has a short half-life because of a rapid liver-mediated conversion to 6-hydroxymelatonin and 6-sulfatoxymelatonin (26).

In our experiments, melatonin protected the heart as late as 45 min after the onset of ligation (Fig. 3). In contrast, melatonin administered intraperitoneally 60 min after ligation (i.e., during the release of artery occlusion) was not protective (Fig. 3). Because it takes 2–3 min for melatonin to enter the blood circulation after intraperitoneal administration, these results indicate that melatonin will not protect the heart if administered after reperfusion and therefore must be delivered to the heart before the release of ligation. This conclusion is supported by our experiment in which melatonin injected directly into the LV right before reperfusion still conferred cardioprotection (Fig. 4). Because the mouse heart is not known to have any collateral flow, this result suggests that melatonin acts during reperfusion. This is a clinically relevant finding, because melatonin could be administered shortly after a heart attack to prevent reperfusion injury.

Our study demonstrates that melatonin is a very effective cardioprotective agent in both mice and rats (Fig. 8). This stands in contrast to a previous study that showed no protective effect of melatonin on myocardial infarct size in rabbits receiving 70 times more melatonin (10 mg/kg) infused intravenously (6). The disparity between the two sets of results may be attributed to a number of differences in experimental conditions, such as the dose and route of melatonin administration. More directly, in the rabbit study, a high percentage of alcohol (1 ml or 0.81 g/kg) was used to dissolve and deliver a pharmacological dose of melatonin. The presence of alcohol above a certain threshold during ischemia has been shown by Krenz et al. (14) to abolish protection from both ischemic preconditioning and mitochondrial ATP-sensitive potassium channel activation in the rabbit model. Thus it is possible that the lack of a cardioprotective effect was due to the masking effect of the alcohol. In the present study, a significantly lower amount of alcohol (67 μl or 0.054 g/kg) was injected intraperitoneally in the animals 30 min before LAD ligation. Therefore, most of the alcohol was metabolized before ischemia, effectively removing the masking effect. Further experiments are needed to resolve this issue.

On the basis of our RT-PCR analysis, Mel1a and Mel1b melatonin receptors are present in the mouse heart (Fig. 6A). It is possible that melatonin’s cardioprotective effect is mediated by its receptors. However, our results with mice deficient for the Mel1a melatonin receptor argue against this possibility (Fig. 6B). In addition, mice treated with 8-M-PDOT, which activates both Mel1a and Mel1b melatonin receptors but does not scavenge free radicals (7), showed no protective effect (Fig. 7). 8-M-PDOT has been shown to be 20-fold more selective for Mel1b vs. Mel1a receptor subtype (7). This observation is in agreement with our previous study demonstrating that melatonin, but not 8-M-PDOT, protects against doxorubicin-induced cardiotoxicity (17). Although the free radical scavenging activity of melatonin was not measured directly in our in vivo study, it was shown previously that melatonin scavenges hydroxyl radicals and can reduce the extent of lipid peroxidation in isolated rat hearts subjected to I/R injury (13). This study, combined with our results with 8-M-PDOT, suggests that melatonin’s cardioprotective effects are not mediated by its receptors but rather by its free radical scavenging activity.
Melatonin protects against myocardial infarction

One potentially puzzling result is the lack of correlation between the plasma levels of melatonin and the extent of protection on infaract size. It could be that the concentration of melatonin in the heart during the first 2 h after intraperitoneal injection is above a putative threshold needed for cardioprotection; if this were true, one would only expect a concentration-dependent effect of melatonin below this threshold. This issue needs to be clarified in future studies, perhaps by determining the cardiac content of melatonin with radioactive melatonin.

In summary, melatonin can confer cardioprotection against I/R injury, most likely through its free radical scavenging activities. It is clear that melatonin could potentially become an ideal agent for clinical treatment of patients with ischemic heart diseases or for patients undergoing cardiovascular surgery. First, based on surface area [M2 = weight (g)2/3 × K × 10−4, where the constant specific for each animal species (K) is 10.5 for mice; Ref. 11] the dose used for mice (150 µg/kg) is equivalent to 0.8 mg/1.8 m2 for humans. This is approximately equal to the typical dose of commercial forms of melatonin that consumers use to restore normal sleep patterns, which indicates that the dose of melatonin necessary to confer cardioprotection will not be toxic to humans. Second, melatonin is a very effective and low-cost cardioprotective agent with very few undesirable side effects. Finally, recent studies show that melatonin treatment prevents ischemia-induced cell death or apoptosis in the central nervous system (19, 33), splanchnic artery (5), and spinal cord (9). Therefore, melatonin treatment could provide protection to the heart as well as to other vital organs when ischemic cardiac arrest is required. In short, melatonin has great clinical potential as a cardioprotective agent, and further experiments in humans should be carried out to explore its possibilities.

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