Physiol. Res. 54: 491-495, 2005

Protective Effects of Melatonin on Myocardial Ischemia-Reperfusion Induced Infarct Size and Oxidative Changes

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Received July 8, 2004
Accepted November 23, 2004
On-line available January 10, 2005

Summary

Free radicals, calcium overloading and loss of membrane phospholipids play an important role in the development of ischemia/reperfusion (I/R) injury. Melatonin is a well-known antioxidant and free radical scavenger. Melatonin may also reduce the intracellular calcium overloading and inhibit lipid peroxidation. This study was designed to investigate the effects of melatonin on the I/R-induced cardiac infarct size in an *in vivo* rat model. We also investigated glutathione (GSH) levels, an antioxidant the levels of which are influenced by oxidative stress, and malondialdehyde (MDA) levels, which is an index of lipid peroxidation. To produce cardiac damage, the left main coronary artery was occluded for 30 min, followed by 120 min reperfusion, in anesthetized rats. Melatonin (10 mg/kg) or vehicle was given 10 min before ischemia *via* the jugular vein. Infarct size, expressed as the percentage of the risk zone, was found significantly greater in I/R group than in the melatonin-treated I/R group. MDA levels were significantly higher, but GSH levels were lower in the I/R group than in the control group. Melatonin significantly reduced the MDA values and increased the GSH levels. These results suggest that oxidative stress contributes to myocardial I/R injury and melatonin administration exerts a mitigating effect on infarct size. Furthermore, the results indicated that melatonin improves the antioxidant capacity of the heart and attenuates the degree of lipid peroxidation after I/R.

Key words

Melatonin • Reperfusion injury • Heart • Infarct size • Glutathione • Malondialdehyde

Introduction

Cardiovascular disease causes morbidity and mortality of people in the developed countries of the world. Coronary artery ischemia-reperfusion (I/R) injury that is known to occur on restoration of coronary flow after a period of myocardial ischemia includes myocardial cell injury and necrosis (Dhalla *et al*. 2000). Reperfusion of ischemic myocardium leads to severe damage, which is indicated by free radicals, intracellular calcium overloading and loss of membrane phospholipids (Maxwell and Lip 1997**,** Dobšák *et al*. 2003). Malondialdehyde (MDA), a stable metabolite of the free radical-mediated lipid peroxidation cascade, is widely used as marker of oxidative stress. Glutathione (GSH) is an important endogenous antioxidant the levels of which are influenced by oxidative stress.

Melatonin is a well-known antioxidant and free

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radical scavenger (Reiter *et al*. 2000, 2001). Besides its function as a broad spectrum free radical scavenger, melatonin may also regulate the intracellular calcium levels (Vaněček 1995) and limits the MDA levels (Dobšák *et al*. 2003). Melatonin has been shown to reduce myocardial I/R injury (Reiter and Tan 2003, Tan *et al*. 1998, Lagneux *et al*. 2000, Dobšák *et al*. 2003). In our previous study, we demonstrated that physiological concentrations of melatonin were important in preventing I/R-induced cardiac infarct size (Sahna *et al*. 2002). On the other hand, melatonin (4 mg/kg) administration also tended to reduce the infarct size, but these changes did not reach statistical significance. Lee *et al*. (2002) showed that melatonin (5 mg/kg) inhibited ventricular arrhythmias and reduced the superoxide production resulting from I/R.

In the present study, we investigated the effects of 10 mg/kg doses of melatonin on the I/R-induced cardiac infarct size in an *in vivo* rat model. Furthermore, we also investigated the GSH and MDA levels, which are widely used as markers of oxidative stress. Effects of melatonin on GSH levels related to I/R in myocardial damage has not yet been well documented.

Methods

Experimental groups

Male Wistar rats weighing 250-300 g were placed in a quiet and temperature (21±2 °C) and humidity (60±5 %) controlled room in which a 12-12 h light-dark cycle was maintained. All experiments were performed between 9:00 and 17:00 h.

Rats were divided into three groups. Control (sham), I/R+vehicle and I/R+melatonin. Vehicle or melatonin (10 mg kg^{-1}) were administered by intravenous injection 10 min before ischemia. Infarct size and tissue GSH and MDA levels measurements were performed in 8 animals for each group. Melatonin (Sigma, St. Louis, MO, U.S.A.) was dissolved in ethanol and further diluted in saline to give a final concentration of 1 %.

All experiments in this study were performed in accordance with the guidelines for animal research from the National Institutes of Health and were approved by the Local Committee on Animal Research.

Ischemia-reperfusion procedure

Rats were anesthetized with urethane (1.2-1.4 g/kg) administered intraperitoneally. The jugular vein and the trachea were cannulated for drug administration and artificial respiration, respectively. Systemic blood pressure (BP) was monitored from the carotid artery by a Harvard model 50-8952 transducer (Harvard Apparatus, Inc., Massachusetts, U.S.A.) and displayed on a Harvard Universal pen recorder (Harvard Apparatus, Inc., Massachusetts, U.S.A.) together with a standard lead-1 ECG.

The chest was opened by a left thoracotomy, followed by sectioning the fourth and fifth ribs, about 2 mm to the left of the sternum. Positive-pressure artificial respiration was started immediately with room air, using a volume of 1.5 ml/100 g body weight at a rate 60 beats/min to maintain normal pCO_2, pO_2, and pH parameters.

After the pericardium was incised, the heart was exteriorized by gentle pressure on the right side of the rib cage. A 6/0 silk suture attached to a 10-mm micropoint reverse-cutting needle was quickly placed under the left main coronary artery. The heart was then carefully replaced in the chest, and the animal was allowed to recover for 20 min. Any animal in which this procedure produced arrhythmias or a sustained decrease in mean arterial BP to less than 70 mm Hg was discarded.

A small plastic snare was threaded through the ligature and placed in contact with the heart. The artery could then be occluded by applying tension to the ligature, and reperfusion was achieved by releasing the tension.

Evaluation of tissue death

The artery was occluded for 30 min and then reperfused for 120 min before the experiment was terminated. These durations of ischemia and reperfusion were already successfully used in the same experimental model (Kaneko *et al.* 2000, Sahna *et al.* 2002). ECG changes, BP, and heart rate (HR) were measured before and during occlusion and reperfusion.

At the end of each *in vivo* study the heart was quickly removed and mounted on a Langendorff apparatus where it was flushed with saline at room temperature for 60 s. The coronary branch was then reoccluded and fluorescent particles (1-10 μm in diameter from Duke Scientific Corp., Palo Alto, CA, USA) were infused into the perfusate to mark the risk zone (the non-fluorescent tissue). The heart was then frozen and a total of 4 tranverse slices, 2 mm in size, from each heart were cut starting from the apex. During this sectioning, a 0.5 mm wide full-thickness transverse sample was taken from the second cut from the apex for biochemical investigations and placed into liquid nitrogen and stored at –70 °C until assayed for biochemical analysis (the levels of MDA and GSH). For evaluation of tissue death, the slices were incubated in 1 % triphenyl tetrazolium chloride (TTC) in pH 7.4 buffer at 37 °C for 20 min. TTC stains from living tissue are of a deep red color while

necrotic tissue is TTC-negative and appears tan. The infarct and risk zone, considered to be the area lacking fluorescence under UV light, were traced. The volume of infarct and the risk zone was determined by planimetry of each tracing and multiplying by the slice thickness. Infarct size expressed as the percentage of the risk zone was measured as described previously (Sahna *et al.* 2002).

Biochemical determination

The heart sample was homogenized in ice-cold 150 mM KCI for determination of the levels of MDA. The MDA concentration of the homogenates was determined spectrophotometrically by measuring the presence of thiobarbituric acid reactive substances (Vermeulen and Baldew 1992). GSH was determined by the spectrophotometric method, which was based on the use of Elman's reagent. Results were expressed as nmol/g tissue.

Statistics

Data are expressed as arithmetic means ± S.E.M.; when $p<0.05$, the difference was considered to be statistically significant. Multiple comparisons between the experimental groups were performed by one-way analysis of variance with the Tukey *post hoc* test.

Results

There was no significant difference in mean arterial blood pressure or heart rate between both group at the beginning or throughout the experiment. Melatonin administration had no significant effect on hemodynamic parameters during ischemia or reperfusion (Table 1).

Table 2 presents the summary of risk zone, infarct size and infarct size/risk zone ratio. Melatonin administration (10 mg kg^{-1}, before ischemia) to rats significantly reduced the infarct size ratio.

MDA levels were significantly higher in the I/R group than in sham-operated control group. Melatonin significantly reduced the increased MDA values (Table 3). GSH levels were lower in the I/R rats than in the controls. Melatonin administration significantly increased the GSH levels.

Table 1. Summary of hemodynamic variables.

Groups	End of stabilization	End of ischemia	Reperfusion (min)		
			30	60	120
BP (mm Hg)					
I/R + vehicle	87 ± 3	73 ± 5	82 ± 5	88 ± 3	84 ± 3
I/R + melatonin (10 mg.kg^{-1})	77 ± 4	68 ± 3	75 ± 1	83 ± 2	80 ± 2
HR (beats min^{-1})					
I/R + vehicle	307 ± 11	360 ± 14	326 ± 13	333 ± 11	330 ± 9
I/R + melatonin (10 mg.kg^{-1})	313 ± 17	320 ± 18	328 ± 11	326 ± 11	324 ± 8

Values are means ± S.E.M. n=8 in each group, ischemia-reperfusion (I/R), mean arterial blood pressure (BP), heart rate (HR).

Table 2. Summary of infarct size data.

Group	Risk zone (cm^3)	Infarct size (cm^3)	Infarct size/ Risk zone (%)
I/R+vehicle	45 ± 3	23 ± 3	50 ± 4
I/R+melatonin (10 mg/kg^{-1})	48 ± 1	16 ± 1[a]	35 ± 1[a]

Values are means ± S.E.M. n=8 in each group. Ischemia-reperfusion (I/R), [a] significant difference resulting from melatonin administration ($p<0.05$).

Table 3. Effects of melatonin and ischemia-reperfusion (I/R) on the heart glutathione (GSH) and malondialdehyde (MDA) levels.

Groups	GSH (nmol/g)	MDA (nmol/g)
Control	675 ± 48	55 ± 5
I/R + vehicle	462 ± 25	141 ± 10[a]
I/R + melatonin	794 ± 140[b]	52 ± 34[c]

Values are means ± S.E.M. n=8 in each group. [a] significantly different from sham-operated group ($p<0.001$), [b] significant difference resulting from melatonin administration, ($p<0.02$), [c] significant difference resulting from melatonin.

Discussion

In this study, we demonstrated that the cardiac infarct size resulting from I/R was significantly reduced by melatonin administration. I/R increased MDA levels comparative to control and melatonin administration significantly reduced the increased MDA values. I/R leads to lower GSH levels and melatonin administration significantly increased the GSH levels. These results suggest that melatonin might reduce I/R-induced damage.

The protective effect of melatonin on I/R-induced infarct size has been demonstrated in the rat heart. Lagneux *et al.* (2000) suggested that melatonin reduced I/R-induced infarct size in the isolated rat heart. In our previous study on pinealectomized rats, we demonstrated that the physiological concentrations of melatonin were important in preventing the changes of I/R-induced cardiac infarct size in *in vivo* rat model (Sahna *et al*. 2002). On the other hand, melatonin administration to rats with intact pineal failed to attenuate significantly the I/R-induced infarct size. We reported that melatonin (4 mg/kg) given to pineal intact rats also tended to reduce the infarct size, but these changes did not reach statistical significance (Sahna *et al*. 2002). Similarly, Szárszoi *et al.* (2001) have shown that low concentration of melatonin had no effect on I/R injury of the isolated perfused rat heart. However, Lee *et al*. (2002) demonstrated that melatonin (5 mg/kg) markedly suppressed ventricular tachycardia and fibrillation and also reduced the superoxide production and lowered myloperoxidase activity (an index of neutrophil infiltration) resulting from I/R. We aimed to investigate the effects of high doses of melatonin on the I/R-induced cardiac infarct size and oxidative stress in an *in vivo* rat model.

It is generally accepted that reperfusion of the heart after a period of ischemia causes oxidative damage that is indicated by generation of free radicals, intracellular calcium overloading and loss of membrane phospholipids. At the onset of reperfusion, the mitochondrial respiratory rate is increased markedly and greater quantities of free radicals are generated. Cardiac myocytes, endothelial cells, and infiltrating neutrophils contribute to this enhanced ROS production. These radicals may exceed the capacity of the cellular intrinsic free radical scavenging systems and lead to cellular dysfunction and death (Lefer and Granger 2000).

Melatonin is known to directly detoxify superoxide-based free radicals and related reactants. Melatonin is capable of direct scavenging a number of oxygen-based reactants, such as hydroxyl radical, superoxide anion radical, and singlet oxygen and neutralized the nitrogen-based reactant peroxynitrite anion (Reiter *et al*. 2000, 2001). Besides its direct free radical scavenging actions, melatonin activates antioxidant enzymes. Some of melatonin's antioxidant actions probably derive from its stimulatory effect on superoxide dismutase, glutathione peroxidase (GSH-Px), glutathione reductase (GSH-Rd), and glucose-6-phosphate dehydrogenase and its inhibitory action on inducible nitric oxide synthase (Reiter and Tan 2003). Experimental evidence has shown that melatonin also promotes the activity of GSH-Rd, thereby helping to maintain high levels of reduced GSH (Hara *et al.* 2001).

In addition to being an effective free radical scavenger, melatonin also decreases intracellular calcium concentrations (Vaněček 1995). Furthermore, it was shown that melatonin can inhibits lipid peroxidation products (Dobšák *et al*. 2003). Kaneko *et al*. (2000) shown that melatonin eliminated hydroxyl radicals and decreased lipid peroxidation induced by I/R in isolated rat heart. In agreement with our findings, Dobšák *et al*. (2003) demonstrated that melatonin limits the MDA level resulting from I/R in isolated working rat heart. Melatonin reduced the I/R damage to other organs such as the kidney (Sahna *et al*. 2003a), liver (Sener *et al*. 2003) and brain (Pei *et al*. 2003) in rats. Similarly, melatonin protects against cardiotoxicity induced by chemotherapeutic drugs, which are often toxic in cardiac tissue (Sahna *et al*. 2003b).

These results showed that oxidative stress contributes to myocardial I/R injury and melatonin administration exerts an infarct size limiting effect. The protective actions of melatonin can be attributed to the reduction of free radical-mediated lipid peroxidation and increased antioxidative defence system. GSH levels, which are an indicator of the antioxidative defence system, were reduced in I/R group, but they were significantly increased in the melatonin-treated group. This is also the first finding that shows the relationship between melatonin and GSH due to myocardial I/R damage. Our findings suggest that melatonin may have considerable effects on infarct size and oxidative changes in the heart resulting from I/R. Consequently, they support the idea that melatonin has cardioprotective effects and it may play a role in the pathophysiology of cardiovascular diseases.

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