Melatonin protects against heart ischemia-reperfusion injury by inhibiting mitochondrial permeability transition pore opening

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Am J Physiol Heart Circ Physiol 297: H1487–H1493, 2009. First published August 14, 2009; doi:10.1152/ajpheart.00163.2009.— Melatonin, a well-known antioxidant, has been shown to protect against ischemia-reperfusion myocardial damage. Mitochondrial permeability transition pore (MPTP) opening is an important event in cardiomyocyte cell death occurring during ischemia-reperfusion and therefore a possible target for cardioprotection. In the present study, we tested the hypothesis that melatonin could protect heart against ischemia-reperfusion injury by inhibiting MPTP opening. Isolated perfused rat hearts were subjected to global ischemia and reperfusion in the presence or absence of melatonin in a Langerdoff apparatus. Melatonin treatment significantly improves the functional recovery of Langerdoff hearts on reperfusion, reduces the infarct size, and decreases necrotic damage as shown by the reduced release of lactate dehydrogenase. Mitochondria isolated from melatonin-treated hearts are less sensitive than mitochondria from reperfused hearts to MPTP opening as demonstrated by their higher resistance to Ca\(^{2+}\). Similar results were obtained following treatment of ischemic-reperfused rat heart with cyclosporine A, a known inhibitor of MPTP opening. In addition, melatonin prevents mitochondrial NAD\(^+\) release and mitochondrial cytochrome c release and, as previously shown, cardiolipin oxidation associated with ischemia-reperfusion. Together, these results demonstrate that melatonin protects heart from reperfusion injury by inhibiting MPTP opening, probably via prevention of cardiolipin peroxidation.

Reperfusion following a prolonged period of ischemia leads to myocardial dysfunction and irreversible damage characterized by cardiomyocyte hypercontracture, reduction of left ventricular pressure, augmented vascular resistance, elevated incidence of ventricular fibrillation. Mitochondria are known to be intimately involved in the processes that lead to cell death following reperfusion, in both necrotic and apoptotic forms of cell death and are therefore potential target for protective intervention (10, 41). Reactive oxygen species (ROS), which are produced mainly at the level of complex I and III of the respiratory chain, are considered an important factor in producing lethal cell injury associated with cardiac ischemia-reperfusion (I/R) (2, 9, 27, 50, 51). Mitochondria isolated from ischemic-reperfused rat hearts exhibit impaired mitochondrial function and depressed respiratory chain activity that are due, in part, to ROS-induced cardiolipin peroxidation (25, 31, 35). ROS production, cardiolipin peroxidation, and respiratory chain impairment are linked to each other to create a vicious cycle that leads to the decline in mitochondrial bioenergetics and subsequent mitochondrial dysfunction associated with I/R (30).

Growing evidence has become available supporting a crucial role of mitochondrial permeability transition in cardiomyocyte cell death occurring during I/R (7, 16, 17). Permeability transition is caused by the opening of the mitochondrial permeability transition pore (MPTP), a multiprotein megachannel connecting the mitochondrial matrix compartment with the cytosol. Massive MPTP opening results in mitochondrial depolarization, swelling and rupture of the external mitochondrial membrane, uncoupling of the respiratory chain, and efflux of cytochrome c and other proapoptotic factors that may lead to either apoptosis or necrosis. It has been suggested that MPTP remains closed during the ischemic period, during which time its opening is inhibited by the lactate-induced acid conditions (15). At reperfusion there is an influx of Ca\(^{2+}\) into the mitochondria, a burst of ROS production and rapid correction of the acidosis. All these factors contribute to increase the opening probability of MPTP in the early minutes of reperfusion following a prolonged period of ischemia. Identification and characterization of agents and interventions that can protect the heart from the damaging effect of MPTP are of considerable importance in attenuating I/R-induced cardiac dysfunction.

Several recent publications present evidence that melatonin and several of its metabolites have significant protecting actions against cardiac damage and altered physiology that occur during I/R (22, 38, 40, 43, 47, 48). In this respect, we have recently reported that melatonin has a strong protective effect against mitochondrial dysfunction associated with cardiac I/R (34). This effect appears to be due, at least in part, to the ability of this compound to preserve the content and integrity of cardiolipin molecules, which play a critical role in mitochondrial bioenergetics (18, 45). Moreover, melatonin has been shown to reduce early changes in the intramitochondrial cardiolipin during apoptosis (20, 26) and also to inhibit the mitochondrial permeability transition (3).

We recently reported that peroxidized cardiolipin behaves as an inducer of the mitochondrial permeability transition in isolated rat heart mitochondria, lowering the threshold of calcium for inducing this process and/or potentiating the effect of this cation in MPTP opening (33). This effect of peroxidized cardiolipin on MPTP is associated with a release of cytochrome c from mitochondria. It is thus conceivable that an increased level of peroxidized cardiolipin upon reperfusion might contribute to MPTP opening associated with cardiac posts ischemic reperfusion.
In view of these observations, we hypothesized that melatonin could protect heart against I/R injury by inhibiting MPTP opening through cardiolipin protection. Here, we use the Langendorff-perfused heart model of I/R to provide the first evidence showing that melatonin treatment can inhibit mitochondrial MPTP opening upon reperfusion in isolated mitochondria and intact heart and investigated the mechanism underlying this effect. Our data imply that melatonin protects heart from reperfusion injury by inhibiting MPTP opening through a mechanism involving the preservation of mitochondrial cardiolipin integrity. In addition, our results support a role of peroxidized cardiolipin in the MPTP opening following cardiac reperfusion.

**METHODS**

**Animal experiments.** Male Wistar rats (250–300 g) were used throughout these studies. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication no. 85-23, revised 1996). Rats received human care in accordance with Italian law (DL-116, 27 January 1992; Institutional license number from Ministero della Sanità: No. 23958-A) and in compliance with European Convention of Animal Care. After intraperitoneal injection of heparin (1,000 IU/kg) and administration of thiopental (50 mg), hearts were removed and then placed in ice-cold Krehs-Henseleit buffer. The aorta was cannulated, and the heart was perfused in retrograde fashion according to Langendorff constant-flow model (10 ml/min) with Krehs-Henseleit buffer, at 37°C, saturated with 95% O2-5% CO2. Hearts were placed in a water-jacketed chamber at 37°C.

The following groups of rat hearts (6 for each group) were employed for these experiments. Group 1 (control) hearts were perfused for 60 min at 37°C with a Krehs-Henseleit solution and used as control group. Group 2 (reperfused) hearts were perfused for 15 min and then subjected to global ischemia for 30 min followed by 15 min of reperfusion. Group 3 (reperfused + melatonin) hearts were perfused for 15 min with a Krehs-Henseleit solution in the presence of 50 μM melatonin and then subjected to global ischemia for 30 min, followed by 15 min of reperfusion with Krehs-Henseleit solution in the presence of 50 μM melatonin. Group 4 (control + melatonin) hearts were perfused for 60 min at 37°C with a Krehs-Henseleit solution in the presence of 50 μM melatonin. Group 5 (reperfused + cyclosporine A) hearts were perfused for 15 min with a Krehs-Henseleit solution in the presence of 0.2 μM cyclosporine A, then subjected to global ischemia for 30 min, followed by 15 min of reperfusion with Krehs-Henseleit solution in the presence of 0.2 μM cyclosporine A. These groups of hearts were used for mitochondrial preparations and experiments. For infarct size determination two other groups were employed. Group 6 (reperfused 120 min) hearts were perfused for 15 min and then subjected to global ischemia for 30 min followed by 120 min of reperfusion. Group 7 (reperfused 120 min + melatonin) hearts were perfused for 15 min with a Krehs-Henseleit solution in the presence of 50 μM melatonin and then subjected to global ischemia for 30 min, followed by 120 min of reperfusion with Krehs-Henseleit solution in the presence of 50 μM melatonin for the first 15 min.

Left ventricular isovolumic pressure was recorded by a strain-gauge pressure transducer (Hewlett-Packard Medical Electronic Division, Model 1280C, Waltham, MA). The left ventricular end-diastolic pressure (LVEDp) was adjusted to 5–10 mmHg.

Left ventricular pressure parameters were recorded just before ischemia and at the end of 15-min reperfusion. The following parameters were measured: LVEDP and left ventricular developed pressure (LVDP).

Samples of perfusate were collected at the end of 15-min reperfusion for the spectrophotometric determination of lactate dehydrogenase (LDH).

**Infarct size evaluation.** The 2,3,5-triphenyltetrazolium chloride staining technique was used to determine infarct size after 120-min reperfusion as previously shown (39). After each experiment, the heart was manually cut into four transverse slices (2–3 mm each). High-resolution slice images were digitally acquired, and then standard planometric analysis (ImageJ 1.32j software National Institutes of Health) was used to determine the infarct area. Since the entire ventricles were at risk from global ischemia, the infarct size was expressed dividing the necrotic area by the total slice area to obtain the percentage of necrosis. Infarct size was calculated as average of values measured on four slices for each heart.

**Isolation of mitochondria.** Rat heart mitochondria were isolated from the whole heart in a medium of 250 mM sucrose, 10 mM Tris-HCl, 1 mM EGTA, pH 7.4, by differential centrifugation of heart homogenates essentially as described previously (32). Mitochondria were resuspended in 250 mM sucrose-10 mM Tris-HCl (pH 7.4) and stored in ice. The yield of mitochondrial proteins (mg/g heart wet wt) within each group of animals was consistent, suggesting minimal variation in the preparations of the mitochondrial fraction.

Mitochondrial protein content was measured by the biuret method using serum albumin as standard.

**Mitochondrial incubation medium.** Unless otherwise specified, incubations of isolated mitochondria were conducted at 25°C in a standard reaction medium containing 150 mM sucrose, 50 mM KCl, 5 mM Tris (pH 7.4), 10 μM EGTA, 1 mM Pi, 2 mM pyruvate, and 5 mM malate, with continuous stirring.

**Calcium retention capacity of mitochondria.** Mitochondrial calcium retention capacity was evaluated following changes in the concentration of free extramitochondrial Ca2+ measured spectrophotometrically at 675–685 nm in the presence of 50 μM Arsenazo III as a free Ca2+ indicator, with an HP 8453 diode array spectrophotometer (44). Mitochondria (0.5 mg/ml) were added to standard incubation medium and loaded with a train of 5 μM Ca2+ pulses at 1-min intervals.

**Measurement of mitochondrial membrane potential.** Membrane potential in intact heart mitochondria was estimated by the safranine O method (1). Accumulation of safranine O in mitochondria is driven by membrane potential (ΔΨ) and results subsequently in decrease of fluorescence. Depolarization results in the release of safranine O from mitochondria and subsequent increase of fluorescence. Freshly isolated mitochondria (0.5 mg protein) were suspended in 3 ml of the standard reaction medium in the presence of 8 μM safranine O, and the formation of membrane potential was induced by the addition of 2 mM pyruvate and 5 mM malate as substrates. Changes of safranine O fluorescence were recorded by use of a Jasco FP-750 spectrophotometer operating at 525 nm excitation and 575 nm emission.

**Detection of mitochondrial NAD+ content.** The content of mitochondrial NAD+ was determined in perchloric extracts essentially as described in Refs. 6 and 11. Briefly, 1 mg of mitochondrial proteins was incubated with 0.1 ml of perchloric acid 21% (vol/vol) for 30 min in an ice-cold bath. Subsequently the suspension was centrifuged at 8,000 × g and the supernatant obtained was neutralized with KOH. The NAD+ present in the extract was determined by measuring the NAD+ dependent lactate dehydrogenase activity fluorometrically at λ ex 340 nm and λ em 460. The reaction medium contained glycine 500 mM, hydrazine 400 mM, pH 9 at 25°C. The reaction was started by the addition of 1-lactate 10 mM.

**Detection of cytochrome c release.** Mitochondrial cytochrome c content was determined by using a 5-mm C4 reverse-phase column (150 × 4.6 mm) on an HP series 1100 HPLC chromatograph. A gradient of 20% acetonitrile in water with trifluoroacetic acid (0.1% vol/vol) to 60% acetonitrile in water with trifluoroacetic acid (0.1% vol/vol) over 12 min with a flow rate of 1 ml/min was used. Absorption at 393 nm was used. To improve the sensitivity of the method, the samples were supplemented with BSA to a final concentration of 25 μM. The amount of cytochrome c was quantitated by the peak area derived from integrating the chromatographic peak (8).
Statistical analysis. Prism 4.00 software (GraphPad Software, San Diego, CA) was used to perform all statistical tests. All the results were expressed as means ± SE, and statistical significance for the comparison was determined by one-way ANOVA followed by Bonferroni correction.

RESULTS

Data regarding the hemodynamic performance of control Langerdoff-perfused hearts and ischemic-reperfused hearts treated or not with melatonin are reported in Table 1. Global normothermic ischemia followed by reperfusion led to a marked decline in the hemodynamic function in the control heart. Pretreatment of heart with melatonin significantly improved functional recovery of the heart during reperfusion as reflected in the greater LVDp and lower LVEDp. No significant difference in heart rate within or between groups was found.

The effect of melatonin administration on the infarct size during reperfusion were measured in isolated rat hearts. As shown in Fig. 1A, melatonin treatment resulted in significant reduction in infarct size. The release of LDH, an indicator of necrotic damage, was significantly increased in reperfused heart than control (Fig. 1B). Melatonin treatment substantially reduced this release.

In the experiment of Fig. 2, we tested the mitochondrial Ca\(^{2+}\) retention capacity, a sensitive and quantitative measure of the ability of mitochondria to open the MPTP in response to accumulated calcium (4, 12). Mitochondria from control perfused heart accumulated 210 ± 18 nmol of Ca\(^{2+}\) per mg protein, after which Ca\(^{2+}\) was spontaneously released (trace a), and this release is indicative of MPTP opening in that it was cyclosporine sensitive (not shown). Mitochondria isolated from I/R heart were less resistant to Ca\(^{2+}\) (90 ± 10 nmol per mg protein) was needed to trigger the opening of the MPTP (trace b). Melatonin treatment of I/R heart restored the tolerance of the MPTP to Ca\(^{2+}\) overload comparable to that of control animals (180 ± 16 nmol per mg protein) (trace c). Melatonin treatment of control perfused heart did not affect the mitochondrial calcium retention capacity (not shown). To assess that the release of Ca\(^{2+}\), following Ca\(^{2+}\) overload, was associated to MPTP opening, we tested the effect of cyclosporine A, a well-known inhibitor of MPTP (15, 16), on Ca\(^{2+}\) overload and release in mitochondria isolated from I/R rat heart. Treatment of I/R heart with 0.2 μM cyclosporine A resulted in a significant increase in Ca\(^{2+}\) overload (205 ± 20 nmol per mg protein), comparable to that of control animals, before this cation could be released from mitochondria, suggesting the involvement of MPTP in the release of Ca\(^{2+}\) observed in our experimental conditions.

It is well known that the MPTP opens at the beginning of the reperfusion and causes collapse of mitochondrial ΔΨ. Therefore, the opening of MPTP was also determined by measuring changes in mitochondrial ΔΨ. Mitochondrial membrane potential was measured by application of safranine O to mitochondria (1). After addition of mitochondria to the cuvette, in

| Table 1. Effect of melatonin on LVDp, LVEDp, and HR in Langerdoff-perfused rat heart subjected to ischemia and reperfusion |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | Control         | Melatonin       | Control         | Melatonin       |
| LVDp, mmHg      | Preischemic     | Preischemic     | Reperfused      | Reperfused      |
|                 | 85.00±6.0       | 90.00±4.0       | 55.00±6.0       | 80.00±5.0\*     |
| LVEDp, mmHg     | 4.00±0.7        | 4.00±0.8        | 50.0±10.0       | 24.00±7.0\*     |
| HR              | 282±12          | 294±10          | 269±13          | 274±14          |

Values are means ± SD of 6 different experiments. Left ventricular developed pressure (LVDp) and left ventricular end-diastolic pressure (LVEDp) were measured as described in METHODS. HR, heart rate. *P < 0.05 vs. reperfused in the absence of melatonin.
the presence of respiratory substrates pyruvate and malate, safranine O was accumulated in mitochondria by negative membrane potential decreasing the fluorescence signal (Fig. 3).

The addition of 30 μM Ca²⁺ to control mitochondria resulted in a transient reduction of ΔΨ due to electrophoretic Ca²⁺ uptake by the uniporter. Approximately 3 min after addition of Ca²⁺, ΔΨ recovered back to its control level and remained stable for at least 10 min (trace a). On the contrary, mitochondria from I/R heart built up a lower potential, compared with the controls, and exhibited a higher sensitivity to Ca²⁺ addition, as demonstrated by the irreversible drop in membrane potential (trace b). This Ca²⁺-induced drop in membrane potential was abolished by cyclosporine A (not shown). Mitochondria isolated from I/R melatonin-treated rats exhibited changes in ΔΨ almost similar to those observed in control heart, indicating the protective effect of melatonin on MPTP opening induced by I/R (trace c).

The opening of MPTP in the intact heart can be measured indirectly following the loss of the mitochondrial NAD⁺. In fact, it has been demonstrated that NAD⁺ content in intact heart is significantly lowered during reperfusion and that this process is prevented by cyclosporine A, reflecting the transition pore opening in situ (6, 11). To confirm the occurrence of MPTP opening in intact heart during the reperfusion and the protective effect of melatonin on this process, we measured NAD⁺ content in mitochondria isolated from control, reperfused, and melatonin-treated reperfused rat hearts. As shown in Fig. 4, the NAD⁺ content was significantly decreased (30%) in mitochondria from I/R heart compared with control heart. Melatonin treatment almost completely prevented this NAD⁺ loss.
It is well established that MPTP opening causes mitochondrial swelling and rupture of the outer mitochondrial membrane. This leads to release of proapoptotic factors including cytochrome c. As an additional index of MPTP opening during I/R and of the protective effect of melatonin on this process, the release of cytochrome c from mitochondria was analyzed. This was assessed measuring the loss of mitochondrial cytochrome c content. For direct evaluation of mitochondrial cytochrome c content we used a very rapid and sensitive method based on reverse-phase HPLC (8). As shown in Fig. 5, a significant loss of cytochrome c (~30%) was observed in mitochondria from ischemic reperfused rats compared with control mitochondria. Melatonin treatment almost completely prevented this loss of cytochrome c. These data show that melatonin treatment significantly and completely inhibits the mitochondrial cytochrome c release normally observed following myocardial I/R.

As previously reported, peroxidized cardiolipin, in the presence of Ca$^{2+}$, induces mitochondrial permeability transition and cytochrome c release in rat heart mitochondria (33). It is thus possible that an increased level of peroxidized cardiolipin might contribute, together with Ca$^{2+}$, to the opening of MPTP induced by I/R and that melatonin could prevent MPTP opening and cytochrome c release by preserving the integrity of cardiolipin molecules. Indeed, in a previous study we have shown that the content of peroxidized cardiolipin significantly increased in mitochondria isolated from I/R heart compared with control heart and that melatonin treatment largely prevented this increase (34).

**DISCUSSION**

Melatonin, an hormonal product of the pineal gland, has been shown to protect against ischemic-reperfusion myocardial damage, although the mechanism by which this compound exerts the cardioprotective effect is not well established (22, 34, 40, 42–43, 45–47). Recent data suggest that some of the cell protective effect of melatonin may be produced through its action at mitochondrial level (24). In line with this, we previously reported that melatonin protects against mitochondrial dysfunction associated with cardiac I/R, by preventing alterations to several parameters involved in mitochondrial bioenergetics (34). The opening of the MPTP is considered a critical event in heart reperfusion injury (7, 16, 17). In addition, it has been reported that melatonin inhibits the MPTP and this contributes to its neuroprotective effect in cerebral ischemia (3). In this study, we examined the mechanistic basis for reported melatonin-mediated cardioprotection following I/R. Specifically, we tested the possibility that the inhibition of MPTP opening during reperfusion might be involved in this cardioprotective effect of melatonin.

The data presented show, for the first time, that the protection against reperfusion injury provided by melatonin treatment of isolated reperfused rat heart is associated with inhibition of the MPTP opening. Thus, in the reperfused heart, the enhanced recovery of hemodynamic functions of the reperfused heart mediated by melatonin treatment as well as the reduction in infarct size and the decreased necrotic damage as shown by the release of LDH were accompanied by the inhibition of MPTP opening detected in situ by the mitochondrial release of NAD$^+$ (6, 11). Furthermore, our results show that mitochondria isolated from melatonin-treated I/R heart were less sensitive than control mitochondria to MPTP opening, as demonstrated by their higher resistance to Ca$^{2+}$ (see Figs. 2 and 3). Similar results were obtained following treatment of I/R rat heart with cyclosporine A, a well-known inhibitor of MPTP opening (15, 16). Altogether, these results demonstrate that melatonin protects against heart I/R injury by inhibiting MPTP opening.

We also investigated the possible mechanism involved in the inhibition of MPTP opening during reperfusion by melatonin treatment. Ca$^{2+}$ overload is considered an important factor responsible for MPTP opening associated with I/R (14). However, measurements of mitochondrial Ca$^{2+}$ in isolated cardiac myocytes imply that it is the mitochondrial Ca$^{2+}$ at the end of ischemia rather than during reperfusion that correlates better with cell injury (29). Furthermore, in isolated heart mitochondria MPTP opening is usually obtained by the addition of relatively high Ca$^{2+}$ concentrations. Such high Ca$^{2+}$ concentrations are hardly achieved in viable cells, suggesting that Ca$^{2+}$ alone does not cause MPTP opening directly and that MPTP is sensitized to Ca$^{2+}$ by additional factors, particularly by ROS (23) or products of their oxidative attack to mitochondrial constituents.

Cardiolipin, a phospholipid component of the inner mitochondrial membrane, is known to be intimately involved in several mitochondrial bioenergetic processes (18, 45) as well as in the regulation of the initial steps of cell death (13). Alterations in the structure and/or content of this phospholipid are responsible for mitochondrial dysfunction in a variety of pathological settings (5a, 25, 30, 31, 35). Cardiolipin molecules are particularly susceptible to ROS attack either because of their high content of unsaturated fatty acids or because their location near to the site of ROS production mainly at the level of complex I and III of the respiratory chain (30, 31, 35). Our previous data showed that treatment of rat heart mitochondria with micromolar concentrations of peroxidized cardiolipin lowers the threshold of Ca$^{2+}$ required to induce matrix swell-
In conclusion, our data support a possible new role of peroxidized cardiolipin in MPTP opening during heart reperfusion and also demonstrate that melatonin, at pharmacological concentrations, strongly protects against cardiac reperfusion injury. The mechanism of this protection appears to be due, at least in part, to the inhibition of MPTP opening via prevention of cardiolipin peroxidation. Therapeutic use of melatonin may provide a useful strategy for the treatment of cardiac reperfusion injury as well as for other cardiovascular disorders with mitochondrial oxidative damage in their etiology.

REFERENCES


