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Melatonin ameliorates calcium homeostasis in myocardial and ischemia–reperfusion injury in chronically hypoxic rats

Abstract: Chronic hypoxia (CH) leads to the deterioration of myocardial functions with impaired calcium handling in the sarcoplasmic reticulum (SR), which may be mediated by oxidative stress. We hypothesized that administration of antioxidant melatonin would protect against cardiac and ischemia–reperfusion (I/R) injury by ameliorating SR calcium handling. Adult Sprague–Dawley rats that had received a daily injection of melatonin or vehicle were exposed to 10% oxygen for 4 wk. The heart of each rat was then dissected and perfused using a Langendorff apparatus. The ratio of heart-to-body weight, ventricular hypertrophy and hematocrit were increased in the hypoxic rats compared with the normoxic controls. Malondialdehyde levels were also increased in the heart of hypoxic rats and were lowered by the treatment of melatonin. The hearts were subjected to left coronary artery ischemia (30 min) followed by 120-min reperfusion. Lactate dehydrogenase leakage before ischemia, during I/R and infarct size of the isolated perfused hearts were significantly elevated in the vehicle-treated hypoxic rats but not in the melatonin-treated rats. Spectrofluorometric studies showed that resting calcium levels and I/R-induced calcium overload in the cardiomyocytes were more significantly altered in the hypoxic rats than the normoxic controls. Also, the hypoxic group had decreased levels of the SR calcium content and reduced amplitude and decay time of electrically induced calcium transients, indicating impaired contractility and SR calcium re-uptake. Moreover, there were reductions in protein expression of calcium handling proteins, markedly shown at the level of SR-Ca^{2+} ATPase (SERCA) in the heart of hypoxic rats. Melatonin treatment significantly mitigated the calcium handling in the hypoxic rats by preserving SERCA expression. The results suggest that melatonin is cardioprotective against CH-induced myocardial injury by improving calcium handling in the SR of cardiomyocytes via an antioxidant mechanism.

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Key words: antioxidant, calcium, cardioprotection, chronic hypoxia, ischemia, melatonin

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Introduction

Myocardial hypoxia can be caused by ischemic hypoxia with a local reduction of coronary blood flow and by systemic hypoxia with lower levels of arterial oxygen. It is well accepted that myocardial injury is closely associated with the severity of ischemic hypoxia relevant to the clinical manifestation of ischemic heart disease. Yet myocardial injury, if any, caused by systemic hypoxia at moderate levels under chronic conditions is still controversial. This may relate to the fact that myocardial function is essentially normal in high-altitude natives, in subjects during ascent and sojourns at high altitudes, and in patients with chronic obstructive pulmonary disease (COPD). Many studies have showed that short-term hypoxia can confer cardioprotection against myocardial infarction, contractile dysfunction and ventricular arrhythmias [1–3]. However, hypoxia in the long term could have adverse effects on myocardial functions, particularly shown by the deterioration of calcium (Ca^{2+}) homeostasis in sarcoplasmic reticulum (SR) [4, 5] and by impaired Gs(alpha) and adenyl cyclase intracellular signaling functions leading to altered myocardial responses to beta-adrenoceptor activation [6].

Recent studies have shown that overproduction of reactive oxygen species (ROS) is a major mechanistic cause of myocardial injury during ischemia/hypoxia–reperfusion (I/R) [7–9]. Such a robust increase in cellular ROS levels could lead to opening of mitochondrial permeability transition pore [10, 11] and impaired intracellular Ca^{2+} homeostasis [12, 13]. Hence, one rationale behind pharmacologically induced cardioprotection against I/R injury was to lower the ROS level during the oxidative insult. However, it is not clear if this pharmacological strategy could be beneficial to myocardial functions under chronically hypoxic conditions.

It is well known that melatonin, as well as its metabolites are potent antioxidants [14, 15] and melatonin is a cardioprotective agent against I/R injuries [16–22]. The
mechanistic effect of melatonin on I/R-induced myocardial infarction is commonly attributed to its antioxidant properties and its ability to scavenge free radical species [23–25] while also stimulating the synthesis of antioxidant enzymes [26–28]. Although recent studies have documented the acute effects of melatonin against myocardial I/R injury, currently there is a lack of information on the chronic effect of melatonin on myocardial functions in prolonged hypoxia (CH). In this study, we examined the hypothesis that chronic administration of melatonin can alleviate CH-induced myocardial injury in rats. Results suggested that the melatonin treatment could reduce the level of oxidative stress and mitigate SR-\(\text{Ca}^{2+}\) handling in the cardiomyocytes leading to improved myocardial functions in the hypoxic rats.

**Material and methods**

**Animals**

Animal care and experimental protocol for this study were approved by the Committee on the Use of Live Animals in Teaching and Research of The University of Hong Kong. The Laboratory Animal Unit of The University of Hong Kong is fully accredited by the Association for Assessment and Accreditation for Laboratory Animal Care (AAALAC International). Male Sprague–Dawley rats weighing about 70 g at age 28 days were randomly divided into normoxic and CH groups as reported previously [4–6]. While the normoxic controls were maintained in room air, CH rats were kept in a 300-L acrylic chamber for normobaric hypoxia in the same room and had free access to water and chow. The oxygen fraction inside the chamber was kept at 10 ± 0.5%, 24 hr per day. The desired oxygen content was established by a mixture of room air and nitrogen that was regulated and monitored by an oxygen analyzer (Vacumetrics Inc., CA, USA) [29]. The rats were exposed to hypoxia for 28 days and were immediately used in the experiments. The animal room was controlled at a constant temperature (22 ± 2°C), humidity and light:dark cycle (lights on 07:00–19:00).

**Drug preparation**

Melatonin (Sigma, St. Louis, MO, USA) solution was prepared fresh before injection by dissolving the indoleamine in absolute ethanol and further dilution with normal saline; the final concentration of ethanol was 2%. As reported previously, melatonin in 10 mg/kg body weight [30–33] or vehicle (2% ethanol in normal saline) was administered intraperitoneally each day. The rats were taken out of the chamber for 30 min for the administration.

**Isolated perfused heart preparation**

Rats from normoxic and CH groups (both melatonin- and vehicle-treated groups) were decapitated and the hearts were quickly removed and placed in ice-cold Krebs–Henseleit (K-H) perfusion buffer before being mounted on the Langendorff apparatus for perfusion at 37°C with K-H buffer at constant pressure (100 cm of H\(_2\)O) and equilibrated with 95% of O\(_2\)/5% CO\(_2\). The buffer contained (in mm) 118.0 NaCl, 4.7 KCl, 1.25 CaCl\(_2\), 1.2 KH\(_2 PO_4\), 1.2 MgSO\(_4\), 25.0 NaHCO\(_3\) and 11.0 glucose. All the hearts were subjected to regional ischemia described previously [34, 35]. Briefly, a snare was formed by placing a silk suture around the left coronary artery of rat heart. The snare was pulled in order to occlude the coronary artery to produce ischemia. Reperfusion was done by releasing the snare. In this present study, the isolated rat hearts were subjected to 30 min of ischemia followed by 120 min of reperfusion which induced myocardial injury.

**Measurement of the area of risk**

To determine the infarct size, the coronary artery was re-occluded at the end of reperfusion and the heart was perfused with 2.5% Evans blue to delineate the area of risk. The hearts were frozen at –70°C, cut into thin slices, which were perpendicular to the septum, from the apex to the base. Then incubated in sodium phosphate buffer containing 1% (w/v) 2,3,5-triphenyl-tetrazolium chloride for 10 min in order to visualize the unstained infarct region. The infarct and the risk zone areas were determined by planimetry with software Image/J from the National Institutes of Health (Bethesda, MD). The infarct size measured was expressed as a percentage of the risk zone.

**Determination of myocardial injury by lactate dehydrogenase (LDH) efflux**

The effluent from each isolated perfused rat heart was collected at 15 min before regional ischemia, 5th min of reperfusion and the LDH was assayed spectrophotometrically by using a kit purchased from Sigma-Aldrich (St. Louis, MO, USA). The LDH activity measured was expressed as units per liter.

**Measurement of lipid peroxidation**

The lipid peroxidation was determined by using a Bioxytech® LPO-586™ kit (OxisResearch, OR, USA). The reaction product was measured spectrophotometrically at 586 nm. Standard curves were constructed with 1,1,3,3-tetraethoxypropane as a standard. The level of malondialdehyde (MDA) in the heart is expressed as \(\mu\)M, while the MDA concentration (\(\mu\)M) in heart was normalized to wet tissue weight (mg) and expressed as \(\mu\)M/mg.

**Preparation of isolated ventricular myocytes**

Single ventricular myocytes were isolated from the normoxic and CH rats by using a collagenase method described previously [35, 36]. After isolation, myocytes were allowed to stabilize for at least 30 min before any experiment.

**Measurement of \([\text{Ca}^{2+}]_i\)**

A spectrofluorometric method with Fura-2/AM as a \(\text{Ca}^{2+}\) indicator was used during the measurement of \([\text{Ca}^{2+}]_i\). Ventricular myocytes from either normoxic or hypoxic rats were incubated with 5 \(\mu\)M Fura-2/AM for 35 min.
Fluorescent signals obtained at 340 nm (F340) and 380 nm (F380) excitation wavelengths were recorded and stored in computer for data processing and analysis. The F340/F380 ratio was used to indicate cytosolic [Ca$^{2+}$]$^i$ in the ventricular myocytes. During the measurement of electrically induced [Ca$^{2+}$]$^i$, transients (E[Ca$^{2+}$]$^i$), myocytes were electrically stimulated at 0.2 Hz, whereas measurement of caffeine-induced [Ca$^{2+}$]$^i$, transients (C[Ca$^{2+}$]$^i$) were done by applying 10 mM caffeine directly to the ventricular myocytes. The amplitude of E[Ca$^{2+}$]$^i$, and C[Ca$^{2+}$]$^i$ were determined as the difference between the resting and the peak [Ca$^{2+}$]$^i$ levels; the time for 50% decay of the transients (T$_{50}$) was used to represent the decay of both transients. During the measurements, the isolated fura-2-loaded cardiomyocytes from each group were incubated for 10 min with non-glucose K-H solution containing 10 mM 2-deoxy-D-glucose and 10 mM sodium dithionite to induce metabolic inhibition and anoxia (M/A). Reperfusion (M/A-R) was followed by incubating the myocytes with normal K-H solution for further 10 min.

Western blotting for SERCA, RyR and NCX

Isolated cardiomyocytes from normoxic and hypoxic groups were collected after collagenase digestion from whole hearts. To detect the expression of SERCA2 and RyR, SR vesicles were obtained by following procedures. Briefly, myocytes were sonicated on ice in the extraction medium containing (in mM): 15 Tris, 10 NaHCO$_3$, 5 Na$_2$HPO$_4$, 250 sucrose and 1 EDTA (pH 7.3). The homogenate were centrifuged for 10 min at 1000 g to remove cellular debris. The supernatant was further centrifuged for 35 min at 20,000 g. Then the pellet was re-suspended in a mixture of 0.6 M KCl and 0.03 M histidine (pH 7.0) and centrifuged for 35 min at 20,000 g. The final pellet was re-suspended in a mixture of 0.25 M sucrose and 0.01 M histidine (pH 7.3) and stored at −70°C. All solutions contained three proteases inhibitors: 1 mg/mL leupeptin, 1 mg/mL aprotinin and 1 mM phenylmethylsulfonyl fluoride (PMSF). For the measurement of NCX, purification of plasma membrane vesicles were carried out as described above. The pellet i.e. sarcolemma-enriched fraction was dissolved in the lysis buffer (0.6 M sucrose and 10 mM imidazole–HCl, pH 7.0) and stored at −70°C. The protein concentration of the samples was quantified by the Bio-Rad protein assay method by using bovine serum albumin (BSA) for the standard curve. Sample proteins (20 μg/lane) were separated in SDS-polyacrylamide gel (10% for SERCA and NCX; 6% for RyR) and transferred electrophoretically to polyvinylidene difluoride membranes (0.2 μm pore size; Bio-Rad) at 4°C in transfer buffer with glycine, Tris and 20% methanol with the Bio-Rad Trans-blot electrophoretic transfer system. After blocking with Tris-buffered saline (TBS; with Tris, NaCl and 0.2% Tween 20) containing 5% non-fat milk, the membranes were incubated overnight at 4°C with the goat anti-SERCA2a polyclonal antibody (1:400 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-RyR2 monoclonal antibody (1:3330 dilution; Affinity BioReagent, Golden, CO, USA), mouse anti-NCX1 monoclonal antibody (1:500 dilution; Abcam, Cambridge, UK) and mouse anti-β-actin monoclonal antibody (1:5000 dilution; Santa Cruz Biotechnology). The second antibody for both protein determinations was anti-goat or anti-mouse antibody conjugated to horseradish peroxidase (1:2000 dilution; Dakocytomation, Denmark A/S) in 5% non-fat milk TBS for 1 hr at room temperature. The protein bands of SERCA, RyR and NCX were detected by the chemiluminescence method (ECL Western blotting detection; Amersham Biosciences).

Statistical analysis

Values are expressed as means ± S.E.M. One-way ANOVA (Turkey’s multiple comparison test) was used to determine the differences among the multiple groups. The significance level was set at $P < 0.05$.

Results

As shown in Fig. 1, the ratio of heart-to-body weight was increased in the CH rats (vehicle- and melatonin-treated
groups were, respectively, 143.8% and 125% of the normoxic control. The ratio of left ventricular weight to the right ventricle and septum in the vehicle-treated hypoxic rat was increased by 10.1% of the normoxic control, but was not different between the melatonin-treated hypoxic rat and the normoxic control (Fig. 1B). Hematocrit was increased by 17.0% and 21.2%, respectively, in the vehicle- and melatonin-treated rats, comparing with the normoxic control (Fig. 1C).

Fig. 2 shows the MDA level in the cardiac tissue, representing the level of lipid peroxidation under oxidative stress. The MDA level significantly increased in the vehicle group, which was 313% of the normoxic control, but the increase was much less at 25% in the melatonin group.

Before ischemia, the resting level of the LDH release from the perfused heart of the vehicle-treated hypoxic rats was significantly elevated by 38.6% of the normoxic control (Fig. 3A). There were no differences in the resting LDH release between the melatonin-treated group and the normoxic control, suggesting that melatonin reduced the CH-induced myocardial injury. In addition, the LDH release during 5 min reperfusion after ischemia was markedly increased in the vehicle group (127% of the normoxic control) but not in the melatonin-treated group (105% of the normoxic control, Fig. 3B). Moreover, the infarct size of the hearts in the melatonin-treated hypoxic rat (101% of the normoxic control) was significantly less than that of the vehicle group (124% of the normoxic control, Fig. 3C).
Before ischemia, the resting level of cytosolic calcium in isolated cardiomyocytes was higher in the hypoxic rats treated with vehicle than those of the melatonin-treated rats and the normoxic control (Fig. 4). Following 10 min MI/A-R, the calcium overloading was worsened in the vehicle group (110% of the resting level), but was significantly less in the melatonin group (106% of the resting level), compared with that of the normoxic control (107% of the resting level).

The amplitude of caffeine-induced \( [Ca^{2+}] \) transients from SR \([37, 38]\), were not different between the corresponding VCH group; \(*P < 0.05\) versus corresponding normoxic group; \(**P < 0.01\) versus corresponding normoxic group; \(P < 0.05\) versus corresponding VCH group; \(P < 0.05\) versus corresponding group before MI/A-R.

Following MI/A-R, the amplitudes were decreased further by 25% and 28% of the levels before MI/A-R, respectively in the normoxic control and vehicle-treated hypoxic rats. However, the decrease was less in the hypoxic rats treated with melatonin (23% of the level before MI/A-R), reflecting that SR-Ca\(^{2+}\) content was much less in the melatonin-treated rats, which was by 16% of the normoxic control. Following MI/A-R, the amplitudes were decreased further by 25% and 28% of the levels before MI/A-R, respectively in the normoxic control and vehicle-treated hypoxic rats. However, the decrease was less in the hypoxic rats treated with melatonin (23% of the level before MI/A-R), reflecting that SR-Ca\(^{2+}\) content was much less in the melatonin-treated rats, which was by 16% of the normoxic control. Following MI/A-R, the amplitudes were decreased further by 25% and 28% of the levels before MI/A-R, respectively in the normoxic control and vehicle-treated hypoxic rats. However, the decrease was less in the hypoxic rats treated with melatonin (23% of the level before MI/A-R), reflecting that SR-Ca\(^{2+}\) content was much less in the melatonin-treated rats, which was by 16% of the normoxic control.

The time to peak \(T_{50}\) of \(E[Ca^{2+}]\) which represents mainly the \(Ca^{2+}\) reuptake to SR via SERCA (>90% \(Ca^{2+}\) in cytoplasm) and partly the extrusion to extracellular space by NCX [42, 43], was longer in the hypoxic rats than that of the normoxic group (by 37% and 23%, respectively, in vehicle- and melatonin-treated rats). During MI/A-R, the values were markedly prolonged in the normoxic control (121% of that before MI/A-R). However, the increase in \(T_{50}\) value was significantly less in the melatonin-treated rats (13% of that before MI/A-R) than that of the hypoxic rats treated with vehicle (23% of that before MI/A-R). Moreover, the time to peak of \(E[Ca^{2+}]\) (Fig. 6C) which indicates the speed of \(Ca^{2+}\) release via RyR of SR [6], was not different between the
hypoxic and normoxic groups before MI/A. During MI/A-R, the value was markedly increased in the normoxic group (9% of the level before MI/A-R) but the increase was not different from the melatonin-treated hypoxic rats.

The cardiac SERCA2a expression (Fig. 7A) was sharply decreased in the vehicle-treated hypoxic rats by 53% of the normoxic control. The reduced expression was significantly less in the hypoxic rats treated with melatonin, which was by 18.8% of the normoxic control. Yet, levels of the protein expression of Na\(^+\)/Ca\(^{2+}\) exchanger (NCX1) (Fig. 7B) and ryanodine receptor (RyR2) (Fig. 7C) in CH rat hearts were not different from those of the normoxic control.

Discussion

The major finding of this study is that melatonin markedly lowered the levels of oxidative stress, [Ca\(^{2+}\)]\(_i\) overload and I/R injury in the myocardium by ameliorating SERCA expression and function for the calcium handling in the SR of cardiomyocytes in CH rats. Results support the contention that antioxidant melatonin and/or its metabolites are cardioprotective against CH-induced myocardial injury.

In consistent to our previous findings [4–6], the CH heart was hypertrophied with diminished [Ca\(^{2+}\)]\(_i\) handling, which imitates the pathogenic development of heart failure. In this study, we found that melatonin treatment was significant in reducing the myocardial injury in CH rats. Hence, levels of the cardiac hypertrophy and resting LDH release were markedly lowered by melatonin treatment in the hypoxic rats. This is likely to be explained by the fact that the level of oxidative stress shown by the MDA level in the cardiac tissue of the melatonin-treated rat was not different from the normoxic control, strongly suggesting a free radical scavenging mechanism mediated by the antioxidant property of melatonin.

Previous studies have reported that melatonin is a cardioprotective agent against myocardial and I/R injuries [16–20, 44]. Multiple mechanisms could explain the antioxidant property of melatonin, including by scavenging oxygen-based free radicals directly [15, 45–49] or indirectly by stimulating antioxidant enzymes [50, 51] and increasing mitochondrial oxidative phosphorylation to lower free radical generations [45, 52–55]. We found that melatonin not only reduced MDA levels, but also lowered the LDH release and infarct size induced by I/R in the CH rat heart. This observation was anticipated because melatonin treatment lessened the oxidative stress in the CH heart and this might improve cardiac tolerance against the oxidative injury induced by ROS overproduction during I/R [56–58]. These results support the idea that the administration of melatonin could be a preventive treatment for the worsening myocardial function and injury under CH conditions.
For the cellular and molecular alterations in the pathogenesis of a failing heart, it is well known that Ca\(^{2+}\) plays an important role in maintaining cardiac functions. The SR and sarcolemma of the cardiomyocytes are major components of normal Ca\(^{2+}\) homeostasis in the heart [59]. When the depolarization of the action potential activates L-type Ca\(^{2+}\) channels, Ca\(^{2+}\) influx occurs to increase [Ca\(^{2+}\)]\(_i\) levels. A small rise in [Ca\(^{2+}\)]\(_i\) triggers Ca\(^{2+}\) release from SR via RyR, by the process known as Ca\(^{2+}\) overload and damaging myocardial contractility in CH rat cardiomyocytes [64–66]. In agreement with our previous findings [6], CH significantly lowered the amplitudes of caffeine- and electrically induced [Ca\(^{2+}\)]\(_i\), suggesting decreased SR Ca\(^{2+}\) content and contractility in CH rat cardiomyocytes.

Moreover, we demonstrated that during MI/A-R, the impaired Ca\(^{2+}\) homeostasis in CH rat cardiomyocytes was more severe with elevated [Ca\(^{2+}\)]\(_i\), overloading level and prolonged decay time (T\(_{50}\)) of E[Ca\(^{2+}\)]\(_i\), indicating deterioration of SR-Ca\(^{2+}\) re-uptake in the CH rat cardiomyocyte. Thus, the alterations in the SR-Ca\(^{2+}\) handling and Ca\(^{2+}\) homeostasis underlying the CH-induced myocardial injury were in parallel to those induced by I/R, but rather developed in a progressive manner under CH conditions. Importantly, melatonin preserved the SR-Ca\(^{2+}\) content and improved contractility in the CH rat cardiomyocyte. Furthermore, the protective effect of melatonin was also effective against the additional oxidative insult imposed by MI/A-R, which are essential to the increased myocardial tolerance against I/R injury. This is in consistent with findings in previous studies suggesting that impaired myocardial and calcium handling functions are improved by the melatonin treatment [16–20, 67].

To address whether alterations in the expression of Ca\(^{2+}\) handling proteins of SR and sarcolemma may account for the ameliorated Ca\(^{2+}\) homeostasis in the CH rat cardiomyocytes, we examined the protein expression of the SERCA, RyR and NCX. Our results showed that the protein expression of SERCA, but not RyR nor NCX, was significantly attenuated in the CH rat heart, supporting that the melatonin treatment [16–20, 67] of antioxidant effect of melatonin in the mechanistic detail of the SERCA regulation awaits further investigation.

In conclusion, we have shown that melatonin was cardioprotective against CH-induced myocardial injury. Numerous other studies have also documented melatonin’s cardioprotective actions under a variety of different situations [68]. Based on current findings, we proposed that the administration of melatonin could be a preventive approach to alleviate the oxidative stress in the heart under...
CH conditions, which is a major pathogenic cause of the alterations in myocardial function leading to subsequent injury and lowered tolerance to I/R injury. The fact that chronic melatonin treatment mitigates the SR-Ca\(^{2+}\) handling and Ca\(^{2+}\) homeostasis in the cardiomyocyte under CH conditions, addresses the cellular mechanism for the cardioprotective effect of melatonin. In addition, the regulation of SERCA expression is involved in the mechanistic effect of melatonin on the improved myocardial function in CH, thus, functionally important for maintaining the replenishment of SR-Ca\(^{2+}\) content and preventing the [Ca\(^{2+}\)\] overload. This provides a cellular basis for the better maintenance of Ca\(^{2+}\) homeostasis and contractility in cardiomyocytes under CH conditions and also the tolerance to I/R-induced myocardial injuries.

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