

Infusion of Melatonin Into the Paraventricular Nucleus Ameliorates Myocardial Ischemia–Reperfusion Injury by Regulating Oxidative Stress and Inflammatory Cytokines

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Abstract: Melatonin, the receptors for which are abundant in the hypothalamic paraventricular nucleus (PVN), can protect the heart from myocardial ischemia–reperfusion (MI/R) injury. The aim of this study was to determine whether the infusion of melatonin into the PVN protects the heart from MI/R injury by suppressing oxidative stress or regulating the balance between proinflammatory cytokines and anti-inflammatory cytokines in MI/R rats. Male Sprague–Dawley rats were treated with a bilateral PVN infusion of melatonin. MI/R operation was performed 1 week after infusion. At the end of the third week after the infusion, all the rats were euthanized. This was followed by immunohistochemistry and immunofluorescence studies of the rats. MI/R rats showed larger infarct size, increased left ventricular (LV) end-diastolic volume, and decreased LV ejection fraction and LV fractional shortening. Moreover, MI/R rats had a higher level of norepinephrine in the plasma, heart, and PVN; higher PVN levels of reactive oxygen species, NOX2, NOX4, IL-1 β , and NF- κ B activity; and lower PVN levels of copper/zinc superoxide dismutase (Cu/Zn-SOD) and IL-10 compared with the sham group. Melatonin infusion in PVN reduced LV end-diastolic volume, norepinephrine, reactive oxygen species, NOX2, NOX4, IL-1 β , and NF- κ B activity, and increased LV ejection fraction, LV fractional shortening, Cu/Zn-SOD, and IL-10. Overall, these results suggest that the infusion of melatonin ameliorates sympathetic nerve activity

and MI/R injury by attenuating oxidative stress and inflammatory cytokines in the PVN of MI/R rats.

Key Words: melatonin, paraventricular nucleus, myocardial ischemia–reperfusion injury, oxidative stress, inflammatory cytokines

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INTRODUCTION

Myocardial ischemia–reperfusion (MI/R) injury is a prominent cause of mortality worldwide.¹ Recent studies have established that melatonin (Mel) can alleviate MI/R injury,^{2,3} and neurohumoral excitation is associated with the progression of MI/R.⁴ In hypertensive rats, the levels of proinflammatory cytokines (PICs) and reactive oxygen species (ROS) are increased both in peripheral tissues and in the paraventricular nucleus (PVN),⁵ a critical central integrative site that regulates sympathetic nerve activity and the pathophysiology of ischemic heart disease.⁶ ROS and PICs in the PVN play important roles in modulating sympathetic nerve activity^{7,8}; hence, brain ROS and PICs are potentially involved in the induction of neurohumoral excitation in ischemic heart disease. Some studies have reported the causes of MI/R injury.^{9,10} Ischemic heart disease is induced by multiple factors, including sympathetic hyperactivity, renin–angiotensin system activation, PIC secretion, and oxidative stress.^{9–13} For example, oxidative stress occurs when there is an imbalance between ROS overproduction and inactivation of the antioxidant defense system, which results in endothelial dysfunction and production of inflammatory cytokines, including interleukin (IL)-1 β and tumor necrosis factor (TNF)- α .¹⁴ Nicotinamide adenine dinucleotide phosphate oxidases (NAD(P)H oxidases) and nitric oxide synthase are critical for ROS production in the cardiovascular system.¹⁵ Excessive ROS plays important roles in the progression of MI/R injury in rats.¹⁶

Some studies have described the cardioprotective role of Mel in MI/R injury. Mel, a circadian hormone with significant antioxidant properties,^{17,18} prevents MI/R injury.^{19–21} Mel interacts efficiently with ROS and reactive nitrogen species, increases antioxidant enzymes, and decreases pro-oxidant enzymes.^{18,22,23} Mel-induced cardioprotective effects may be receptor-dependent, and its antiadrenergic actions contribute significantly to its potential to inhibit molecular damage in cardiomyocytes in MI/R

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injury.²⁴ Furthermore, Mel inhibits the upregulation of IL-1 β and NF- κ B but increases the production of IL-10.²⁵ Subsequent studies have reported that NF- κ B activation in the PVN leads to sympathetic excitation, which is associated with increases in PICs and oxidative stress in the PVN.²⁶ However, to the best of our knowledge, the effects of Mel on PVN function have not been thoroughly investigated.

Accordingly, in this study, we aimed to elucidate the protective effects of Mel on MI/R injury by investigating the effects of PVN infusion of Mel in MI/R model rats.

METHODS

Animals

Experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health and approved by the Xi'an Jiaotong University Committee on Animal Care. Healthy adult male Sprague–Dawley rats (weighing 220–285 g), from Air Force Medical University laboratory animal center, were housed in a controlled room (12:12-h light–dark cycle) with ad libitum access to standard rat chow and tap water. All subsequent surgeries were performed under anesthesia and aseptic conditions.

General Experimental Protocol

The rats were randomly assigned to 4 groups: (1) sham + PVN vehicle, (2) sham + PVN Mel, (3) MI/R + PVN vehicle, and (4) MI/R + PVN Mel. Samples from individual rats for each group were analyzed.

PVN cannulae were implanted bilaterally, as described previously,²⁷ and osmotic mini-pumps (Alzet Model 1004; infusion rate of 0.11 μ L/h; Durect Corporation, Cupertino, CA) were connected to the PVN cannulae for chronic infusion of Mel (0.025 μ g/h; Sigma-Aldrich, St. Louis, MO) or vehicle (artificial cerebrospinal fluid) for 1 week.²⁸ One week later, surgery for MI/R was performed, as previously described.¹⁹ After rats were anesthetized, surgery for myocardial ischemia was performed by ligating the left anterior descending coronary artery. Thirty minutes after ischemia, some MI/R rats underwent reperfusion for 6 hours to assess the size of the myocardial infarct, whereas some other MI/R rats were euthanized 2 weeks later for analysis of protein expression and cardiac function. The sham group underwent the same surgical procedures, except that the suture passed under the left coronary artery was untied. Thereafter, all rats were euthanized, and blood and brain tissue specimens were harvested for molecular, immunofluorescence, and immunohistochemical analyses.

Bilateral Implantation of PVN Cannulae for Chronic Infusion

Bilateral PVN cannulae were implanted, as described previously.^{29,30} After induction of anesthesia with 3% pentobarbital sodium (30 mg/kg, intraperitoneal injection), the rats were placed in a stereotaxic apparatus (Stoelting WPI, Wood Dale, IL), and a stainless steel cannula was implanted bilaterally into the PVN. The stereotaxic coordinates for PVN

were as follows: 1.8 mm caudal from bregma, 0.4 mm lateral to the midline, and 7.9 mm ventral to the dorsal surface. The osmotic mini-pump filled with Mel or vehicle was implanted subcutaneously at the dorsa of the neck and connected to the PVN cannulae. Penicillin was used to prevent infections. Thereafter, the locations of the cannulae were determined and only animals with discernible bilateral PVN infusion sites were used in the final analyses. The success rate of bilateral PVN cannulae implantation was 71%.

Surgical Procedure for MI/R Injury

In brief, rats were anesthetized with ketamine + xylazine (90 and 10 mg/kg i.p., respectively) to induce MI/R injury by ligating the left anterior descending coronary artery. These rats were endotracheally intubated and mechanically ventilated with ambient air at a respiratory rate of 50–55/min and a tidal volume of 2.5 mL. Under aseptic conditions, left thoracotomy was performed to expose the heart. The pericardium was opened, and the heart was exteriorized. The left anterior descending coronary artery was ligated between the pulmonary outflow tract and the left atrium for 30 minutes. Thereafter, the rope was untied. The heart was returned to the chest cavity, the lungs were reinflated, and the chest incision was closed. Sham rats were treated similarly but did not undergo coronary artery occlusion. After completion of the surgical procedures, rats were removed from the ventilator and extubated. Postsurgical animals were administered benzathine penicillin (30,000 units intramuscularly) and lidocaine (2 mg intramuscularly every 4 hours for 2 doses). After myocardial reperfusion for 6 hours, some rats were euthanized to assess the size of the myocardial infarct.

Determination of Myocardial Infarct Size

At 6 hours after surgery, the ligature of the coronary artery was tied again and 2% Evans blue (1 mL) dye was injected into the left ventricle. The dye was distributed uniformly, except in the section previously perfused by the occluded coronary artery [area at risk (AAR)]. The heart was immediately excised, frozen at -80°C , and sliced into 1-mm-thick sections perpendicular to the longitudinal axis of the heart. The slices were incubated in 1% TTC in phosphate buffer (pH 7.4) at 37°C for 20 minutes and then photographed with a digital camera. The Evans blue-stained area (normal myocardium), TTC-stained area (red, ischemic but viable myocardium), and gray area (infarct myocardium) were measured digitally using Image Pro-Plus software (Media Cybernetics, Rockville, MD). The myocardial infarct size was expressed as a percentage of the infarct area (INF) divided by the total AAR (INF/AAR \times 100%).

Echocardiographic Assessment

Echocardiographic measurements were performed 2 weeks after reperfusion, as previously described.¹⁹ The rats were anesthetized with ketamine (25 mg/kg, intraperitoneally). Thereafter, left ventricular (LV) function, including the LV ejection fraction (LVEF), LV fractional shortening (LVFS), and LV end-diastolic volume (LVEDV) was determined using computer algorithms.

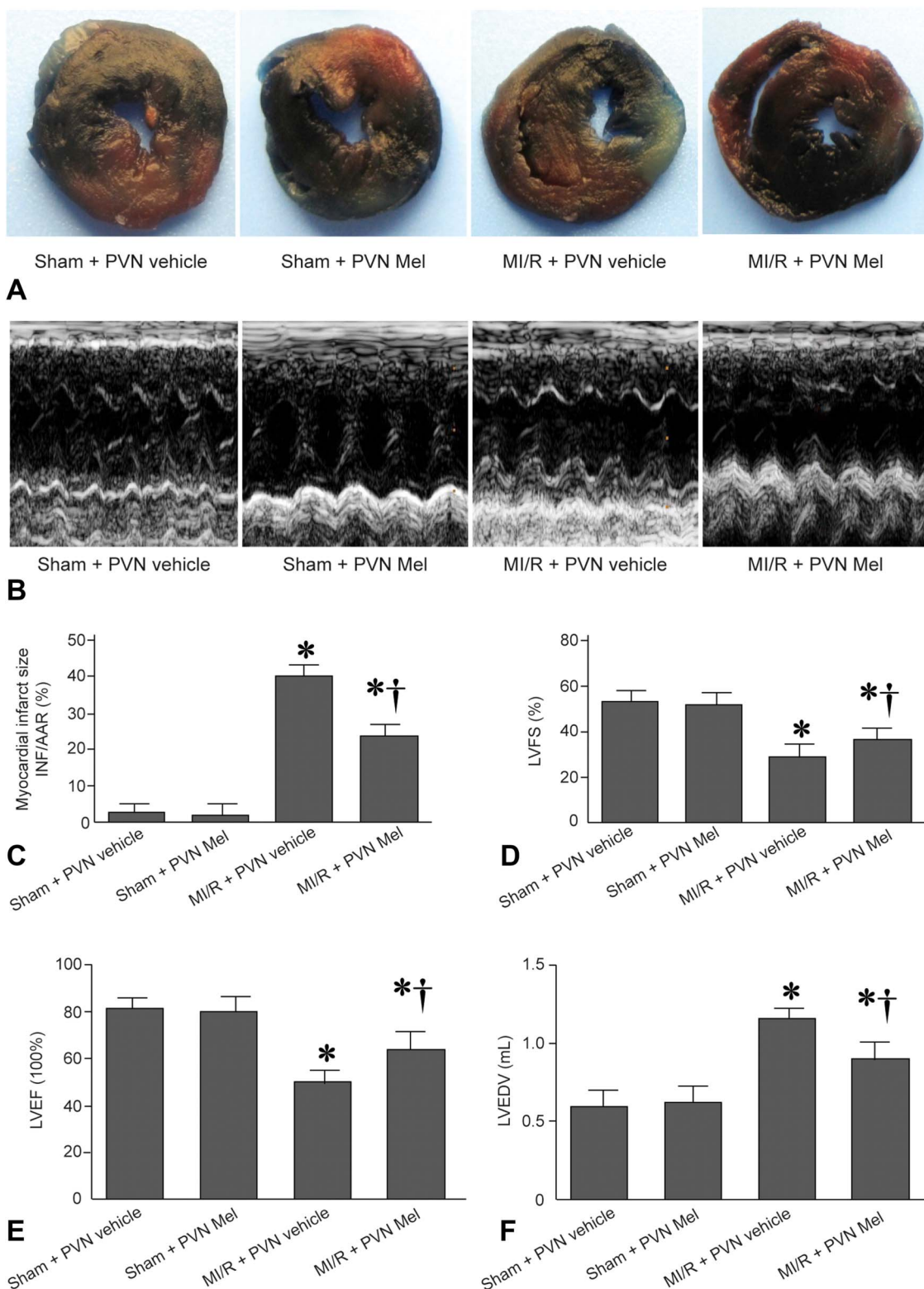


FIGURE 1. Melatonin ameliorated MI/R injury by reducing myocardial infarct size and improving cardiac functional recovery. A, Evans blue/TTC double staining was performed 6 hours after reperfusion. The blue-stained portion indicates the nonischemic, normal region; the red-stained portion indicates I/R; and the negative-stained portion indicates the I/R infarcted region. B, Representative M-mode echocardiographic images. Cardiac function was assessed by using echocardiography 2 weeks after reperfusion. C, Myocardial infarct size expressed as percentage of the AAR. D, LVFS. E, LVEF. F, LVEDV. The results are expressed as mean values ± SEMs (n = 7). *P < 0.05 versus the sham group; †P < 0.05 MI/R + PVN Mel versus MI/R + PVN vehicle.

TABLE 1. Echocardiographic Findings

Measurement	Sham + PVN Vehicle	Sham + PVN Mel	MI/R + PVN Vehicle	MI/R + PVN Mel
HR (bpm)	329 ± 19	326 ± 8	399 ± 11*	364 ± 10*†
LVEF (%)	81.6 ± 6.5	80.3 ± 8.1	51.6 ± 5.6*	63.2 ± 5.8*†
LVFS (%)	51.3 ± 8.0	52.2 ± 6.4	30.3 ± 4.6*	38.7 ± 5.5*†
LVEDV (mL)	0.55 ± 0.09	0.58 ± 0.06	1.21 ± 0.17*	0.88 ± 0.13*†

Echocardiographic measurements were performed 2 weeks after reperfusion. Values are expressed as mean values ± SEMs (n = 7).

*P < 0.05 versus the sham group.

†P < 0.05 MI/R + PVN Mel versus MI/R + PVN vehicle.

HR, heart rate.

Blood Sampling

Two weeks after reperfusion, the rats were anesthetized with pentobarbital sodium (30 mg/kg, intraperitoneal injection) and decapitated. Plasma samples were collected through cardiac puncture and stored at -80°C to measure norepinephrine (NE) levels.

Quantification of NE in the PVN and Plasma

Microdissection was performed to isolate the PVN, as described previously.³¹ The tissues were harvested bilaterally from the PVN of individual rats. Microdissected PVN tissues were stored at -80°C until analysis. NE in the PVN and plasma was assayed using high-performance liquid chromatography with electrochemical detection, as previously described.³²

Immunofluorescence and Immunohistochemical Analyses

The procedures for immunofluorescence and immunohistochemical analyses were conducted, as described previously.³³ The brains were sliced into 18-µm-thick sections from the bregma to the lambda, using a cryostat (CM1850; Leica, Heidelberg, Germany). Some of these PVN tissues were stored at -80°C until analysis. Primary anti-IL-10 (sc-365858, dilution 1:300), anti-IL-1β (sc-52012, dilution 1:200), anti-NOX2 (gp91phox) (sc-130543, dilution 1:300), and anti-NOX4 (p47phox) (sc-17844, dilution 1:400) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). ROS levels in the PVN were measured through staining with

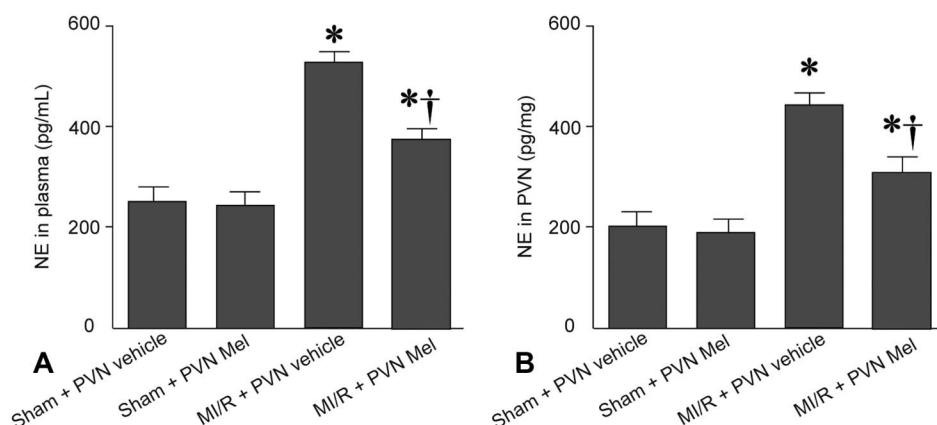
fluorescently labeled dihydroethidium (DHE; Beyotime, Shanghai, China). For each animal, positive neurons within bilateral borders of the PVN were manually enumerated in 3 consecutive sections, and average values were determined.

Quantification of NF-κB Activity and Cytokines

An NF-κB active enzyme-linked immunosorbent assay (ELISA) kit (Active Motif, Shanghai, China) was used to quantify NF-κB p65 activity in accordance with the manufacturer's instructions.

Extraction and data analysis were performed in accordance with the manufacturer's instructions. Microdissected PVN tissues were lysed in extract buffer, triturated, and centrifuged at 12,000g for 20 seconds at 4°C. The nuclear pellets were resuspended in extract buffer and eliminated via centrifugation (12,000g for 20 seconds at 4°C). The nuclear extract of PVN tissue was obtained at 3–5 mg/mL. After preparing the nuclear extracts, they were placed in wells coated with an oligonucleotide containing a NF-κB consensus binding site. Nuclear extracts were incubated in a plate coated with the NF-κB probe. The primary antibody against NF-κB was incubated for 1 hour, followed by incubation with a horseradish peroxidase-conjugated secondary antibody for 1 hour. After substrate addition, the absorbance was read using a Thermo Scientific Microplate Reader at 450 nm. NF-κB p65 from nuclear extracts was 0.5–1 µg per well, as determined through a standard curve and upon normalization of protein content.

FIGURE 2. Effects of PVN infusion of melatonin on plasma (A) and PVN (B) levels of NE in MI/R rats. Values are expressed as mean ± SEMs (n = 7). *P < 0.05 versus the sham group; †P < 0.05 MI/R + PVN Mel versus MI/R + PVN vehicle.



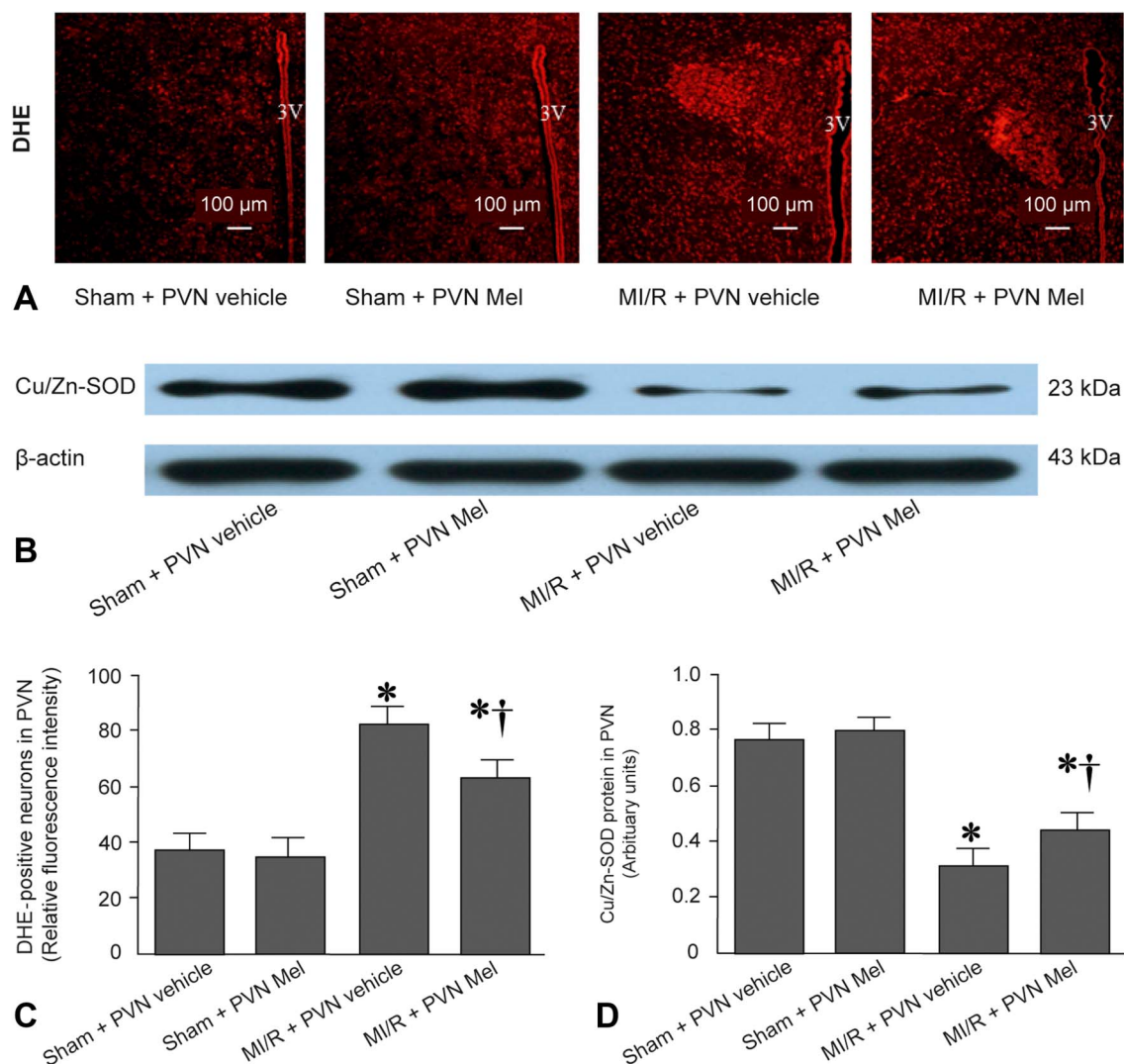


FIGURE 3. Effects of PVN infusion of melatonin or vehicle on PVN levels of superoxide and Cu/Zn-SOD proteins in MI/R rats. A, Representative immunofluorescence image of fluorescent-labeled dihydroethidium (DHE) in the PVN in different groups. B, Representative immunoblots of Cu/Zn-SOD in the PVN in different groups. C, Densitometric analysis of the immunofluorescent intensity of DHE in the PVN in different groups. D, Densitometric analysis of protein expression of Cu/Zn-SOD in the PVN in different groups. Values are expressed as mean ± SEM (n = 7). *P < 0.05 versus the sham group; †P < 0.05 MI/R + PVN Mel versus MI/R + PVN vehicle.

Tissue cytokine levels were determined using ELISA (Biosource International Inc, Camarillo, CA), as previously described.^{33,34} In accordance with the manufacturer’s instructions, microdissected PVN tissues were also lysed in extract buffer, triturated, and centrifuged at 12,000g for 20 seconds at 4°C. Thereafter, the standards or sample diluents were added and incubated in appropriate wells of microtiter plates pre-coated with primary antibodies for 1 hour and HRP-conjugated secondary antibody for 1 hour. The conjugate was added, and plates were incubated for 1 hour at 37°C and washed. The reactions were terminated with stop solution, and absorbance was read at 450 nm for tissue cytokine measurements, using a microtiter plate reader (MK3; Thermo Fisher Scientific). Each sample was quantified using a standard curve and upon normalization of protein content.

Western Blot Analysis

Western blotting was performed, as described previously.²⁶ PVN tissue homogenates were subjected to Western blot analysis to determine protein levels using the following primary antibodies: anti-NOX2 (sc-130543, dilution 1:500, 60 kDa), anti-NOX4 (sc-17844, dilution 1:200, 67 kDa), anti-IL-10 (sc-365858, dilution 1:100, 19 kDa), anti-IL-1β (sc-52012, dilution 1:100, 31 kDa), and anti-Cu/Zn-SOD (sc-101523, dilution 1:300, 17 kDa) antibodies (Santa Cruz Biotechnology). Band densities were analyzed using the NIH ImageJ software (NIH, Bethesda, MD).

Data Analysis

Analysis of data was conducted using the SPSS 11.5 statistical software. Data are presented as mean ± SEM values. Echocardiographic data were analyzed using repeated-measures

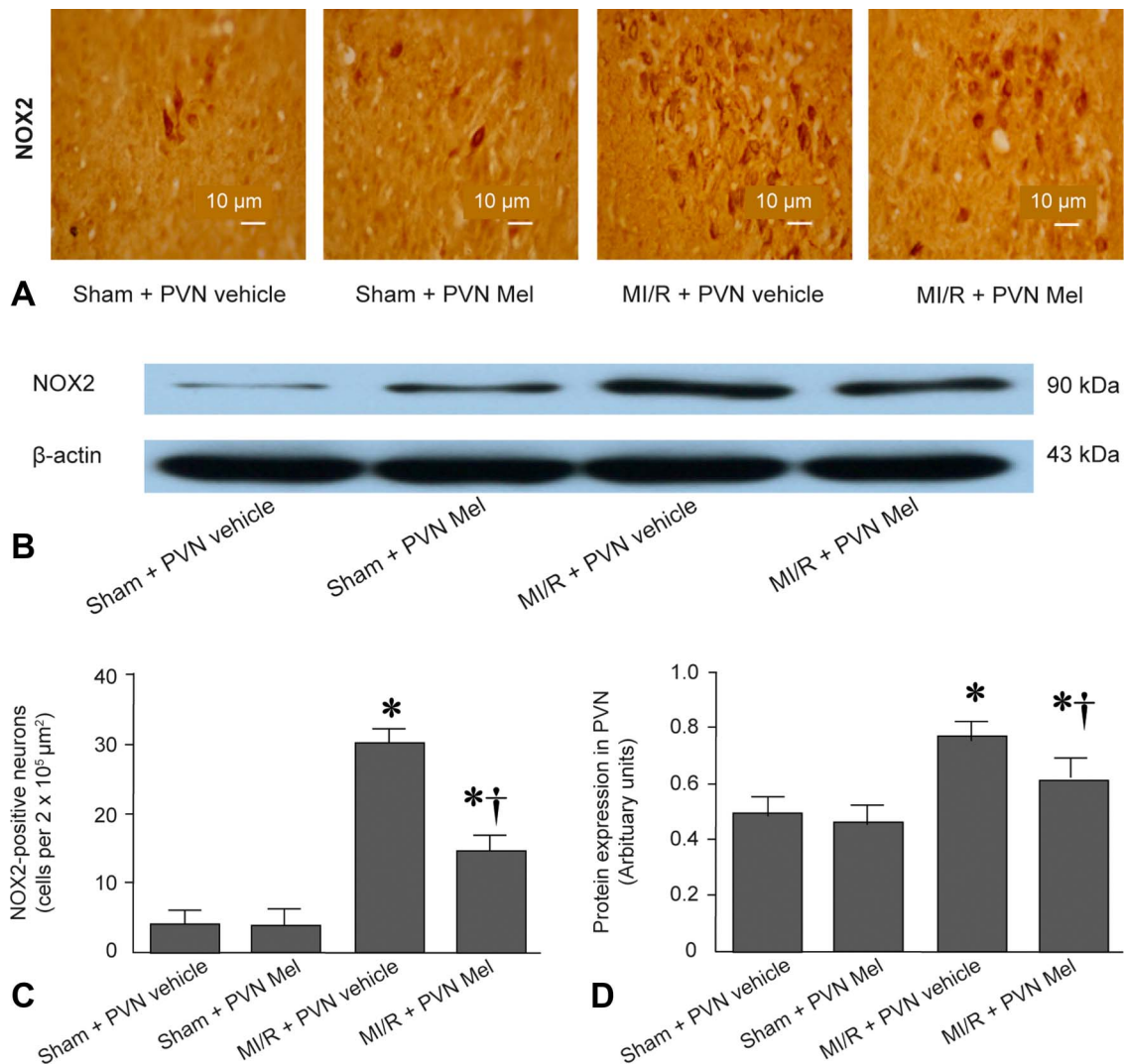


FIGURE 4. Effects of PVN infusion of melatonin on PVN levels of NOX2 in MI/R rats. **A**, Representative immunohistochemical staining of NOX2 in the PVN in different groups. **B**, Representative immunoblots of NOX2 in the PVN in different groups. **C**, Densitometric analysis of immunohistochemical intensity of NOX2 in the PVN in different groups. **D**, Densitometric analysis of protein expression of NOX2 in the PVN in different groups. Values are expressed as mean values ± SEMs (n = 7). *P < 0.05 versus the sham group; †P < 0.05 MI/R + PVN Mel versus MI/R + PVN vehicle.

analysis of variance. Other data were analyzed by analysis of variance, followed by Tukey’s post hoc tests. Results with P values less than 0.05 were considered statistically significant.

RESULTS

PVN Infusion With Mel Ameliorated MI/R Damage

First, we evaluated the effects of Mel treatment on the hearts of rats in the sham group. Bilateral PVN infusion of Mel displayed no significant effects on LVEF, LVFS, LVEDV, and myocardial infarct size compared with the vehicle in the sham group (P > 0.05). By contrast, bilateral PVN infusion with Mel in the reperfusion group decreased the myocardial infarct size in comparison with that in the MI/R operation group

(Figs. 1A, C, P < 0.05). After 2 weeks, LVEF and LVFS in the Mel treatment group were lower than those in the MI/R operation group (Figs. 1E, D, P < 0.05). In addition, LVEDV was significantly higher in the MI/R + PVN Mel group than in the MI/R + PVN vehicle group (Fig. 1F, P < 0.05). Together, these results show that Mel treatment attenuated MI/R-induced injury (Figs. 1B, D–F and Table 1).

PVN Infusion With Mel Suppressed NE in MI/R Rats

NE is an indirect marker of sympathetic activity and is usually measured using high-performance liquid chromatography. MI/R rats exhibited significantly higher plasma (542 ± 19 vs. 254 ± 13 pg/mL, respectively; P < 0.05, Fig. 2A) and PVN levels of NE (458 ± 34 vs. 206 ± 32 pg/mg, respectively; P < 0.05, Fig. 2B) than that in the sham group. However, 2 weeks

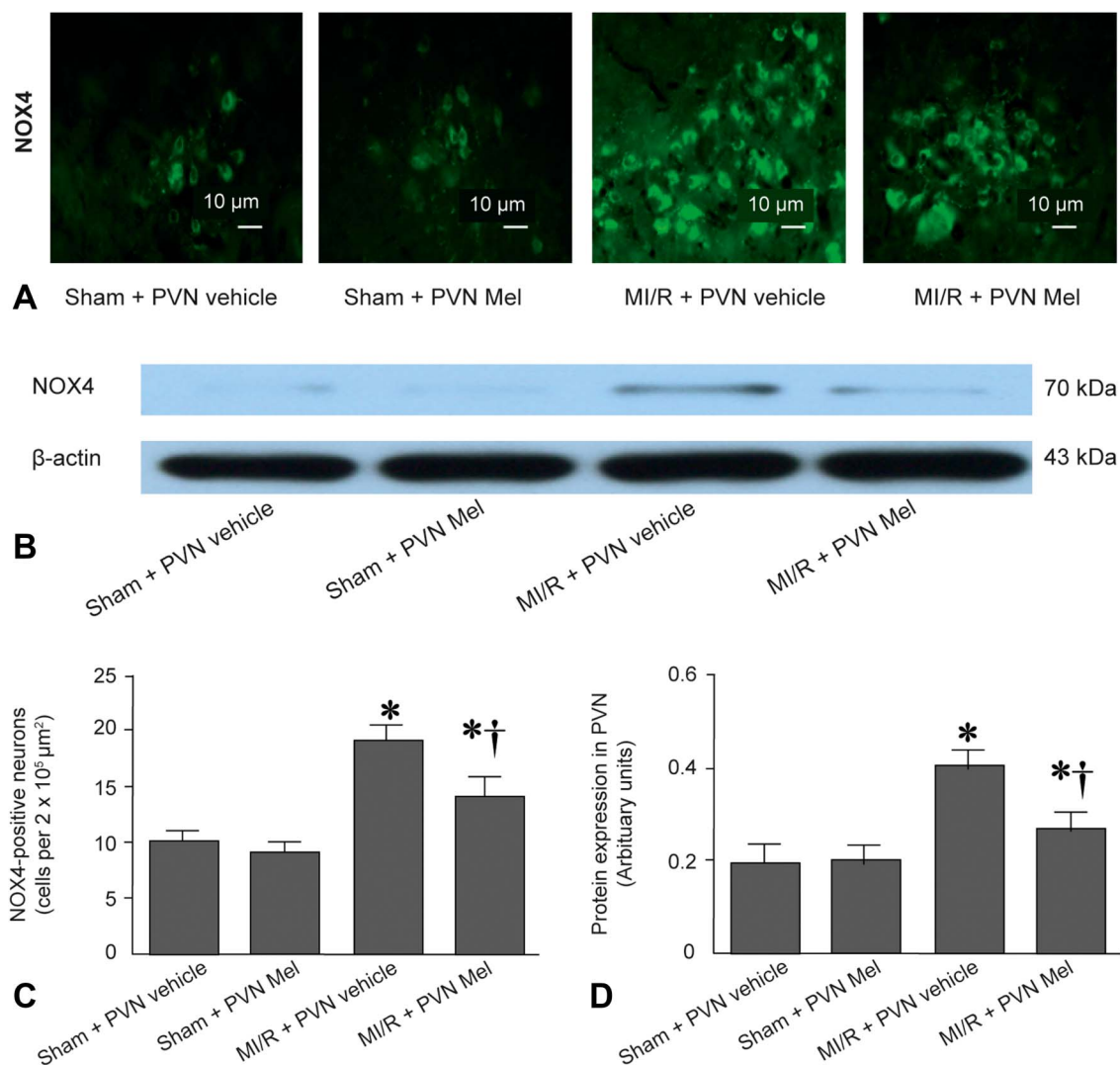


FIGURE 5. Effects of PVN infusion of melatonin on PVN levels of NOX4 in MI/R rats. **A**, Representative immunohistochemical staining of NOX4 in the PVN of the sham and MI/R groups. **B**, Representative immunoblots of NOX4 in the PVN of the sham and MI/R groups. **C**, Densitometric analysis of immunohistochemical intensity of NOX4 in the PVN of the sham and MI/R groups. **D**, Densitometric analysis of protein expression of NOX4 in the PVN of the sham and MI/R groups. Values are expressed as mean values ± SEMs (n = 7). **P* < 0.05 versus the sham group; †*P* < 0.05 MI/R + PVN Mel versus MI/R + PVN vehicle.

after PVN infusion with Mel, levels of NE in the PVN (391 ± 20 vs. 458 ± 34 pg/mg, respectively; *P* < 0.05, Fig. 2B) and plasma (312 ± 17 vs. 542 ± 19 pg/mL, respectively; *P* < 0.05, Fig. 2A) were suppressed in MI/R rats (Fig. 2).

PVN Infusion With Mel Attenuated the Expression of Oxidative Stress Markers and Cu/Zn-SOD Protein in MI/R Rats

Immunofluorescence staining was performed to quantify DHE activity for the assessment of superoxide levels in the PVN, and Western blotting was performed to quantify Cu/Zn-SOD protein expression to reflect the activity of antioxidant enzymes. The MI/R group displayed a significant increase in ROS levels (85 ± 5.3 vs. 42 ± 4.8 cells/1 × 10⁴ μm², respectively; *P* < 0.05, Figs. 3A, C) and significantly reduced Cu/Zn-SOD protein

expression (0.31 ± 0.03 vs. 0.79 ± 0.11; *P* < 0.05, Figs. 3B, D) in the PVN group compared with those in the sham group. However, 2 weeks after PVN infusion with Mel, these changes were attenuated in MI/R rats (Fig. 3, *P* < 0.05).

PVN Infusion With Mel Influenced NOX2 Expression in MI/R Rats

Western blotting and immunohistochemical analysis revealed that MI/R rats had more NOX2-positive neurons (32 ± 8 vs. 4 ± 1 cells/2 × 10⁵ μm², respectively; *P* < 0.05, Figs. 4A, C) and higher NOX2 protein expression (0.76 ± 0.15 vs. 0.51 ± 0.10, respectively; *P* < 0.05, Figs. 4B, D) in the PVN group than in the sham group; however, 2 weeks after PVN infusion with Mel, the number of NOX2-positive neurons (14 ± 4 vs. 32 ± 8 cells/2 × 10⁵ μm², respectively; *P* < 0.05, Figs. 4A, C) and the expression of NOX2 protein

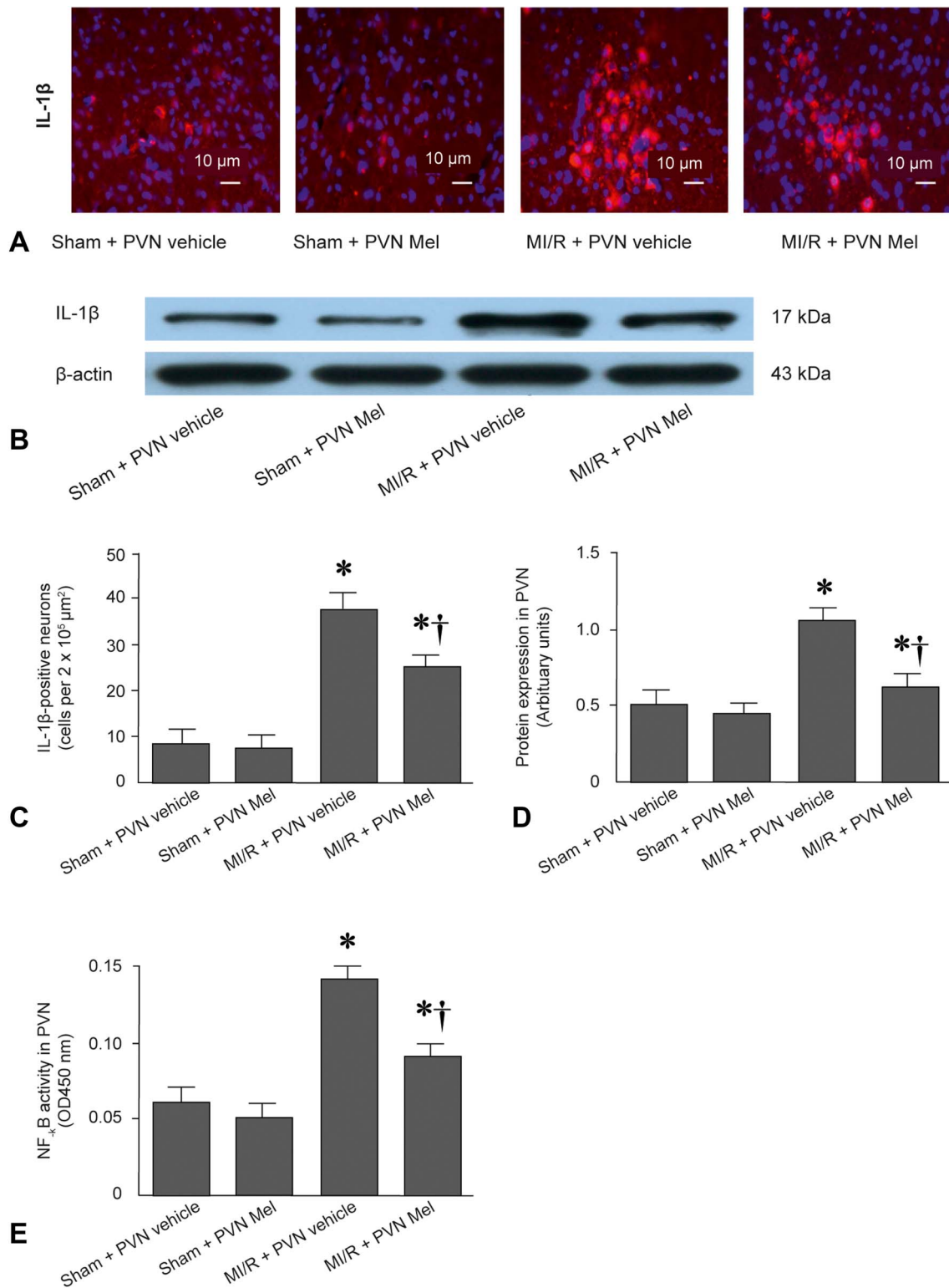


FIGURE 6. Effects of PVN infusion of melatonin on PVN levels of IL-1β and NF-κB p65 in MI/R rats. **A**, Representative immunohistochemical staining of IL-1β in the PVN of the sham and MI/R groups. **B**, Representative immunoblots of IL-1β in the PVN of the sham and MI/R groups. **C**, Densitometric analysis of immunohistochemical intensity of IL-1β in the PVN of the sham and MI/R groups. **D**, Densitometric analysis of protein expression of IL-1β in the PVN of the sham and MI/R groups. **E**, Effects of PVN infusion of melatonin on NF-κB p65 activation in the PVN of the sham and MI/R groups. Values are expressed as mean values ± SEMs (n = 7). *P < 0.05 versus the sham group; †P < 0.05 MI/R + PVN Mel versus MI/R + PVN vehicle.

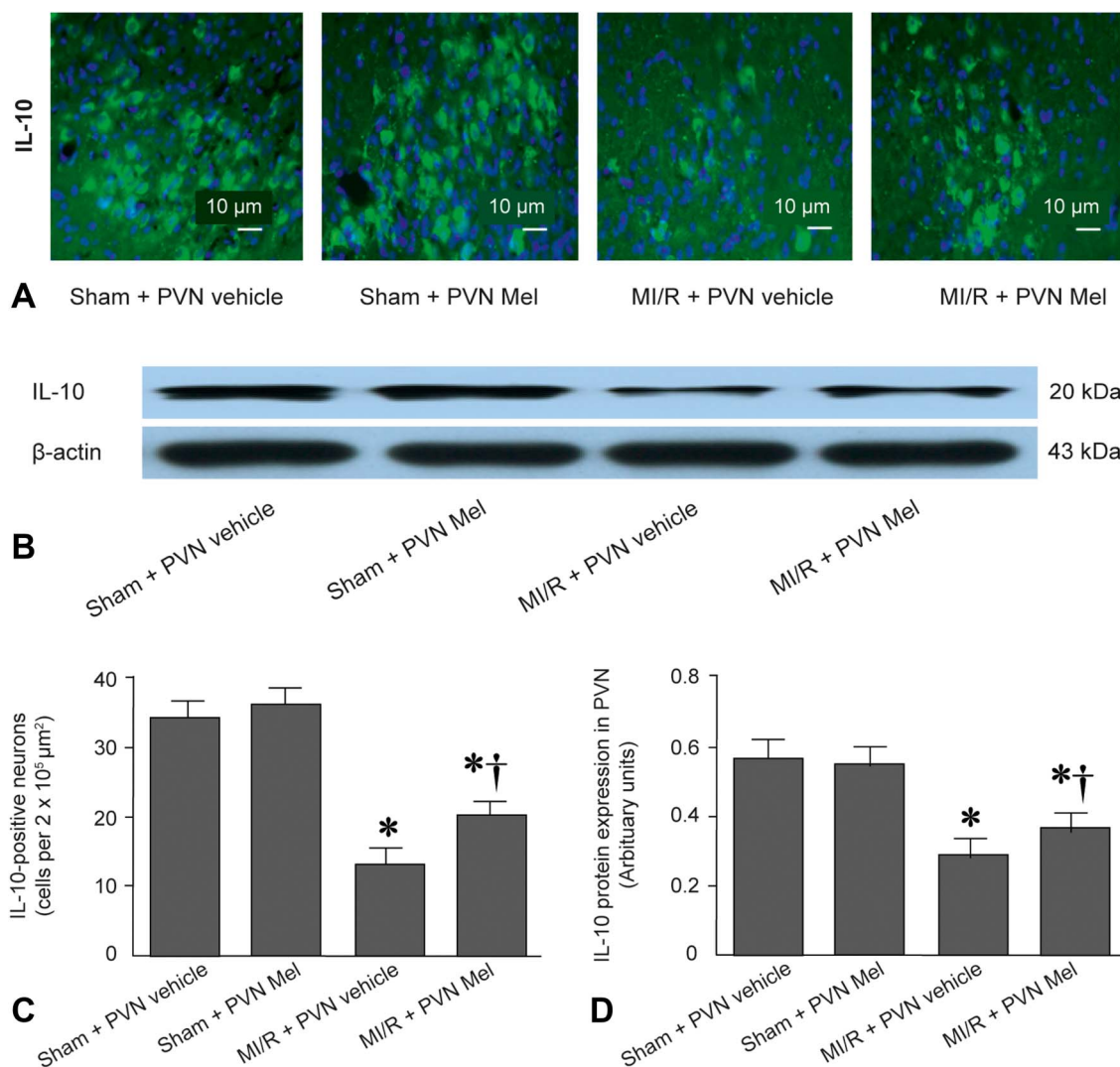


FIGURE 7. Effects of PVN infusion of melatonin on PVN levels of IL-10 in MI/R rats. A, Representative immunohistochemical staining of IL-10 in the PVN of the sham and MI/R groups. B, Representative immunoblots of IL-10 in the PVN of the sham and MI/R groups. C, Densitometric analysis of immunohistochemical intensity of IL-10 in the PVN of the sham and MI/R groups. D, Densitometric analysis of protein expression of IL-10 in the PVN of the sham and MI/R groups. Values are expressed as mean values ± SEMs (n = 7). *P < 0.05 versus the sham group; †P < 0.05 MI/R + PVN Mel versus MI/R + PVN vehicle.

(0.61 ± 0.14 vs. 0.76 ± 0.15, respectively; P < 0.05, Figs. 4B, D) were significantly reduced in MI/R rats (Fig. 4).

PVN Infusion With Mel Influenced NOX4 Expression in MI/R Rats

Western blotting and immunofluorescence analyses revealed that MI/R rats contained a significantly greater number of NOX4-positive neurons (18 ± 2 vs. 9 ± 1 cells/2 × 10⁵ μm², respectively; P < 0.05, Figs. 5A, C) and displayed significantly higher NOX4 protein expression levels (0.41 ± 0.10 vs. 0.19 ± 0.07, respectively; P < 0.05, Figs. 5B, D) in the PVN group than in the sham group. Two weeks after PVN infusion with Mel, NOX4-positive neurons (14 ± 1 vs. 18 ± 2 cells/2 × 10⁵ μm², P < 0.05, Figs. 5A, C) and NOX4 protein expression (0.26 ± 0.10 vs. 0.41 ± 0.10, respectively; P < 0.05, Figs. 5B, D) were decreased in MI/R rats (Fig. 5).

PVN Infusion With Mel Reduced IL-1β and NF-κB Activation in MI/R Rats

The MI/R group reported a significant increase in IL-1β (38 ± 2 vs. 8 ± 1 cells/2 × 10⁵ μm², respectively; P < 0.05, Figs. 6A, C) and NF-κB (Fig. 6E, P < 0.05) levels in the PVN group in comparison with the sham group. Two weeks after PVN infusion with Mel, IL-1β (24 ± 3 vs. 38 ± 2 cells/2 × 10⁵ μm², respectively; P < 0.05, Figs. 6A, C) and NF-κB levels were significantly reduced in the PVN of MI/R rats (Figs. 6A–E).

PVN Infusion With Mel Increased IL-10 Activation in MI/R Rats

MI/R rats reported a significant reduction in the number of IL-10-positive neurons (11 ± 2 vs. 34 ± 3 cells/2 × 10⁵ μm², respectively; P < 0.05, Figs. 7A, C) and downregulated

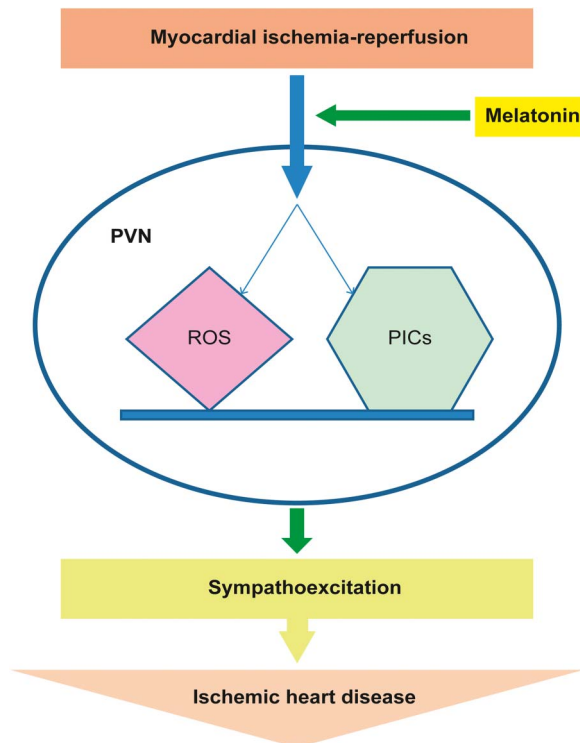


FIGURE 8. A schematic illustration of the proposed mechanism underlying the effects of melatonin on regulating PICs and ROS within the hypothalamic PVN in MI/R rats.

IL-10 protein expression (0.30 ± 0.06 vs. 0.57 ± 0.05 , respectively; $P < 0.05$, Figs. 7B, D) in comparison with the sham group. Two weeks after PVN infusion with Mel, the number of IL-10–positive neurons (21 ± 2 vs. 11 ± 2 cells/ $2 \times 10^5 \mu\text{m}^2$, respectively; $P < 0.05$, Figs. 7A, C) and IL-10 protein levels (0.37 ± 0.03 vs. 0.30 ± 0.06 , respectively; $P < 0.05$, Figs. 7B, D) was significantly increased in the PVN of MI/R rats (Fig. 7).

DISCUSSION

This study is the first, to the best of our knowledge, to show that excessive production of oxidative stress and inflammatory cytokines in the PVN is involved in MI/R injury, PVN infusion with Mel upregulates antioxidant enzymes (Cu/Zn-SOD) and downregulates NOX2 and NOX4 in the PVN, and PVN infusion with Mel suppresses proinflammatory factors IL-1 β and NF- κ B p65, upregulates the anti-inflammatory factor IL-10 in the PVN, and reduces the levels of NE in the PVN of MI/R rats. Together, our findings suggest that PVN infusion with Mel ameliorates MI/R injury.

Literature reviews have reported that interactions between autonomic nervous system dysfunction and excessive production of NE are critical in MI/R injury. Previous studies have reported that after myocardial ischemia, the regulation of PVN neuronal activity significantly alters peripheral sympathetic nerve activity and improves cardiac function in MI rats.^{31,35} Furthermore, Mel significantly decreases peripheral sympathetic nerve activity in hypertensive

rats.³⁶ Moreover, Mel exerts several biological effects and can penetrate the blood–brain barrier.³⁷ Moreover, Mel receptors are widely expressed in the brain and are abundant in the hypothalamic PVN.³⁷ Based on the effects of Mel in the peripheral sympathetic nerve activity, this study shows that NE levels in the PVN and plasma were significantly higher in MI/R rats than in the sham rats and PVN infusion with Mel attenuated the elevation in NE in the PVN and plasma in MI/R rats. Furthermore, we previously reported that NE is increased in ischemia-induced cardiac failure rather than in control rats.³⁸ Moreover, previous studies have indicated that corticotropin-releasing hormone, a physiological marker associated with the activation of the hypothalamo–pituitary–adrenal axis, is upregulated in the PVN.³⁹ Our studies indicate that myocardial infarction upregulates corticotropin-releasing hormone in the PVN of HF rats.^{34,40}

Moreover, numerous excitatory and inhibitory neurotransmitters in the PVN influence its neuronal activity, including glutamate, NE, and gamma-aminobutyric acid (GABA). Kang et al⁴¹ reported that rats with ischemia-induced cardiac failure had higher levels of NE and glutamate and lower levels of GABA in the PVN. Western blotting analysis indicated that HF rats have higher TH levels and lower GAD67 levels in the PVN in comparison with SHAM rats. Continuous intracerebroventricular infusion of the cytokine blockers pentoxifylline (PTX) and etanercept (ETN) attenuates HF-induced reductions in the levels of NE, glutamate, and tyrosine hydroxylase and increases levels of GABA and GAD67 in the PVN. This study is the first to show that proinflammatory cytokines and NF- κ B modulate neurotransmitters in the PVN and contribute to sympathoexcitation in rats with ischemia-induced cardiac failure.³⁸ Therefore, this study speculates that bilateral PVN chronic infusion of melatonin in MI/R rats attenuates NF- κ B activation and downregulates inflammatory cytokines, thus increasing levels of glutamate, NE, and TH and decreasing levels of GABA and GAD67 in the PVN in MI/R rats.

Anker et al⁴² reported that in comparison with control subjects, patients with chronic cardiac failure had increased NE, epinephrine, cortisol, and growth hormone levels. Ammar et al⁴³ reported that plasma NE and cortisol levels increased significantly in patients with I/R injury, concurrent with the present and previous findings. These findings suggest that PVN infusion with Mel potentially regulates neurotransmitters, reduces sympathetic nerve overactivation, and ameliorates MI/R injury.

This suppressive effect of Mel on sympathetic nerve overactivation may be associated with the regulation of oxidative stress and inflammatory factors. Recent studies have reported that the protective effects of Mel are primarily associated with antioxidant generation, reduced cardiolipin peroxidation, and reduced mitochondrial ROS release.^{44,45} Mel efficiently interacts with various ROS, thus upregulating antioxidant enzymes and downregulating pro-oxidant enzymes.¹⁴ NAD(P)H subunits in the PVN, particularly NOX2 and NOX4, are the primary sources of ROS. In the hypothalamus, high levels of ROS are important factors that enhance the excitability of peripheral sympathetic nerves, and blocking the formation of central ROS can significantly

reduce sympathetic nerve activity.⁵ SOD, an antioxidant enzyme, is the first line of defense against ROS-induced oxidative tissue injury.⁴⁶ In this study, NOX2 and NOX4 were significantly upregulated in the PVN of rats in the MI/R group, and the antioxidant enzyme Cu/Zn-SOD was downregulated in comparison with the sham group. However, PVN infusion with Mel upregulated Cu/Zn-SOD and downregulated NOX2 and NOX4. The present results indicate that Mel in the PVN may decrease sympathetic excitation and ameliorate MI/R injury by triggering the overproduction of SOD and inhibiting the ROS pathway.

Furthermore, recent studies have reported that activation of NF- κ B in the PVN contributes to oxidative stress and sympathetic excitation in a rat model of cardiac failure.⁴⁷ Other studies have reported that high levels of ROS induce NF- κ B activation and then upregulate IL-1 β , thus exacerbating oxidative stress.¹⁴ This study shows that chronic PVN infusion with Mel downregulates NF- κ B and IL-1 β and upregulates IL-10 in the PVN of MI/R rats. Therefore, our findings indicate that PVN infusion with Mel may reduce sympathetic nerve activity and ameliorate MI/R injury by downregulating NF- κ B and IL-1 β and upregulating IL-10 in the PVN of MI/R rats.

CONCLUSIONS

This study shows that PVN infusion with Mel attenuated SOD downregulation and NOX2 and NOX4 upregulation in the PVN of MI/R rats. PVN infusion with Mel attenuated IL-10 downregulation and an increase in NF- κ B, IL-1 β , and NE levels in the PVN of MI/R rats. The present results suggest that PVN infusion with Mel potentially protects against MI/R injury by attenuating oxidative stress, reducing the generation of PICs, and inhibiting sympathetic nerve activity (Fig. 8). Our findings provide important insights into the therapeutic value of using Mel for treating MI/R injury, presumably by its potential antioxidative and anti-inflammatory activities in the PVN and provide a novel approach to treat the central nervous system upon MI/R injury.

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