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Original Article

Role of morphine and sufentanil in myocardial ischemia/reperfusion induced ventricular arrhythmias

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Abstract: In this study, we investigated the preconditioning effects of the classical opioid morphine and the novel opioid receptor agonist sufentanil on myocardial ischemia/reperfusion (I/R)-induced ventricular arrhythmias and their possible modes of action. Rats were divided into six groups: sham operation (C), I/R model (I/R), morphine (M) and sufentanil (S) preconditioned, and morphine (MPA) and sufentanil (SPA) preconditioned plus the p38 MAPK inhibitor SB203580. All groups (except C) received I/R, with morphine and sufentanil administered intravenously before left coronary artery ligation, and SB203580 administered before preconditioning. Hemodynamic indices (heart rate, mean arterial blood pressure and rate pressure product) were decreased in all groups compared to C ($P < 0.05$) but increased significantly in M, S, MPA and SPA ($P < 0.05$) compared with I/R; there were no significant differences between M and S, and between MPA and SPA. Analysis of arrhythmias by electrocardiography showed a similar trend. Western blotting and/or immunohistochemistry revealed that myocardiocytes in I/R expressed high levels of p38 MAPK and phosphorylated p38 MAPK, with decreased levels in M, S, MPA and SPA compared to C, while expression of Cx43 and p-Cx43 showed opposing patterns to that of p38 MAPK. Morphine and sufentanil may protect against myocardial I/R injury via Cx43 phosphorylation, but not via p38 MAPK.

Keywords: Morphine, sufentanil, myocardial ischemia/reperfusion, ventricular arrhythmia, p38 MAPK, Cx43

Introduction

Myocardial ischemia is a major cause of death in most countries worldwide [1]. Currently, coronary artery reperfusion is the most effective method to reduce myocardial ischemia and mortality [2]; however, myocardial reperfusion after ischemia has been shown to cause cardiac damage and other complications. Growing evidence from both animal experiments and clinical observations indicate that apoptosis plays a key role in myocardial ischemia/reperfusion (I/R) injury [3, 4].

Morphine is one of the most commonly used opioids due to its strong analgesic effects. Several studies have shown that morphine exerts protective effects against myocardial I/R-induced cardiac injury [5, 6]. Although the pathway is not yet established, activation of the MAPK cascade has been implicated in the cardioprotection [7]. Fryer et al. [8] suggested that p38 MAPK is an integral component of opioid-induced delayed cardioprotection. Sufentanil is a selective synthetic μ-opioid receptor agonist drug and shows unique pharmacodynamic and pharmacokinetic properties. It has hemodynamic stability and exerts strong analgesic effects without accumulation [9]. Due to these characteristics, sufentanil is widely used in various types of cardiac surgery.

Post-ischemic arrhythmia is a common cause of death during I/R injury. Connexin 43 (Cx43) has been shown to play a vital role in post-ischemic fatal arrhythmias and is less activated (by phosphorylation) after myocardial ischemia [10].

Various intracellular signaling pathways are thought to play a critical role in the myocardial response to ischemia and remodeling. Multiple

mitogen-activated protein kinases (MAPKs) are activated during ischemia and may contribute to the structural and functional changes. Three of the five major MAPK cascades have been studied extensively in the heart: extracellular signal-regulated kinase (ERK1 and ERK2) [11], c-Jun N-terminal kinases (JNK1 and JNK2) [12] and p38 mitogen-activated protein kinase (p38 MAPK) [13].

It was reported that the occurrence of arrhythmias is closely connected with cardiac gap junction protein expression, which regulates intercellular communication [14]. Connexin 43 (Cx43), the major gap junction protein, plays an important role in the electrical activity of ventricular myocytes by phosphorylation [15, 16].

In this study, we investigated the effects of morphine and sufentanil on myocardial I/R-induced arrhythmias and explored the involvement of Cx43 and p38-mitogen-activated protein kinase phosphorylation (p38 MAPK) in the mechanism.

Materials and methods

Animals

In this study, healthy male Sprague-Dawley rats (SPF Grade; aged 8 to 12 weeks; weight, 250-330 g) were provided by the Laboratory Animal Center, Ningxia Medical University (China). The protocols were approved by the Institutional Animal Care and Use Committee of Ningxia Medical University.

Experimental grouping

All rats used in the experiment were numbered and randomly divided into six groups (n = 6 per group). In the sham operation group (C), left thoracotomy was performed, and a 6-0 non-invasive suture line was placed around the left anterior descending (LAD) coronary artery without ligation. In the ischemia/reperfusion group (I/R) group, rats were subjected to LAD coronary artery ligation for 30 min followed by reperfusion for 120 min. In the morphine preconditioning group (M), 0.3 mg/kg morphine (Northeast Pharmaceutical Group Shenyang No. 1 Pharmaceutical Co., Ltd.) was administered three times intravenously via a venous pump before LAD coronary artery ligation (Circulation Research, 1996, 78 (6): 1100-1104). In the sufentanil preconditioning group (S), 3 μg/kg sufentanil (Yichang Humanwell Pharmaceutical Co., Ltd) [17] was administered using the same protocol. There was a 5-min interval between each drug administration [18, 19], and the subsequent ligation procedure resembled that used in the I/R group. In the morphine and p38 MAPK inhibitor group (MPA) and sufentanil and p38 MAPK receptor blocker group (SPA), 2 mg/kg [SB203580 (SB, p38 MAPK inhibitor; Abcam, Cambridge, MA, USA)] [20] was administered intravenously 10 min before morphine and sufentanil preconditioning, and the subsequent procedure resembled that used in the group M.

Preparation of myocardial I/R model [21]

All the rats underwent a 12-h fasting period before the operation, with free access to water. Anesthesia was performed by intraperitoneal injection of 3% pentobarbital sodium (40 mg/kg), and lead II electrocardiograms (ECG) were recorded during the experiment using a BL-420S Biological Data Acquisition and Analysis System (Chengdu TME Technology, China). The rats were connected to a small animal ventilator after tracheal intubation with breathing supported under normal atmospheric pressure. A respiratory rate of 60-70 bpm and a tidal volume 2-3 ml/100 g were recorded. An intravenous catheter was placed in the right carotid artery and connected to an energy converter to record arterial blood pressure. The left femoral vein was cannulated for the administration of drug or vehicle. Taking the left main coronary vein as the landmark, a 6-0 non-invasive suture line was placed 1-2 mm below the left auricle and covered by a polyethylene tube (length 1.5 cm; diameter 0.2 mm). Successful LAD ligation was confirmed by observation of cyanotic myocardial tissue below the ligature, with weakened movement and ECG showing significant ST-elevation. On releasing the ligature, successful reperfusion was confirmed by the observation of ST-depression due to reactive hyperemia in the local myocardium. The body temperature was maintained at 37-38°C by the hot plate method during surgery.

Monitoring of hemodynamic changes

Hemodynamic changes in recipients were recorded at the following time-points: 10 min before ischemia (T0), immediately after isch-

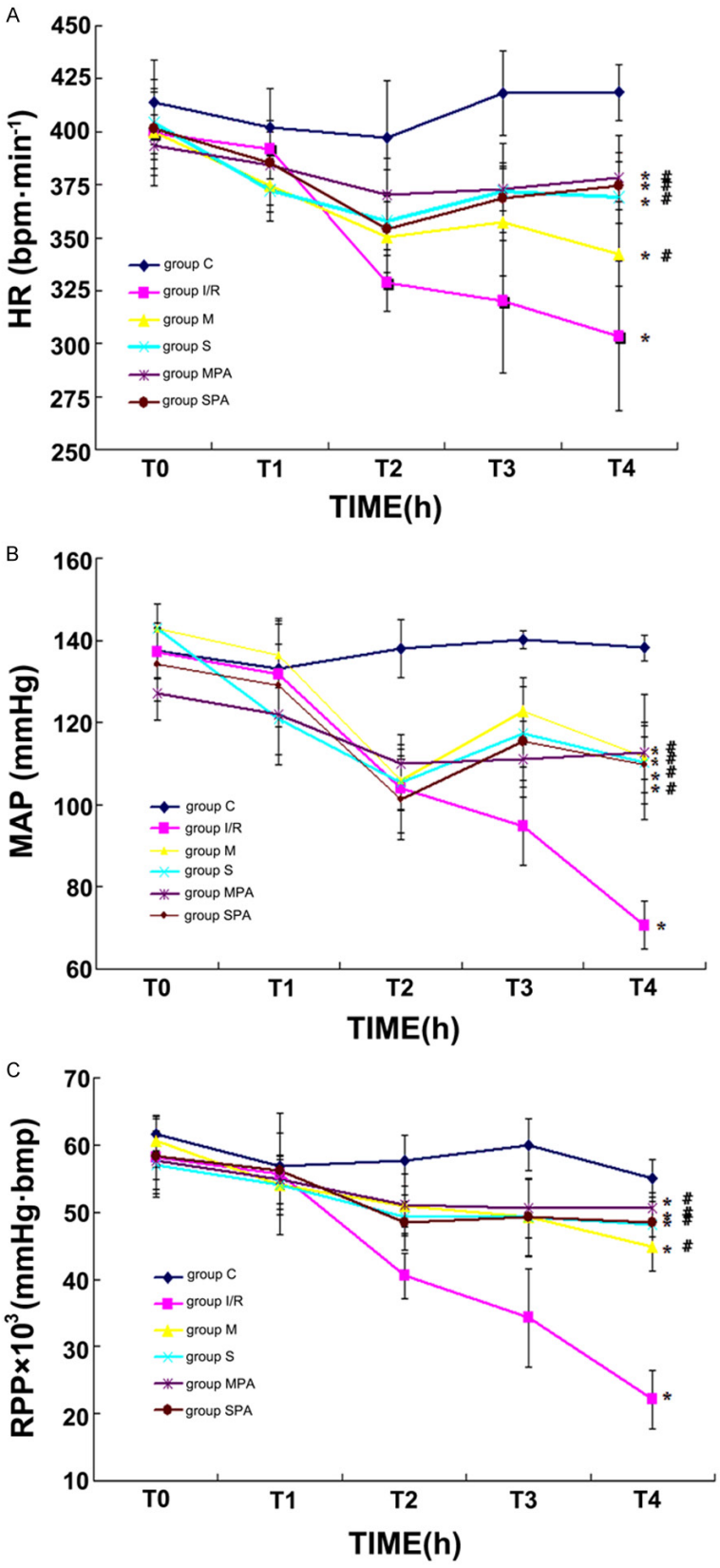

Figure 1. Hemodynamics for each group. A. The results of HR. B. The results of MAP. C. The results of RPP (*$P < 0.05$ versus the group C; #$P < 0.05$ versus the group I/R).

emia (T1), 30 min after ischemia (T2), 30 min after reperfusion (T3), and 120 min after reperfusion (T4). The heart rate (HR) and mean arterial blood pressure (MAP) were recorded and the product of systolic blood pressure (SBP) and HR were calculated as the rate pressure product (RPP).

Arrhythmia determination

The electrocardiogram was scored for arrhythmia according to the system described by Wang et al. [22]: 0, no ventricular arrhythmia; 1, accidental ventricular extrasystole (less than 3 times within 1 min); 2, frequent ventricular extrasystole (3 times or more within 1 min); 3, accidental ventricular tachycardia (less than 3 times within 1 min); 4, frequent ventricular tachycardia (3 times or more within 1 min) or accidental ventricular fibrillation (less than 3 times within 1 min); 5, frequent ventricular fibrillation (3 times or more within 1 min) or death.

Immunohistochemical analysis of total Cx43 protein expression

Rats were euthanized at the end of the experiment and the heart was immediately removed and washed with cold normal saline. The tissue was dried and sliced longitudinally into 5-6 myocardial tissue samples (approximately 2 mm in thickness). The second part of myocardial tissue sample was embedded in paraffin. After deparaffinization and rehydration, tissues were sliced into sections with 4 μm thickness and then stained immunohistochemically for Cx43 protein expression using an anti-Cx43 anti-

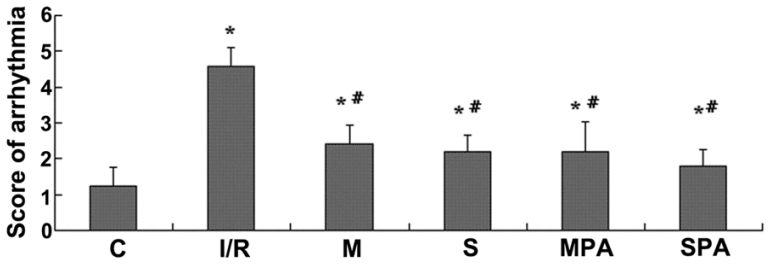

Figure 2. The score of arrhythmia ($^{*}P < 0.05$ versus the group C; $^{\#}P < 0.05$ versus the group I/R).

body (Abcam) and an SABC IHC kit (Wuhan Boster Bioengineering Co., Ltd). Immunoreactivity was detected by DAB substrate development. The sections were then subjected to hematoxylin counterstaining, dehydration, clearing, and sealing with neutral balsam. Positive staining (brown and yellow) was observed in the gap junctions of the myocardium under a microscope (Zeiss, Oberkochen, Germany). Five random visual fields were selected and photographed for each section.

Western blot analysis

The tissue was stored at -80°C prior to analysis. The total protein of frozen tissue was extracted and the total protein content was quantified using the BCA method. Samples of total protein were added to 5× SDS sample buffer (4:1) for separation by SDS-PAGE (10% gel) followed by transfer to PVDF membranes. The immunoblots were blocked for 1 h with 5% nonfat dried milk in Tris-buffered saline containing 0.1% Tween 20 (pH 8.0) and probed overnight at 4°C with the primary detection antibodies specific for p38 MAPK (Cell Signaling Technology, Danvers, MA, USA), phospho-p38 MAPK (Cell Signaling Technology), Cx43 (Abcam), phospho-Cx43-Ser368 (Abcam). GAPDH was probed using an anti-GAPDH (Abcam) as an internal standard. The Cx43 antibody detected total Cx43 protein (39-44 kDa) and phosphorylated Cx43 (S368) (42-46 kDa). Immunoreactivity was visualized by Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific, MA, USA).

Statistical analysis

SPSS17.0 (IBM, NY, USA) was used to analyze the data. Quantitative data are shown as the mean value ± standard deviation (SD). Differences among multiple groups were analyzed using ANOVA and differences between two groups were analyzed by LSD post-hoc tests. $P < 0.05$ indicated a statistically significant difference.

Results

Assessment of hemodynamic changes

The HR, MAP and RPP of group I/R at timepoints T2, T3 and T4 were all decreased in comparison with those of group C ($P < 0.05$), and these values were all increased in groups M, S, MPA and SPA ($P < 0.05$). There were no significant differences between the groups M, S, MPA and SPA. The HR, MAP and RPP of group I/R were decreased at T2, T3 and T4 compared with the values at T0 ($P < 0.05$), while no significant differences were found in groups M, S, MPA and SPA (**Figure 1**).

Results of ECG and occurrence of arrhythmias during I/R

Ventricular arrhythmias, such as ventricular extrasystole, tachycardia and fibrillation, appeared in all other groups during the process of I/R, especially in group I/R. As the reperfusion continued, myocardial blood circulation was improved and hence, the occurrence of arrhythmias was reduced. Compared with group C, groups I/R, M, S, MPA and SPA, all had significantly higher arrhythmia scores ($P < 0.05$). In contrast, the arrhythmia scores were lower in groups M, S, MPA and SPA than those in group I/R ($P < 0.05$). However, there were no significant differences between groups M, S, MPA and SPA (**Figure 2**).

IHC analysis of Cx43 protein expression in myocardial tissue

Positive staining of Cx43 protein was evenly distributed in cord-like and cluster-like patterns in normal myocardial tissue (group C). Apparent end-to-end and end-to-side junctions with intercalated disks were observed. Compared with group C, Cx43-positive staining was distributed irregularly as scattered vague dots in group I/R. In groups M, S, MPA and SPA, the distribution

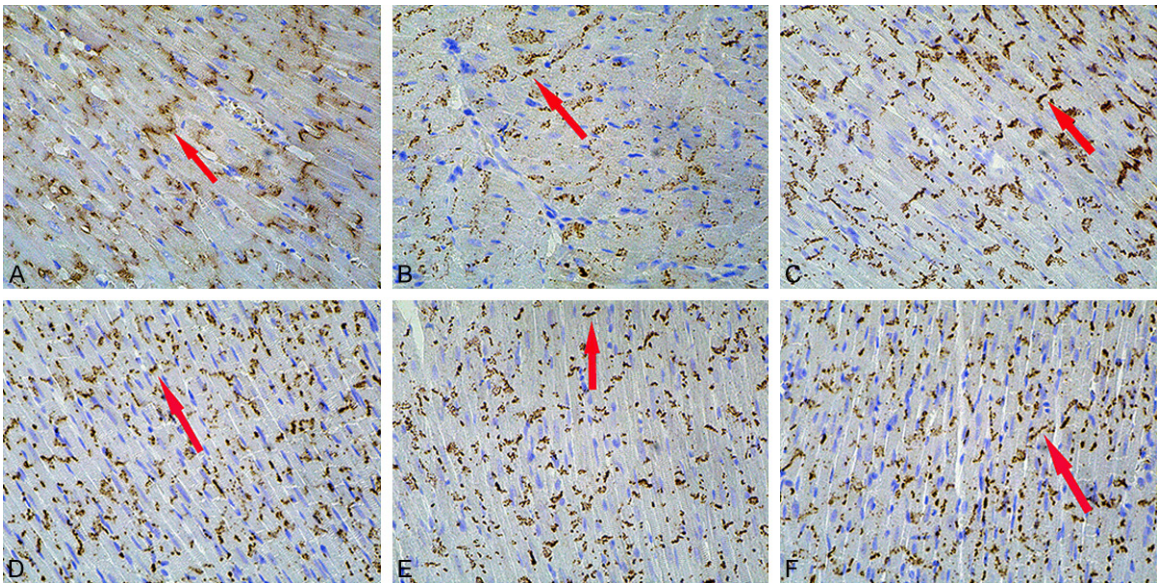

Figure 3. Immunohistochemical detection of total Cx43 gap junction expression (SABC ×400). A. Group C; B. Group I/R; C. Group M; D. Group S; E. Group MPA; F. Group SPA. Arrows indicate the different patterns of Cx43 staining.

was comparatively even, and similar to that observed in group C (**Figure 3**).

Western blot analysis

Compared with group C, the expression levels of p38 MAPK and p-p38 MPAK in group I/R were increased sharply, but were decreased in groups M, S, MPA and SPA (**Figure 4A** and **4C**).

Analysis of the effects of the MAPK signal pathway on Cx43 phosphorylation showed that phosphorylation of Cx43 at the Ser368 site (p-Cx43-Ser368) was significantly decreased in group I/R compared with that in group C. Groups M, S, MPA and SPA showed less phosphorylation of Cx43 at Ser368, although the levels were still higher than those observed in group I/R. However, the levels in groups M, S, MPA and SPA were similar (**Figure 4B** and **4D**).

Discussion

In the current study, administration of morphine or sufentanil 30 min before ischemia ameliorated the I/R-induced arrhythmia, and caused only minor fluctuations in HR and MAP. Furthermore, morphine and sufentanil induced increased p-Cx43 levels, although this effect may not be mediated via the p38 MAPK pathway.

Morphine preconditioning ameliorated the I/R-induced arrhythmia, with little fluctuation in HR and MAP. The results for groups M and MPA were not similar because morphine induced p-Cx43 levels, but not by inhibiting the p38 MAPK pathway. The specific mechanism is unclear and requires further in-depth studies for clarification.

Dephosphorylation of Cx43 induces uncoupling of gap junctions [23], which leads to cardiac rhythm disturbances and fatal arrhythmia [24]. It is well known that Cx43 is dephosphorylated in cardiac I/R models in pigs [25], rabbits [26], and rats [27]. Dephosphorylated Cx43 increases the permeability of gap junctions, ultimately leading to Ca^{2+} overload and cardiac myocyte destruction [28].

In this study, the arrhythmia scores were high due to decreased p-Cx43 levels after I/R. However, after the treatment with sufentanil, the p-Cx43 levels were increased, with simultaneously decreased arrhythmia scores. Despantez et al. [29] showed that downregulation of Cx43 induced disturbances in impulse propagation. Zhou et al. [30] also demonstrated that the anti-arrhythmic effects were related to Cx43 protein expression. Our results were consistent with these studies. We then investigated the effects of Cx43 protein expression.

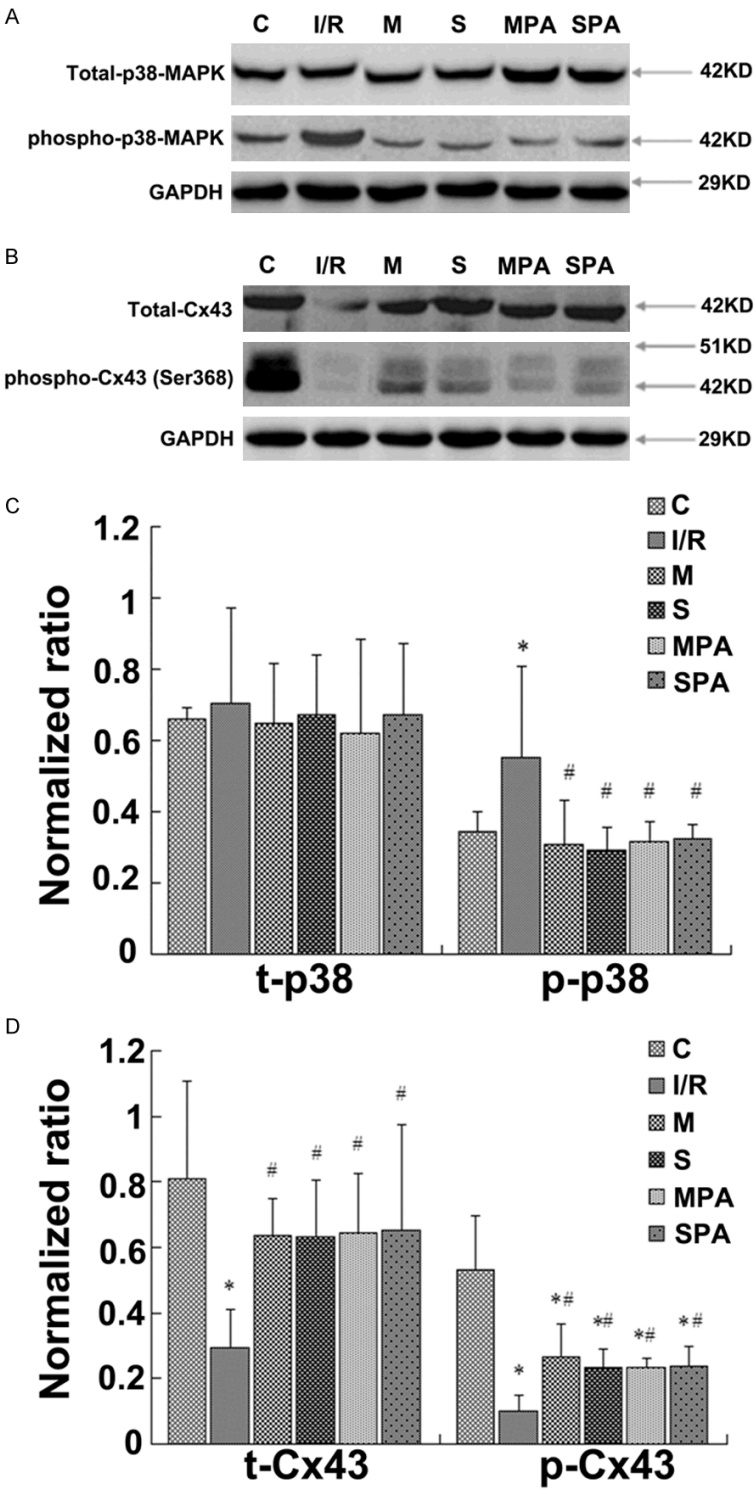

Figure 4. Western blot analysis of p38-MAPK and Cx43. (A) Total-p38-MAPK and phospho-p38-MAPK in heart tissue. (B) Total-Cx43 and phospho-Cx43 (Ser368) in heart tissue. (C and D) Quantitation of WB results of (A and B). $^{*}P < 0.05$ versus the group C; $^{\#}P < 0.05$ versus the group I/R.

The MAPK family primarily consists of extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK), and p38. p38 MAPK is activated by phosphorylation caused by ischemia, stress, radiation and pro-inflammatory cytokines [31]. However, p-p38 MAPK blocks the connection between gap junctions, which causes cardiac disturbances. See et al. [2] found that p38 was activated in I/R and exacerbated cardiac injury. This may be due to the generation of reactive oxygen species (ROS) and osmotic stress. Kaiser et al. [32] also demonstrated that the activated p38 MAPK plays an important role in I/R-induced myocardial injury and dysfunction. Active phosphorylated p38 MAPK enters the nucleus, where it influences transcription factors, gene transcription, and protein synthesis resulting in changes in cytoskeletal structure and mediating cell proliferation, differentiation, and apoptosis [33, 34]. In other words, p-p38 MAPK is the active form of p38 in the MAPK signaling pathway.

In this study, we assessed the effectiveness of SB203580 in inhibiting p38 MAPK activity by measuring p-p38 levels. We found that p-p38 MAPK was increased after I/R, and decreased by treatment with sufentanil, with a similar trend observed in the effects on arrhythmia scores. Although SB203580, a highly selective p38 inhibitor, was used before sufentanil treatment, p-p38 MAPK and p-Cx43 expression was not affected. Thus, we conclude that sufentanil acted on Cx43, but not via the p38 MAPK pathway.

This study is not without limitations. First, selective p38 inhibitor, SB203580, does not

mediate complete inhibition [35] and although p38-α and p38-β are sensitive to SB203580 inhibition, p38-γ and p38-δ are not. In addition, the effects were analyzed only 2 h after reperfusion, and the long-term effects of sufentanil remain to be elucidated in further studies.

Conclusion

Our study demonstrates that preconditioning with morphine or sufentanil attenuated myocardial I/R-induced ventricular arrhythmia in rats. The cardioprotective effects of morphine and sufentanil may be mediated by upregulating p-Cx43, not by the p38 MAPK pathway.

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Disclosure of conflict of interest

None.

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