Sufentanil protects the rat myocardium against ischemia–reperfusion injury via activation of the ERK1/2 pathway

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Abstract Sufentanil, a lipophilic opioid, is the most frequently used clinical drug for ischemic heart disease. The effects of sufentanil on MAPK signaling in ischemic heart disease were explored. The effects of sufentanil on ischemia–reperfusion (IR)-induced myocardial injury in a rat model were examined. The serum levels of CK, LDH, MDA and SOD, and the activities of Na^+/K^+ -ATPase and $\text{Ca}^{2+}/\text{Mg}^{2+}$ -ATPase were measured. The levels of total and phosphorylated ERK1/2, JNK, and p38 were measured by western blotting in the heart, and the myocardial H9C2 cell line was studied. Using the Cell Counting Kit-8, the growth rate of H9C2 cells affected by sufentanil was studied. The serum levels of CK, LDH and MDA were higher in the IR group than in the SO and SUF groups. The SOD level, as well as the activities of Na^+/K^+ -ATPase and $\text{Ca}^{2+}/\text{Mg}^{2+}$ -ATPase, were lower in the SO and SUF groups than in the IR group. The phosphorylated ERK1/2 level was

lower in the IR group than in the SO and SUF groups. The growth rate of H9C2 cells increased with the concentration of sufentanil and the exposure time. The phosphorylated ERK level was upregulated by 4–12 h of sufentanil exposure, indicating that the effects were time-dependent. Furthermore, an inhibition of ERK signaling by chemical inhibition suppressed the sufentanil-mediated increase in the growth rate of H9C2 cells. Sufentanil appears to be beneficial for cases of worsening ischemic heart disease. Further studies are necessary before a clinical application is considered.

Keywords Sufentanil · Ischemia · MAPK · ERK1/2

Introduction

Ischemic heart disease, especially acute myocardial infarction (AMI), a primary myocardial condition characterized by the loss of cardiomyocytes and the prevalence of fibroblasts, is a major cause of heart failure. Early reperfusion is an absolute prerequisite for the survival of the ischemic myocardium (Wang et al. 2013; Vicencio et al. 2015). However, reperfusion has been referred to as a “double-edged sword” because reperfusion itself may lead to increased myocardial injury beyond that generated by ischemia, which results in a spectrum of reperfusion-associated

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pathologies collectively defined as reperfusion injury (Strutyns'kyi et al. 2012; Shi et al. 2014).

Several mechanisms have been proposed to cause reperfusion injury, including the formation of oxygen free radicals (OFR), calcium overload, neutrophil-mediated myocardial and endothelial injury (Zhai et al. 2015; Yoshioka et al. 2012), progressive decline in microvascular flow to the reperfused myocardium, and depletion of the high-energy phosphate store (Liu et al. 2014).

Cellular signaling, which is essential for the ability of cells to respond to the environment, integrates external cues to intracellular mediators and effectors (Zhou et al. 2015; Zhang et al. 2015). Mitogen-activated protein kinase (MAPK) activation constitutes a paradigm of intracellular signaling. For example, p38, a protein of the MAPK family, was initially identified as a transducer of the response to inflammatory and environmental stress conditions (Wang et al. 2015). Phosphorylation of threonine and tyrosine residues within p38 results in a conformational change that increases the accessibility of the active site and enhances catalysis (Wang et al. 2015; Treusch et al. 2015). Furthermore, ERKs are activated in response to various cytokines and growth factors and mediate primarily mitogenic and anti-apoptotic signals (Teng et al. 2016). Studies have also shown that MAPKs are involved in the proliferation of myocardial cells (Takac and Samaj 2015).

Sufentanil, a lipophilic opioid, is the most frequently used clinical drug for anaesthesia. It has a high selectivity for the $\mu 1$ receptor but a low affinity for the δ receptor (Hu et al. 2013). Due to its strong liposolubility and high adherence ratio to plasma protein in human, sufentanil displays its powerful analgesic effects, which is 5–10 times higher than fentanyl and much higher than morphine (Wu et al. 2014). But, the function of Sufentanil on the cardiac cell during ischemic heart disease was no known. In this study, the effects of sufentanil on MAPK signaling in ischemic heart disease was explored.

Materials and methods

Unless otherwise specified, all chemicals and reagents were purchased from Sigma Chemical Company (St. Louis, MO, USA). Antibodies to IgG, GAPDH, ERK1/2, JNK, p90^{tsk}, phospho-ERK1/2, phospho-

JNK and phospho-p90^{tsk} (Ser380) were purchased from Abcam (Cambridge, MA, USA). U0126, an highly selective inhibitor of phospho-ERK1/2, was also purchased from Abcam.

Cardiac ischemia–reperfusion model

Wistar rats were provided by Weitong Lihua (Beijing, China). Sixty rats (body weight, 200 ± 30 g) of both sexes were randomly assigned to three groups as follows: sham operation group (SO group), ischemia–reperfusion group (IR group), and IR group treated with sufentanil (SUF group). Before starting the experiment, the rats were abstained from food for 12 h, but free to drink water at any time. After the animals were intubated, mechanical ventilation was achieved with a positive rodent respirator using atmospheric air at a tidal volume of 5 mL and a rate of approximately 50 breaths/min. The heart was exposed through a left thoracotomy in the third intercostal space. A 6.0 silk non-traumatic suture was passed through the epicardial layer around the major branch of the left coronary artery, approximately 2 mm from its point of origin. A plastic button with a diameter of approximately 5 mm was threaded through the ligature and placed in contact with the heart. The ends were passed through a small vinyl tube and exteriorized. This method is very convenient for producing ischemia–reperfusion injury during late preconditioning (24 h after surgery and acute preconditioning). Cardiac function was monitored throughout the experiment with an electrocardiogram (ECG). The surgical mortality rate (1–3%) was very low. Rats of the SO group were continuously perfused for 150 min. Rats of the IR group were kept ischemic for 30 min, and then reperfused for 120 min. Rats of the SUF group were kept ischemic for 30 min, and then treated with sufentanil. At the end of the 24-h reperfusion period, the rats were re-anesthetized, the coronary artery was re-occluded at the initial site of occlusion, and the heart was obtained. The animal protocol was approved by the Chongqing Medical University Experimental Animal Management Committee.

Biochemical analysis

The serum levels of creatine kinase (CK) and lactate dehydrogenase (LDH) were measured using commercially available kits. The lipid peroxidation level in the

heart homogenate was measured as MDA, the end product of lipid peroxidation that reacts with thiobarbituric acid (TBA) as the TBA reactive substance (TBARS) to produce a red-colored complex with a peak absorbance at 532 nm according to Buege and Aust (1978). To precipitate proteins, the supernatant (125 mL) was homogenized by sonication with TBS (50 mL) and butylhydroxytoluene (TCA-BHT, 125 mL), followed by centrifugation ($1000\times g$, 10 min, 4 °C). The supernatant (200 mL) was mixed with 0.6 M HCl (40 mL), TBA was dissolved in 0.7 M Tris (160 mL) and the mixture was heated at 80 °C for 10 min. The absorbance of the resultant supernatant was measured at 530 nm. The amount of TBARS was calculated by using an extinction coefficient. SOD activity was measured according to the method of McCord and Fridovich (1968), which is based on the production of superoxide radicals during the conversion of xanthine to uric acid by xanthine oxidase and the inhibition of cytochrome C reduction. One unit of SOD activity was defined as the amount of SOD that produces 50% inhibition of cytochrome C reduction. The activities of CAT and GPx were determined following the methods of Greenwald (1985). Glutathione reductase (GR) activity was assayed as described by Carlberg and Mannervik (1975), with minor modifications that included measuring the oxidation of NADPH at 340 nm. The reaction mixture consisted of 0.1 M sodium phosphate buffer (pH 7.5), 1 mM EDTA, 0.63 mM NADPH and 0.15 mM GSSG.

Western blotting

Cell proteins were separated by 12% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked, and then incubated with primary antibodies overnight at 4 °C. Subsequently, the membranes were washed three times with PBS, and then incubated with peroxidase-conjugated secondary antibodies. Immunoreactive bands were detected with ECL reagents (Pierce, Rockford, IL, USA).

Cell cultures

Rat cardiomyocytes H9c2 (2-1) cells (Cell Bank of Chinese Academy of Sciences, Shanghai, China) were cultured in an incubator at 37 °C with 5% CO₂. The culture media was DMEM containing 15% fetal bovine serum (Gibco, Carlsbad, CA, USA).

Cell growth rate assay

The Cell Counting Kit-8 (CCK-8) kit (Dojindo Laboratories, Kumamoto, Japan) was used to determine cell viability and proliferation. Briefly, cells were seeded in 96-well plates (3000 cells/well, four replicates) and incubated with different final concentrations of sufentanil (0, 5, 10, 20 µM) at different time (12, 24, 36, and 48 h). The cells were replenished with a medium containing CCK-8 solution (10 µL CCK-8 in 100 µL medium) and incubated for 2 h, after which the absorbance at 450 nm was measured using a microplate reader (Bio-Tek Instruments, Winooski, VT, USA). The growth rate of the cells was calculated. % growth rate = (mean experimental absorbance/mean control absorbance) $\times$ 100.

Statistical analysis

Statistics were carried out by one-way analysis of variance (ANOVA) using GraphPad Prism software (version 5.0, GraphPad Software, La Jolla, CA, USA). Data are presented as mean $\pm$ SD.

Results

Levels of CK, LDH, MDA and SOD and the activities of Na⁺-K⁺ + -ATPase and Ca²⁺-Mg²⁺-ATPase

The serum levels of CK and LDH were significantly higher in the IR group than in the SO and SUF groups ($P < 0.05$, Fig. 1a, b). The MDA level in the myocardium was significantly higher in the IR group than in the SO and SUF groups ($P < 0.05$, Fig. 1c). The SOD level in the myocardium was significantly lower in the IR group than in the SO and SUF groups ($P < 0.05$, Fig. 1d). The activities of Na⁺-K⁺-ATPase and Ca²⁺-Mg²⁺-ATPase were significantly lower in the IR group than in the SO and SUF groups ($P < 0.05$, Fig. 1e, f).

Effects of sufentanil on MAPK signaling in the myocardium

To assess MAPK activity, the levels of total and phosphorylated ERK1/2, JNK and p38 were measured in the IR, SO and SUF groups by western

Fig. 1 Serum levels of CK, LDH, MDA and SOD, and the activities of Na⁺-K⁺-ATPase and Ca²⁺-Mg²⁺-ATPase. **a** Serum level of CK. **b** Serum level of LDH. **c** Expression level of MDA in the myocardium. **d** Expression level of SOD in

the myocardium. **e** Activity of Na⁺-K⁺-ATPase in the myocardium. **f** Activity of Ca²⁺-Mg²⁺-ATPase in the myocardium. *Significantly different from the SO group ($P < 0.05$)

blotting (Fig. 2). The total and phosphorylated JNK and phosphorylated p38 levels were not changed in the three groups. However, the phosphorylated ERK1/2 level was lower in the IR group than in

the SO and SUF groups ($P < 0.05$), indicating that ERK1/2 is a key protein mediating the effects of sufentanil. The levels of the other proteins, however, were unchanged.

Fig. 2 Effects of sufentanil on MAPK signaling in the SO, IR and SUF groups. **a** Representative western blots showing the levels of total and phosphorylated ERK, JNK and p38. **b** Histograms summarizing the results shown in **a**. Results are expressed as mean $\pm$ S.D ($n = 5$). *Significantly different from the SO group ($P < 0.05$)

Effects of sufentanil on the growth rate of H9C2 cells

To investigate the growth-mediating effects of sufentanil on the myocardium, the myocardial H9C2 cell line was used. The growth rate of H9C2 cells increased with the concentration of sufentanil and the exposure time. The growth rate was highest after treating H9C2 cells with 10 μ M sufentanil for 36 h (Fig. 3).

Effects of sufentanil on MAPK activity in H9C2 cells

Our results showed that the effects of sufentanil in the myocardium were largely mediated by ERK1/2. To assess MAPK activity in H9C2 cells, the levels of total and phosphorylated ERK1/2, JNK and p38 were

Fig. 3 The growth rate of H9C2 cells after exposure to sufentanil (0, 5, 10 and 20 μ M) for 12, 24, 36 and 48 h

measured at 0, 4, 8 and 12 h by western blotting. The total and phosphorylated JNK and phosphorylated p38 levels were not changed. The phosphorylated ERK1/2 level was higher at 0, 4 and 8 h ($P < 0.05$)

Fig. 4 Effects of sufentanil on MAPK signaling in H9C2 cells. **a** Representative western blots showing the levels of total and phosphorylated ERK, JNK and p38. **b** Histograms summarizing the results shown in **a**. Results are expressed as mean $\pm$ S.D ($n = 5$). *Significantly different from the 0 h data point ($P < 0.05$), # Significantly different from the 4 h data point ($P < 0.05$)

than at time points beyond 8 h (Fig. 4), suggesting that ERK1/2 accelerates the growth rate of H9C2 cells cultured in the presence of sufentanil. To test this hypothesis, a specific inhibitor of ERK1/2 phosphorylation, U0126, was used to inhibit the ERK1/2 level. Upon inhibition of the phosphorylated ERK1/2 level, the levels of the other proteins were unchanged (Fig. 5a, b). In the presence of U0126 and 10 μ M sufentanil, the growth rate of H9C2 cells was unchanged (Fig. 5c), and the growth rate was significantly lower than in the presence of sufentanil alone.

Discussion

In the present study, changes in the haemodynamic parameters supported ischemia–reperfusion injury in the normal rat heart (Li et al. 2016). The enzymes routinely measured in the clinical laboratory for the purpose of diagnosing and monitoring myocardial infarction included creatine kinase (CK) and lactate dehydrogenase (LDH). CK and LDH isoenzyme determinations are useful when there is a question about the tissue source of elevated enzyme activity (Zhang et al. 2012). These enzymes are present in sufficiently high quantities in myocardial tissue so that the death of a relatively small portion of tissue results in a substantial increase in measurable enzyme activity in the serum (Zhang et al. 2012). The relatively high levels of CK and LDH in cardiac tissue indicates that measuring the activities of these enzymes in serum is very useful in the diagnosis and prognosis of myocardial infarction (Zhang et al. 2012; Xu et al. 2013). In this study, the serum levels of LDH and CK were higher in the IR group than in the SO and SUF groups. In the SUF group, there was a decrease in the release of intracellular enzymes (CK and LDH) from ischemic hearts, revealing that cell membranes were protected from damage. Na^+/K^+ -ATPase (a Na, K-pump) is an integral membrane protein that maintains normal physiological Na^+ and K^+ gradients by catalyzing and transporting these ions across the plasma membrane. The enzyme is comprised of α and β subunits, both of which are essential for ATPase and ion pumping functions (Zhang et al. 2012; Xu et al. 2013). Myocardial Na^+/K^+ -ATPase and $\text{Ca}^{2+}/\text{Mg}^{2+}$ -ATPase activities were decreased in the IR group. Our findings are in agreement with previous observations showing that the membrane abnormalities in

Fig. 5 Effects of sufentanil on MAPK activity after p-ERK1/2 was inhibited by U0126. **a** Representative western blots showing the levels of total and phosphorylated ERK, JNK and p38. **b** Histograms summarizing the results shown in **a**. Results are expressed as mean $\pm$ S.D (n = 5). *Significantly different from the 0 h data point ($P < 0.05$). **c** The growth rate of H9C2 cells cultured in the presence or absence of U0126

Na^+/K^+ -ATPase, $\text{Na}^+/\text{Ca}^{2+}$ exchange and Ca^{2+} -pump activities led to intracellular calcium overload in experimental rat models of myocardial ischemia–

reperfusion. Early experimental findings have demonstrated the increased formation of oxygen-derived free radicals (oxy-radicals) in the myocardium during post-ischemia–reperfusion. Consistent with these reports, our findings showed that myocardial IR injury was accompanied by decreases in myocardial ATP production and antioxidant levels/activities, which are indirect indices of mitochondrial function and antioxidant status, respectively (Zhang et al. 2012; Xu et al. 2013; Zhang et al. 2011). The SOD level was significantly higher in the SUF group than in the other groups, indicating that sufentanil may alleviate oxidative injury in the IR group.

We found that sufentanil accelerates the growth rate of H9C2 cells through activation of the ERK1/2 branch of the MAPK pathway. The optimal sufentanil concentration to obtain maximum growth was 10 μM of sufentanil for 36 h. To gain further insight into the mechanism by which sufentanil accelerates the growth rate of H9C2 cells, we evaluated MAPK activity. The MAPKs are a superfamily of serine/threonine kinases that includes ERK, JNK and p38. These kinases are involved primarily in the activation of nuclear transcription factors that control cell proliferation, differentiation and apoptosis (Delhanty et al. 2006). Our results suggest that sufentanil accelerates the growth rate of H9C2 cells via the ERK signaling pathway, and not through the activation of JNK or p38. We found that 4–12 h of sufentanil treatment was required for ERK phosphorylation, and that the stimulus was time-dependent. Furthermore, an inhibition of the ERK level by chemical inhibition suppressed the sufentanil-mediated increase in the growth rate of H9C2 cells.

Overall, our results provide evidence that sufentanil increases the growth rate of H9C2 cells through an ERK-dependent pathway. Sufentanil appears to be beneficial for cases of worsening ischemic heart disease. Further studies are necessary before a clinical application can be considered.

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