

Butorphanol attenuates myocardial ischemia reperfusion injury through inhibiting mitochondria-mediated apoptosis in mice

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Abstract. – OBJECTIVE: To investigate the role of the opioid receptors agonist butorphanol on mice myocardial ischemia reperfusion (I/R) injury.

MATERIALS AND METHODS: The left anterior descending of coronary artery was ligatured for 30 min and then reperfusion for 6 h was performed to mimic the mouse myocardial I/R injury. All mice were randomly divided into three groups: sham group, I/R group and I/R + butorphanol group. Blood samples were collected for the measurement of cardiac troponin I (CTnI) and creatine kinase MB (CK-MB) levels. The infarct size was stained by triphenyltetrazolium chloride. The mitochondria morphology was observed by electron microscopy. The expressions of cleaved caspase-9 and -3, p38, ERK and JNK were detected by Western blot.

RESULTS: The myocardial infarct size, serum CK-MB and CTnI levels, expression of cleaved caspase-9 and -3, phosphorylation of p38 and JNK were all increased in the I/R group compared with the sham group (all $p < 0.01$). Butorphanol reduced the myocardial infarct size, serum CTnI and CK-MB levels, expression of cleaved caspase-9 and -3, and phosphorylation levels of p38 and JNK (all $p < 0.01$). The number of mitochondria and the individual mitochondrial cross-sectional areas were decreased in the I/R mice compared with the sham-operated mice (all $p < 0.01$). Butorphanol reversed these changes in mitochondrial morphology (all $p < 0.01$).

CONCLUSIONS: Butorphanol attenuates myocardial I/R injury through reducing cardiomyocyte apoptosis by inhibiting mitochondria-mediated apoptotic pathway, and blockage of p38 and JNK phosphorylation.

Key Words:

Butorphanol, Ischemia reperfusion injury, Apoptosis, Mitochondria, Mitogen-activated protein kinase.

Introduction

Ischemic heart diseases, such as coronary artery disease and acute coronary syndrome, have been the leading cause of cardiovascular morbidity and mortality. Cardiac myocyte apoptosis plays a key role in myocardial ischemia/reperfusion (I/R) injury¹. In contrast, inhibition of apoptosis has a powerful effect on cardioprotective. Activation of opioid receptor has been reported to be significantly associated with myocardial protection². The opioid receptor family consists of three members: κ , δ , and μ ³. Many studies^{4,5} have revealed that κ - and δ -, but not μ -receptors, are expressed in cardiomyocytes, and the activation of κ - and δ -receptors can attenuate myocardial I/R injury. Butorphanol is a novel opioid receptor agonist, which possesses a high affinity for κ -receptor and partially activation on δ -receptor. Pharmacological researches^{6,7} demonstrated that the administration of butorphanol has the effect of analgesia and relaxation. However, to date, there are few reports focused on butorphanol-induced cardioprotective in myocardial I/R injury. Recently, Wu et al⁸ reported that butorphanol post-conditioning could attenuate myocardial I/R injury through activating κ -receptor in rats. However, the molecular mechanism involved in butorphanol-induced cardioprotective in myocardial I/R injury has not yet been fully elucidated.

Thus, we created a myocardial I/R mouse model to investigate the role and molecular mechanism of butorphanol in myocardial protection.

Materials and Methods

Reagents

Butorphanol injection was obtained from Hengrui Medicine Co., Ltd., (Lianyungang, China). Serum cardiac troponin I (CTnI) and creatine kinase MB (CK-MB) detection kits were obtained from Jiancheng Bioengineering Research (Nanjing, China). All antibodies were provided by Cell Signaling Technology (CST, Danvers, MA, USA). Total protein extraction kits were provided by KeyGen Biotechnology (Nanjing, China).

Ethics Statements

8-10 weeks old and 20-30 g body weight male C57BL/6 mice were provided by the Experimental Animal Center of Jiangsu Province (Nanjing, China). Animal experiments followed the Guide for Laboratory Animals published by the NIH. Mice were fed with standard mouse food and water, and were kept under 12:12 hour light/dark cycles with 22°C. This study was approved by the Animal Ethics Committee of Dalian Medical University Animal Center.

Mice Model of Myocardial Ischemia/Reperfusion Injury

The mice were anesthetized by pentobarbital (50 mg/kg, intraperitoneal injection), intubated and ventilated. A left horizontal incision was made at the third intercostal space. To achieve I/R injury, a 8-0 silk suture was tied around both the left anterior descending (LAD) and a silicon tube. Myocardial ischemia was measured by the electrocardiograph performance (ST elevated). The sham-operated mice underwent an identical surgical procedure without ligation. After ischemia for 30 min, the silicon tube was removed to achieve reperfusion for 6 h. There were three groups: (1) sham group; (2) I/R group, sodium chloride was injected intramuscularly at the start of reperfusion; (3) I/R + butorphanol (B) group, butorphanol (50 µg/kg) was injected intramuscularly at the start of reperfusion. $n = 15$ in each group. After reperfusion, the heart samples were harvested and then stored at -70°C.

Assessment of Myocardial Infarct Size

Evans blue dye was perfused into the aorta to demarcate the ischemic area-at-risk. Hearts

were excised and sliced. The heart sections were stained with 1% triphenyltetrazolium chloride at 37°C for 20 min and fixed with 4% paraformaldehyde at room temperature for 8 h. Infarcted myocardium was carefully separated from the non-infarcted myocardium and weighed. The infarct size is presented as a percentage of the ischemic area-at-risk.

Measurement of Serum Myocardial Enzymes

The enzyme-labeled immunosorbent assay was used to measure the serum CK-MB and CTn I levels. After 6 h of reperfusion, blood samples were collected and centrifuged, then transferred to Eppendorf (EP) tubes. The following steps were performed according to the instructions of detection kits.

Electron Microscopy Analysis

The performance of electron microscopy analysis was described as previously⁹. Electron microscopy images of mitochondria were collected for analysis. PhotoshopCS5.0 software was used to analysis the electron microscopy images and to measure both the number and the size of the myocardial mitochondria.

Western Blotting

Western blot analysis was used to determine the expression of protein extracted from the I/R myocardium. Briefly, gel electrophoresis was performed to separate the different molecular weight proteins and then transferred onto polyvinylidene difluoride membranes. These membranes were incubated with anti-cleaved caspase9, anti-cleaved caspase3, anti-p-p38, anti-p38, anti-p-ERK1/2, anti-ERK1/2, anti-p-JNK and anti-JNK for overnight at 4°C. After incubating with these primary antibodies, the membranes were washed in Tris-buffered saline and Tween 20 (TBST-20). After that, they were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody at room temperature for another 2 h. Western blot detection kit and ImageJ software (NIH) were used to measure the blot signal and density.

Statistical Analysis

SPSS 19.0 software (IBM, Armonk, NY, USA) was used for statistical analysis. All results were presented as means $\pm$ standard deviations (SD). Differences among different groups were analyzed by one-way analysis of variance. Differ-

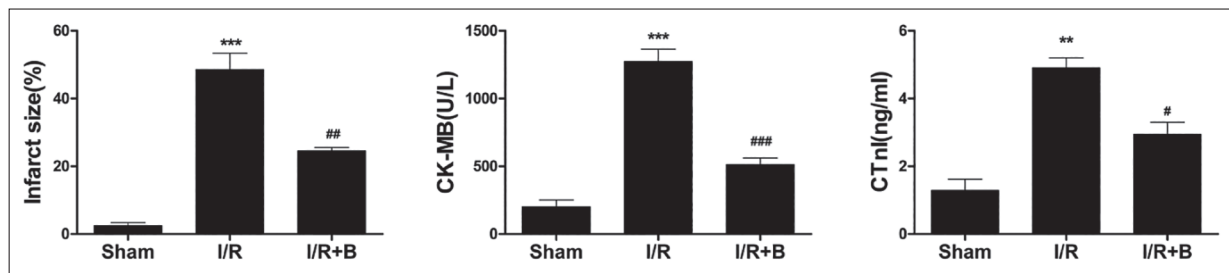


Figure 1. Effect of butorphanol on infarct size and myocardial enzymes. CTnI: cardiac troponin I; CK-MB: creatine kinase MB; I/R: ischemia/reperfusion; B: butorphanol. ** $p < 0.01$ and *** $p < 0.001$ vs. sham; # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ vs. I/R.

ences between two groups were analyzed by Student's t -test. A value of $p < 0.05$ was considered as statistically significant.

Results

Butorphanol Reduced Myocardial I/R-induced Increase in Infarct Size and Myocardial Enzymes

As shown in Figure 1, compared with the sham group, the myocardial infarct size and serum CTn I and CK-MB levels were significantly

increased in I/R group ($p < 0.001$; $p < 0.001$; $p < 0.001$, respectively). Butorphanol significantly reduced the myocardial infarct size and serum CTn I and CK-MB levels in contrast to the I/R group ($p < 0.001$; $p < 0.001$; $p < 0.001$, respectively).

Butorphanol Inhibited Myocardial I/R-induced Mitochondrial Apoptosis

Myocardial mitochondrial morphology was observed via electron microscopy (Figure 2). Compared with sham group, the mitochondria in the I/R group were smaller and more disor-

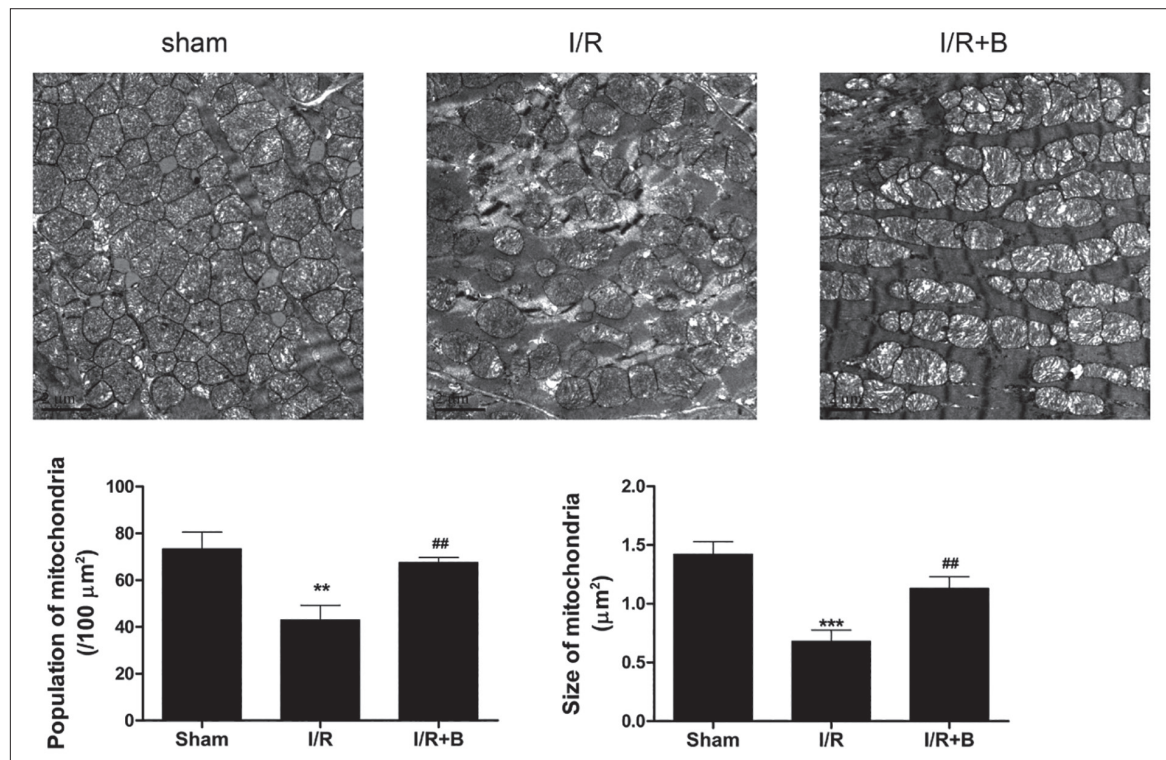


Figure 2. Effect of butorphanol on mitochondrial morphology in I/R myocardium. I/R: ischemia/reperfusion (magnification of 10,000 $\times$) (B indicates butorphanol). ** $p < 0.01$ and *** $p < 0.001$ vs. sham; ## $p < 0.01$ vs. I/R.

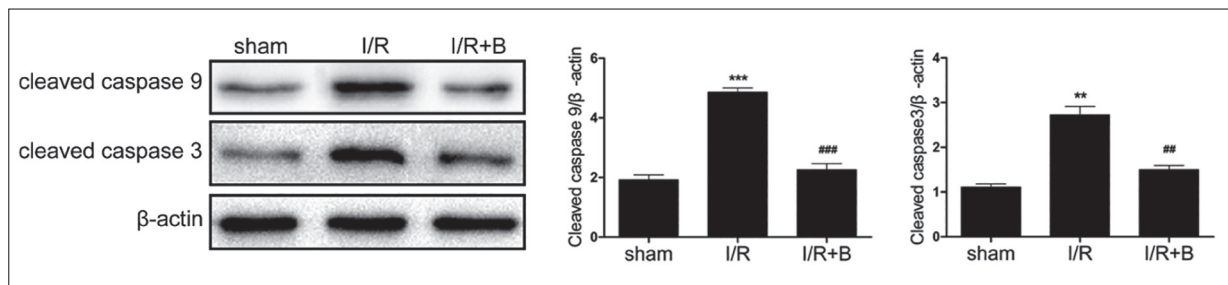


Figure 3. Effect of butorphanol on the expression of cleaved caspase-9 and -3 in the I/R myocardium. I/R: ischemia/reperfusion; B: butorphanol. ** $p < 0.01$ and *** $p < 0.001$ vs. sham; ## $p < 0.01$ and ### $p < 0.001$ vs. I/R.

ganized. Both the number and size of mitochondria per area were significantly decreased in the I/R group ($p < 0.01$; $p < 0.001$, respectively). Butorphanol administration substantially reversed these changes in mitochondrial morphology ($p < 0.01$; $p < 0.01$, respectively). The expression of cleaved caspase-9 and -3 was markedly increased in the I/R mice compared with the sham mice ($p < 0.001$; $p < 0.01$, respectively). Butorphanol administration significantly decreased the expression of cleaved caspase-9 and -3 in the I/R mice ($p < 0.001$; $p < 0.01$, respectively) (Figure 3).

Butorphanol Inhibited Myocardial I/R-induced p38 and JNK Phosphorylation

Compared with the sham group, the phosphorylation levels of p38 and JNK were both significantly increased in the I/R group ($p < 0.001$; $p < 0.001$, respectively). However, myocardial I/R did not induce the phosphorylation of ERK1/2 (Figure 4). Butorphanol administration prevented

the increases in p38 and JNK phosphorylation induced by I/R injury ($p < 0.01$; $p < 0.01$, respectively).

Discussion

We focused on the cardioprotective effect of butorphanol in a mouse myocardial I/R injury model. Our results shown that butorphanol administration markedly attenuated I/R injury, as confirmed by decrease in infarct size and myocardial enzymes levels. This protective effect was associated with the reduction in mitochondria-mediated apoptosis and the inhibition of p38 and JNK phosphorylation.

It is now known that activation of κ - and δ -receptors are significantly associated with the cardioprotection². Butorphanol is a novel opioid receptor agonist, which possesses a high affinity for κ -receptor and partially activation on δ -receptor. Wu et al⁸ reported that butorphanol post-conditioning could attenuate myocardial I/R

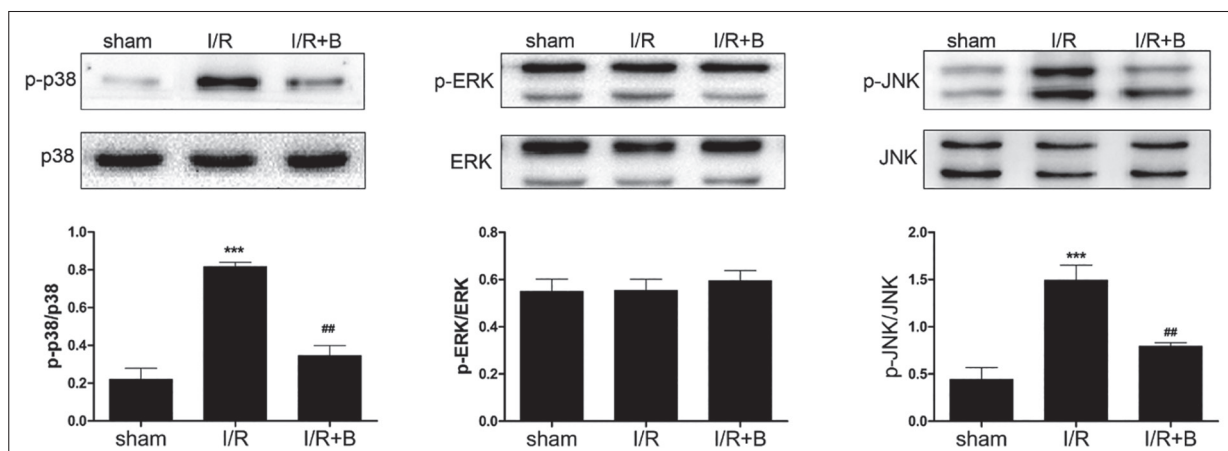


Figure 4. Effect of butorphanol on the phosphorylation of p38, ERK and JNK in the I/R myocardium. I/R: ischemia/reperfusion; B: butorphanol. *** $p < 0.001$ vs. sham; ## $p < 0.01$ vs. I/R.

injury, and this protective effect could be abolished by κ -receptor antagonist nor-binaltorphimine. Down-stream signal targets that involved in opioid-induced cardioprotection include mitochondria and MAPK pathway^{10,11}. Mitochondria have been considered as the central organelles of cardiomyocyte apoptosis, and the change of mitochondrial morphology reflects the state of cardiomyocytes⁹. Mitochondria-mediated apoptosis has been reported as an important mechanism involved in myocardial I/R injury¹². The mitochondria injury can promote cytochrome c to diffuse from the mitochondria into the cytoplasm, thereby activating caspase-9, which ultimately cleaves and activates the key executioner caspase-3, resulting in apoptosis^{13,14}. Consistent with these studies, we demonstrated that the mitochondria became smaller and more disorganized, and the expression of cleaved caspase-9 and -3 was significantly increased in the I/R myocardium. Butorphanol administration reserved mitochondrial morphology and inhibited the mitochondria-dependent apoptosis by reducing the expression of cleaved caspase-9 and -3. p38, extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) are the three components of the mitogen-activated protein kinase (MAPK) signaling pathway¹⁵. p38 and JNK have been viewed as two major effectors that involved in mitochondria-dependent apoptosis¹⁶. The activated JNK and p38 can inhibit the anti-apoptotic role of Bcl-2 via phosphorylating Bcl-2^{17,18}. Moreover, Kim et al¹⁹ reported that the activation of p38 could facilitate apoptosis by increasing the expression of the pro-apoptotic factor Bax. Donovan et al²⁰ observed that phosphorylated JNK could facilitate the mitochondria-mediated apoptosis by activating the pro-apoptotic factors Bad and Bid. Conversely, the cardiomyocyte apoptosis was significantly inhibited by the specific inhibitors of JNK and p38 (SP600125 and SB203580) in response to numerous stimuli *in vitro*¹⁶. We found that the phosphorylation levels of p38 and JNK were significantly increased in the I/R myocardium. Butorphanol suppressed the phosphorylation of p38 and JNK induced by myocardial I/R injury. Fryer et al²¹ reported that ERK phosphorylation was involved in opioid-induced delayed myocardial protection. However, we showed that ERK was not activated in I/R myocardium and ERK phosphorylation was not involved in butorphanol-induced myocardial protection. It may be due to the butorphanol was administrated at the onset of reperfusion.

Conclusions

We showed the protective effect of butorphanol on myocardial I/R injury. Overall, butorphanol attenuates myocardial I/R injury through reducing cardiomyocyte apoptosis via inhibition of mitochondria-mediated apoptosis, and blockage of p38 and JNK phosphorylation. The limitation of the current work is the lack of research on the cellular mechanism. Thus, future research is needed to investigate the potential therapeutic target of butorphanol in cardiomyocytes *in vitro*.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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