

Original Article

Fentanyl combined with butorphanol protects myocardial ischemia/reperfusion injury via κ -opioid receptor-mediated Nrf2-ARE signaling

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Abstract: Aims: To observe the combined effects of fentanyl and butorphanol on myocardial ischemia/reperfusion (I/R) injury, and whether the κ -opioid receptor (KOR)-mediated nuclear factor, erythroid 2-like 2 (Nrf2)-antioxidant response elements (ARE) signaling was involved in these effects was explored. Methods: We constructed a rat model of myocardial I/R injury, and the rats were administered with fentanyl, butorphanol and KOR antagonist nor-binaltorphimine (Nor-BNI). The myocardial infarct size was determined using Evans Blue and triphenyltetrazolium chloride staining. The activities of malondialdehyde (MDA) and superoxide dismutase (SOD) were measured using commercial kits. Western blot analysis was used to examine the activation of Nrf2-ARE signaling. Results: The combined use of fentanyl and butorphanol showed enhanced effects on decreasing the myocardial infarct size after myocardial I/R injury in comparison to the use of fentanyl. Compared with the fentanyl group, MDA activity was significantly decreased, while the SOD activity was significantly increased in the fentanyl+butorphanol group. Fentanyl enhanced nuclear Nrf2 protein expression, and increased the expression of the downstream genes, including NAD(P)H quinone oxidoreductase (NQO1) and heme oxygenase-1 (HO-1). The effect of fentanyl on Nrf2-ARE signaling was enhanced by the combined use of butorphanol. However, the combined effects of fentanyl and butorphanol on Nrf2-ARE signaling were blocked by Nor-BNI. Conclusion: This study provided the first evidence that fentanyl combined with butorphanol could activate Nrf2-ARE signaling to counteract oxidative stress, contributing to the protection of myocardial I/R injury. The enhanced activation of Nrf2-ARE signaling by combined use of fentanyl and butorphanol was mediated by KOR.

Keywords: Fentanyl, butorphanol, myocardial ischemia/reperfusion injury, opioid receptor, Nrf2-ARE signaling

Introduction

Myocardial ischemia/reperfusion (I/R) injury is a major clinical problem in the patients suffering from myocardial ischemia, circulatory arrest or cardiac surgery [1-3]. In recent years, some opioid receptor (OR) agonists have been administered successfully to protect against experimental I/R injury.

Fentanyl is the most widely used opioid drugs in clinic. It has a preferential affinity for μ -OR (MOR), and it also interacts with δ -OR (DOR) and κ -OR (KOR) [4]. Butorphanol is a synthetic selective OR agonist which exhibits agonist activity primarily at the KOR. It has been dem-

onstrated in rodent studies that both fentanyl and butorphanol could protect the heart against myocardial I/R injury [5-7]. At present, the application of fentanyl combined with butorphanol is very common in clinic. However, to date, there have been no reports of the combined effects of fentanyl and butorphanol on myocardial I/R injury.

Oxidative stress plays important roles in the pathogenesis of myocardial I/R injury [8]. Nuclear factor, erythroid 2-like 2 (Nrf2) is an important component of responses to oxidative stress [9-16]. Under stressfull conditions, the level of Nrf2 was dramatically increased, and Nrf2 is phosphorylated and translocated to the

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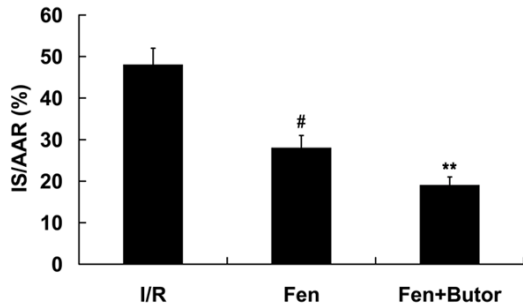


Figure 1. Combined effects of fentanyl (Fen) and butorphanol (Butor) on myocardial infarct size after myocardial I/R injury. IS: infarct size; AAR: area at risk; I/R: ischemia/reperfusion. [#] $P < 0.01$ compared with the I/R group; ^{**} $P < 0.05$ compared with the Fen group.

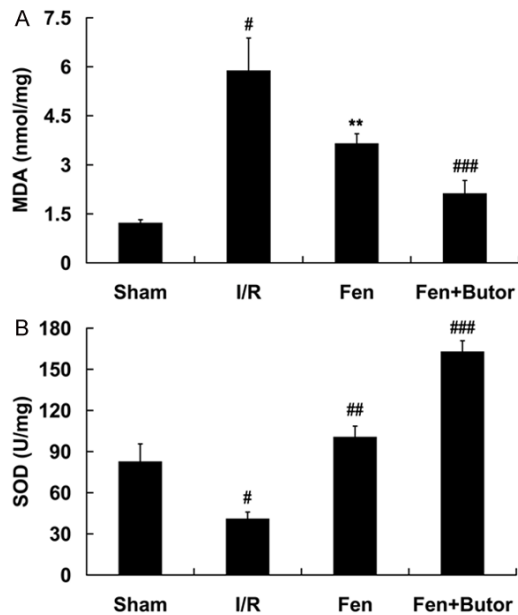


Figure 2. Combined effects of fentanyl and butorphanol on oxidative stress after myocardial IR injury. A. Levels of MDA. B. Levels of SOD. I/R: ischemia/reperfusion; Fen, fentanyl; Butor, butorphanol; MDA, malondialdehyde; SOD, superoxide dismutase. [#] $P < 0.01$ compared with the sham group; ^{**} $P < 0.05$ and ^{###} $P < 0.01$ compared with the I/R group; ^{##} $P < 0.01$ compared with the Fen group.

nucleus [17]. Once there, it can bind to antioxidant response elements (ARE) and regulates the coordinated upregulation of genes in response to oxidative stress [9].

In the present study, we firstly investigated the combined effects of fentanyl and butorphanol on myocardial I/R injury. Furthermore, whether the OR-mediated Nrf2-ARE signaling was involved in these effects was explored.

Materials and methods

Construction of rat IR model and drug treatment

The male Sprague-Dawley (SD) rats (250-350 g) were purchased from the Experimental Animal Center of Wenzhou Medical College. The rats were kept under constant environmental conditions (12 hour light/dark cycles), and fed on a laboratory diet with water. This study was approved by the Ethics Committee of The Second Affiliated Hospital of Wenzhou Medical University, and all the animal experiments were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals. To construct myocardial IR model, the rats were anesthetized with sodium pentobarbital intraperitoneally (40 mg/kg; Sigma, St. Louis, MO, USA), and intubated, ventilated artificially with a respirator. Electrocardiograph (ECG) probes were applied to the limbs to produce an ECG image. Following a left thoracic incision, a 6-0 silk suture with a slipknot was placed around the left anterior descending (LAD) coronary artery to induce myocardial ischemia. 30 min later, the slipknot was released for reperfusion for 120 min. The rats in the sham group underwent the same procedures without occlusion of the LAD. For drug treatment, fentanyl (50 $\mu\text{g}/\text{kg}$; Yichang Humanwell Pharmaceutical Co., Ltd, Yichang, Hubei, China), butorphanol (50 $\mu\text{g}/\text{kg}$; Jiangsu Hengrui Medicine Co., Ltd, Lianyungang, Jiangsu, China) and nor-binaltorphimine (Nor-BNI) (2 mg/kg; Sigma) were administered intravenously at the time of reperfusion. The rats in the I/R group was administered with sodium chloride intravenously at the onset of reperfusion.

Measurement of myocardial infarct size

1% Evans Blue solution (Sigma) was injected into the heart, and the heart was then harvested to refrigerate at -4°C overnight. The heart was sliced into sections about 2-mm thick and incubated with 1% triphenyltetrazolium chloride (Sigma) at 37°C . After staining, the slices were fixed in 4% paraformaldehyde. The area at risk (AAR) was determined as the region lacking blue staining. Infarct size was expressed as a percentage of the AAR.

Measurement of malondialdehyde (MDA) and superoxide dismutase (SOD)

The activities of MDA and SOD were determined using the commercial kits purchased from

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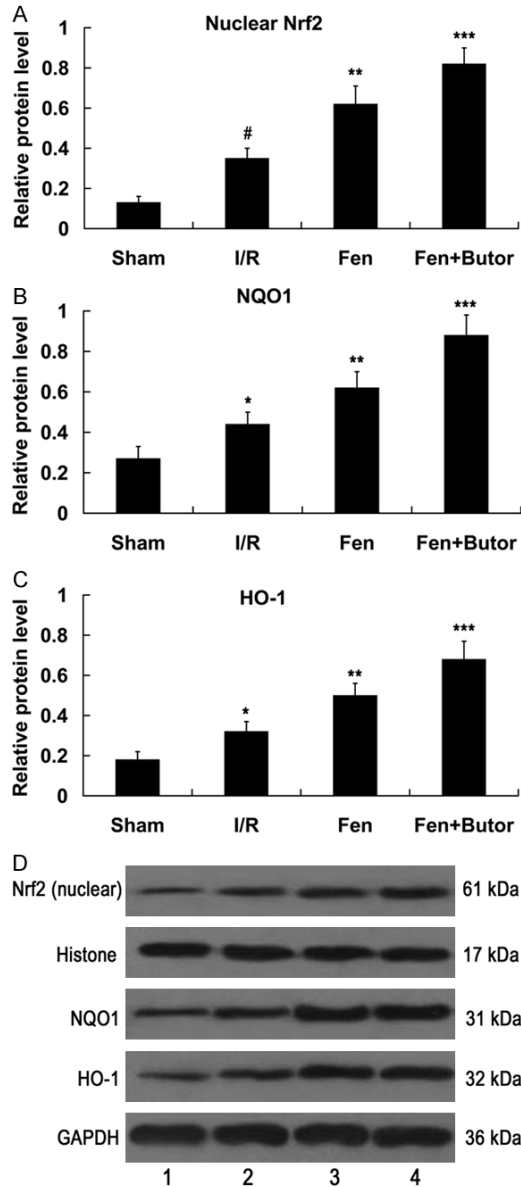


Figure 3. Combined effects of fentanyl and butorphanol on Nrf2-ARE signaling after myocardial IR injury. A. Relative protein level of nuclear Nrf2. Histone was used as the control. B. Relative protein level of NQO1. GAPDH was used as the control. C. Relative protein level of nuclear HO-1. GAPDH was used as the control. D. Representative western blot pictures. Lane 1, sham; lane 2, I/R; lane 3, Fen; lane 4, Fen+Butor. I/R: ischemia/reperfusion; Fen, fentanyl; Butor, butorphanol; Nrf2, nuclear factor, erythroid 2-like 2; ARE, antioxidant response elements; NQO1, NAD(P)H quinone oxidoreductase; HO-1, heme oxygenase-1. ^{*} $P < 0.05$ and [#] $P < 0.01$ compared with the sham group; ^{**} $P < 0.05$ compared with the I/R group; ^{***} $P < 0.05$ compared with the Fen group.

Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China) according to the manufacturer's instructions. The tissues were

weighed and homogenated. The level of MDA was determined using the thiobarbituric acid (TBA) method. A spectrophotometer was used to detect the absorbance at 532 nm for MDA and 550 nm for SOD.

Western blot

Total proteins were extracted using the Total Protein Extraction Kit (Sangon Biotech, Shanghai, China). Nrf2 nuclear fraction was obtained using the Nucleoprotein Extraction Kit (Sangon Biotech). The protein samples were separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis, and transferred onto a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). After blocking with 3% bovine serum albumin (Amresco Inc., Solon, OH, USA), the membranes were incubated with the primary antibodies, including rabbit polyclonal to Nrf2 (1:200), mouse monoclonal to histone (1:800), mouse monoclonal to NAD(P)H quinone oxidoreductase (NQO1) (1:400), mouse monoclonal to heme oxygenase-1 (HO-1) (1:400) and mouse monoclonal to GAPDH (1:400) (all from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), at 37°C for 2 h. After washing with TBST, the membranes were then incubated with the horseradish peroxidase-conjugated secondary antibody at 37°C for 1 h. The signals were visualized by enhanced chemiluminescence substrate (Thermo Fisher, Waltham, MA, USA).

Statistical analysis

All the data are expressed as the mean \pm standard. They were analyzed by SPSS 19.0 statistical software (IBM corp., Armonk, NY, USA) using the Student's t-test. P values less than 0.05 were considered statistically significant.

Results

Combined effects of fentanyl and butorphanol on myocardial infarct size after myocardial I/R injury

The rat myocardial I/R model was constructed, and the effects of fentanyl and butorphanol on myocardial infarct size were examined. As shown in **Figure 1**, the infarct size (IS)/AAR was $48 \pm 4\%$ in I/R group. Fentanyl treatment significantly decreased the IS/AAR to $28 \pm 3\%$ after myocardial I/R injury. Furthermore, significantly decreased IS/AAR was observed in fentanyl+butorphanol group compared with the fentanyl group.

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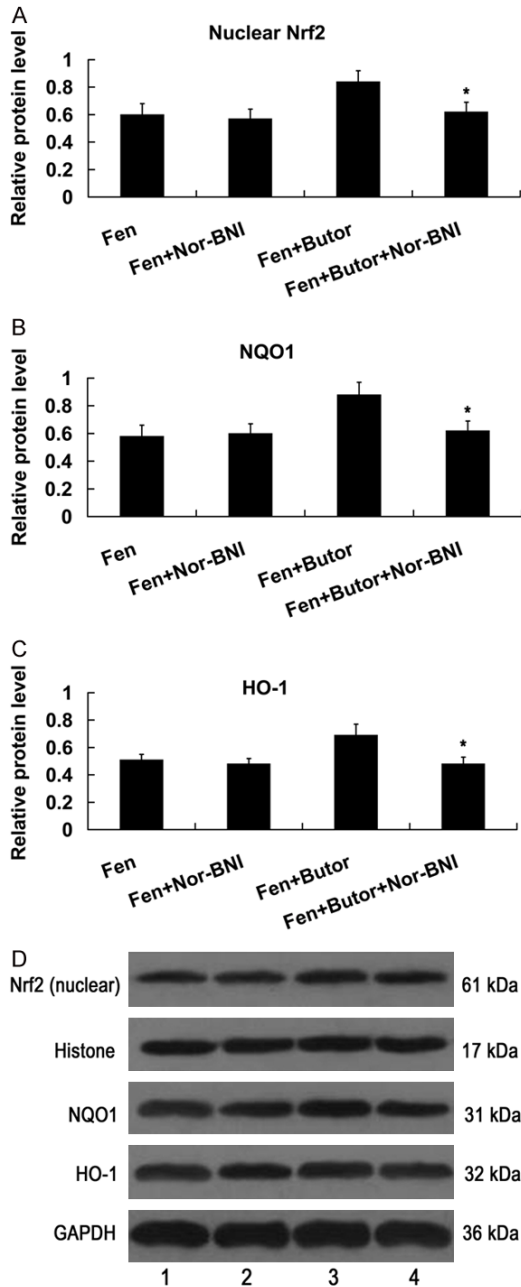


Figure 4. Effects of KOR antagonist on Nrf2-ARE signaling after myocardial IR injury. A. Relative protein level of nuclear Nrf2. Histone was used as the control. B. Relative protein level of NQO1. GAPDH was used as the control. C. Relative protein level of nuclear HO-1. GAPDH was used as the control. D. Representative western blot pictures. Lane 1, Fen; lane 2, Fen+Nor-BNI; lane 3, Fen+Butor; lane 4, Fen+Butor+Nor-BNI. KOR, κ -opioid receptor; I/R: ischemia/reperfusion; Fen, fentanyl; Butor, butorphanol; Nor-BNI, nor-binaltorphimine; Nrf2, nuclear factor, erythroid 2-like 2; ARE, antioxidant response elements; NQO1, NAD(P)H quinone oxidoreductase; HO-1, heme oxygenase-1. * $P < 0.05$ compared with the Fen+Butor group.

Combined effects of fentanyl and butorphanol on oxidative stress after myocardial IR injury

The activities of MDA and SOD in the ischaemic area were measured to investigate the combined effects of fentanyl and butorphanol on oxidative stress after myocardial I/R injury. As expected, compared with the sham group, the activity of MDA was significantly increased, and the SOD activity was significantly decreased after myocardial I/R injury. The activity of MDA in the fentanyl group was less than that in the I/R group, while the SOD activity in the fentanyl group was higher than that in the I/R group. In addition, compared with the fentanyl group, the activity of MDA was significantly decreased, while the SOD activity was significantly increased in the fentanyl+butorphanol group (Figure 2).

Combined effects of fentanyl and butorphanol on Nrf2-ARE signaling after myocardial IR injury

To investigate the combined effects of fentanyl and butorphanol on Nrf2-ARE signaling after myocardial IR injury, the expression of nuclear Nrf2, NQO1 and HO-1 in the ischaemic area were determined by western blot analysis. As shown in Figure 3, myocardial I/R injury triggered Nrf2-ARE signaling, as evidenced by the increased expression of nuclear Nrf2 and downstream genes, including NQO1 and HO-1 in the I/R group compared with the sham group. Fentanyl could enhance nuclear Nrf2 protein expression, and increase the expression of the downstream genes. Compared with the treatment of fentanyl, combined treatment of fentanyl and butorphanol further increased the expression of nuclear Nrf2 and its downstream genes.

Effects of KOR antagonist on Nrf2-ARE signaling after myocardial IR injury

To investigate whether KOR mediates the combined effects of fentanyl and butorphanol on Nrf2-ARE signaling after myocardial I/R injury, the rats were administrated with KOR antagonist Nor-BNI. As shown in Figure 4, Nor-BNI did not show any effects on the expression of nuclear Nrf2, NQO1 and HO-1 in the ischaemic area of rats treated with fentanyl. However, the combined effects of fentanyl and butorphanol on Nrf2-ARE signaling were blocked by Nor-BNI.

Discussion

The combination of various interventions to obtain enhanced therapeutic effects is always an important area of research focus. The combined use of fentanyl and butorphanol is very common in clinic. In the present study, we firstly investigated the combined effects of fentanyl and butorphanol on myocardial I/R injury. The results indicated that combined use of fentanyl and butorphanol enhanced the myocardial protection against I/R injury.

Oxidative stress has been demonstrated to be one of the molecular mechanisms related to ischemic/hypoxia reperfusion injury [8]. Excessive reactive oxygen species (ROS) were produced and the detoxifying enzyme activities of intracellular antioxidants were decreased during oxidative stress. MDA and SOD are two important indicators of oxidative stress. MDA is one of the end-products in the lipid peroxidation process. It is cytotoxic, and often been used to assess oxygen-derived free radical-mediated injury [18]. SOD catalyses the dismutation of superoxide anion, and it is one of the two isozymes responsible for destroying free superoxide radicals in the body. Consistent with the previous studies [19-21], oxidative stress was induced in myocardial IR injury, as demonstrated by increased MDA and decreased SOD in the I/R group compared with the sham group. The antioxidative property of fentanyl is well known in oxidative stress in a rat model of obstructive jaundice [22]. The study by Kang *et al* indicated that butorphanol exerts some antioxidant activity in vitro [23]. In the present study, we found that fentanyl also showed antioxidant activity in a rat model of myocardial I/R injury. Furthermore, combined fentanyl and butorphanol produced enhanced antioxidative effects against myocardial I/R injury.

Nrf2 is a transcription factor controls a web of antioxidant pathways to counterbalance the physiological and pathophysiological outcomes of oxidative stress [24]. It activates genes which contain ARE in their promoters to counteract oxidative injury. HO-1 and NQO1 are important ARE-regulated genes [25]. In the present study, we found myocardial I/R injury triggered Nrf2-ARE signaling, as evidenced by the increased expression of nuclear Nrf2 and downstream genes, including NQO1 and HO-1. Furthermore, we firstly demonstrated that fentanyl could

enhance Nrf2 protein expression and translocation, and increase the expression of the downstream genes. Moreover, this effect was enhanced by the combined use of butorphanol.

The opioid receptors consists of four major subtypes: μ , δ , κ and opioid receptor like-1 (ORL1) [26]. They belong to G-protein coupled receptor superfamily with opioids as ligand [27-29]. All the μ -, δ - and κ -opioid receptors have been suggested to play critical roles in opioid-induced cardioprotection [30-32]. In the rat myocardium, DOR and KOR but not MOR are expressed [33]. Fentanyl is one of the OR agonists, and it has effects on the brain, heart, and liver [34]. Fentanyl is considered to exert its protective effect on myocardial IR injury via DOR activation [5, 6]. Butorphanol targets the heart, mainly via KOR activation. The study by Wu *et al* confirmed that butorphanol post-conditioning provided cardioprotection against myocardial I/R injury, and the KOR was involved in this effect [7]. Protein kinase C (PKC) is the downstream signaling effector of opioid-induced cardioprotection. Nrf2 is the target for PKC regulation. Phosphorylation of Nrf2 at Ser40 by PKC leads to the release of Nrf2 from Keap1, and it is critical for the nuclear translocation of Nrf2 in response to oxidative stress [35, 36]. Based on these, we hypothesized that KOR mediates the enhanced effects of fentanyl combined with butorphanol on Nrf2-ARE signaling after myocardial IR injury. As expected, we found that the combined effects of fentanyl and butorphanol on Nrf2-ARE signaling were blocked by KOR antagonist Nor-BNI.

In conclusion, this study provided the first evidence that the combination of fentanyl and butorphanol obtained enhanced cardioprotection against I/R injury. Fentanyl combined with butorphanol could activate Nrf2-ARE signaling to counteract oxidative stress, thus contributing to the protection of myocardial I/R injury. The enhanced activation of Nrf2-ARE signaling by combined use of fentanyl and butorphanol was mediated by KOR. This study provided the molecular mechanism underlying the combined effects of fentanyl and butorphanol on myocardial I/R injury, and suggested that the combination of fentanyl and butorphanol is more efficient in cardioprotection than fentanyl.

Disclosure of conflict of interest

None.

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