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Remifentanil preconditioning protects rat cardiomyocytes against hypoxia-reoxygenation injury via δ-opioid receptor mediated activation of PI3K/Akt and ERK pathways

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ABSTRACT

Remifentanil preconditioning has been demonstrated to reduce myocardial ischemia reperfusion injury in rat hearts, while the mechanisms are not fully understood. This study investigated the protective effects of remifentanil against hypoxia-reoxygenation injury in adult rat cardiomyocytes and the mechanisms involving opioid receptors and downstream phosphatidylinositol-3-kinase/protein kinase B (PI3K/Akt) and extracellular signal-regulated kinase (ERK) signaling pathways. Adult rat cardiomyocytes were pretreated with remifentanil at different concentrations and then subjected to 90 min hypoxia followed by 120 min reoxygenation. The δ- (naltrindole), κ- (nor-binaltorphimine), or μ-opioid receptor antagonist (CTOP), as well as ERK inhibitor (PD98059) or PI3K inhibitor (wortmannin) was added before remifentanil preconditioning, respectively. Remifentanil showed significant protective effects against hypoxia-reoxygenation injury by increasing cell survival (Trypan blue staining) while reducing LDH activity and cell apoptosis (Hoechst staining). These effects were markedly reversed by naltrindole and were partially blocked by nor-binaltorphimine. Pretreatment of either PD98059 or wortmannin also abolished the protective effects of remifentanil. Following remifentanil preconditioning, the phosphorylation level of Akt reached peak at 10 min of reoxygenation. ERK phosphorylation, however, was subsequently enhanced at 120 min of reoxygenation. The phosphorylation levels of Akt and ERK were both blocked by naltrindole, but not nor-binaltorphimine or CTOP. Wortmannin inhibited the phosphorylation of both Akt and ERK, whereas PD98059 suppressed the phosphorylation of ERK only. In conclusion, our results suggested that remifentanil protected adult rat cardiomyocytes from hypoxia-reoxygenation injury and its effects appears to be dependent on the δ-opioid receptor mediated activation of PI3K/Akt and subsequent ERK signaling pathways.

1. Introduction

Myocardial ischemia-reperfusion (I/R) injury is the phenomenon that the restoration of blood flow to the ischemic area causes additional cell death which paradoxically reduces the beneficial effects of reperfusion. This form of myocardial injury can induce cardiomyocytes death and increase infarct size by itself, which may be associated with the increase rate of death after an acute myocardial infarction and the incidence of post-infarction heart failure (Yellon and Hausenloy, 2007). Numerous treatment strategies have been investigated to protect the myocardium against I/R injury. Ischemic preconditioning (IPC) is well known as a powerful innate protective mechanism, although it is generally clinically impractical due to its invasive and mechanical nature. Comparatively, pharmacologic techniques are more attractive in terms of practical application, avoiding mechanical ischemia.

Remifentanil, a fentanyl derivative, is an ultra-short acting opioid that possesses a high affinity for μ-opioid receptor and a relative lower affinity for δ- and κ-opioid receptors (James et al., 1991). Unlike the conventional opioids, remifentanil is rapidly metabolized by nonspecific blood and tissue esterases, independent of hepatic biotransformation and renal excretion for elimination. These unique pharmacokinetics characteristics make it attractive for intraoperative titration and fast recovery (Irwin and Wong, 2015). In view of the increasing use of remifentanil for general anesthesia during cardiac or non-cardiac surgeries, it is important to known whether remifentanil offers cardioprotection

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against cardiac I/R injury as other opioids do, as well as the underlying mechanisms. Prior studies performed by our research group are the first to report the cardioprotection conferred by remifentanil preconditioning, which mimicked IPC effects against myocardial I/R injury in the intact and isolated rat hearts (Zhang et al., 2004, 2005). Remifentanil also conferred delay cardioprotection in rat hearts 12–36 h after administration (Yu et al., 2007). In two clinical trials, remifentanil was evidenced to reduce cardiac injury markers in patients undergoing cardiopulmonary bypass (Xu et al., 2009; Wong et al., 2010a). The reduction of myocardial infarct size induced by remifentanil was mediated *via* cardiac δ- and κ-opioid receptors (Zhang et al., 2005; Wong et al., 2010b) and extracardiac μ-opioid receptor (Zhang et al., 2004; Yu et al., 2007).

It is known that the excitation of opioid receptors lead to activation of downstream signal kinase cascades thereby mediating the anti-apoptotic effects of morphine and other selective opioid receptor agonists (Tegeder and Geisslinger, 2004). Quite a few researches have manifested the involvement of phosphatidylinositol-3-kinase (PI3K)/protein kinase B (Akt) (Gross et al., 2005; Cohen et al., 2007; Peart et al., 2008) or extracellular signal regulated kinase (ERK) (Fryer et al., 2001; Ikeda et al., 2006; Kim et al., 2011) signaling pathways in opioids or opioid receptor agonists mediated cardioprotection. It has been reported that ERK and anti-apoptotic pathways might be important mediators of remifentanil-induced cardioprotection (Kim et al., 2010), the exact mechanisms, however, are not fully understood. Therefore, the present study was designed to investigate whether reminfentanil exerted cardioprotection *via* opioid receptor mediated activation of PI3K/Akt and ERK pathways in primary cultured adult rat ventricular myocytes.

2. Materials and methods

2.1. Animals

Adult male Sprague-Dawley rats weighing 300 ± 50 g were obtained from the Anhui (China) Laboratory Animal Center. The animals were maintained on a 12 h light/dark cycle at an ambient temperature of 22 ± 2 °C, with food and water available *ad libitum*. All experimental protocols were approved by the Institutional Animal Care and Use Committee of Anhui Medical University, and adhered strictly to the NIH Guide for the Care and Use of Laboratory Animals.

2.2. Isolation of adult rat ventricular myocytes

The adult rat ventricular myocytes were isolated according to a previously established method with minor modifications (Kaur et al., 2006). Following administration of heparin sodium (300 IU), rat hearts were rapidly excised under sodium pentobarbital (Sigma, USA) anesthesia. After the excision, rat hearts were mounted on a modified Langendorff perfusion apparatus with perfusion of calcium-free Krebs buffer containing 110 mM NaCl, 2.6 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, and 11 mM glucose (pH 7.4) for 5 min. This perfusion was then switched to digestion buffer (perfusion buffer containing 0.8 mg/ml collagenase II and 50 μM calcium) for about 30 min until the heart appeared swollen, pale and flaccid. The ventricular tissues were chopped into small pieces and then gently dispersed into cell suspension using plastic transfer pipettes. After that, the cells were filtered through a nylon mesh and washed with stopping buffer (perfusion buffer containing 10% newborn bovine serum (NBS)). The calcium concentration was gradually increased to 1.25 mM. After isolation, the cardiomyocytes were cultured in dishes precoated with sterilized matrigel (BD bioscience, USA) in M199 medium containing 2 mg/ml bovine serum albumin (sigma, USA), 10 mM 2,3-butanedione monoxime (sigma, USA), 10 units/ml penicillin and 10 mg/L streptomycin in culture incubators at 37 °C in an atmosphere of 95% air, 5% CO_2. 2 h after plating, the culture medium was changed to remove unattached dead cells and the viable myocytes were incubated overnight.

2.3. Treatment of adult rat cardiomyocytes

The cultured rat cardiomyocytes were used in the following experimental protocol of 8 groups: control (CON) group, hypoxia-reoxygenation (H/R) group, hypoxia preconditioning (HPC) group, remifentanil preconditioning (RPC) groups at different concentrations (0.1, 0.3, 1.0, 3.0, 10.0 μM). The concentrations of remifentanil (Yichang Humanwell Pharmaceutical Co., Ltd. China) were chosen according to a previous study (Kim et al., 2012) and preliminary experiments. Cardiomyocytes in the CON group were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA) containing 10% NBS in a normoxic atmosphere. To induce hypoxia-reoxygenation injury, cells were cultured in glucose and serum-free medium and exposed to 95% N_2+5% CO_2 for 90 min using a hypoxia chamber (Stem cell Scientific, Canada) followed by 120 min reoxygenation under normal culture conditions. Hypoxia preconditioning was implemented by 10 min hypoxia followed by 30 min reoxygenation prior to 90 min/120 min hypoxia-reoxygenation injury. The cells in remifentanil preconditioning groups were pretreated with reminfentanil at different concentrations for 10 min followed by 30 min drug-free incubation before hypoxia-reoxygenation injury.

In another series of experiments, δ-opioid receptor antagonist naltrindole (NTD, 5 μM, Calbiochem), κ-opioid receptor antagonist nor-binaltorphimine (nor-BNI, 5 μM, Sigma), μ-opioid receptor antagonist CTOP (5 μM, Sigma), ERK inhibitor (PD98059, 30 μM, Cell Signaling Technology), PI3K inhibitor (Wortmannin, 0.1 μM, Cell Signaling Technology) or vehicle (dimethylsulfoxide, DMSO, the final concentration was less than 0.01%) were added for a period of 10 min before remifentanil (1 μM) treatment until the end of remifentanil preconditioning and followed by hypoxia-reoxygenation injury. The opioid receptor antagonists, PD98059 or wortmannin were also added alone without remifentanil preconditioning before hypoxia-reoxygenation as a control.

2.4. Determination of cell viability and injury

Cell viability was determined by Trypan blue exclusion method based on the staining trypsinized cells after treatment with 0.4% trypan blue dye. The number of viable (unstained) and non-viable (blue stained) cardiac myocytes in ten random microscopic fields of each dish was recorded. Cell viability was expressed as percentage of viable cells *versus* total cells (Dhingra et al., 2011). The extent of cell injury was determined by detecting lactate dehydrogenase (LDH) release in the culture medium using an LDH kit (Nanjing Jiancheng Biological Product, China) according to the manufacturer's instructions.

2.5. Detection of cell apoptosis

Cardiomyocytes were fixed with paraformaldehyde and then stained with 1 μg/ml Hoechst 33342 (Sigma, USA), a membrane-permeable nuclear dye. The cells were examined under an Olympus fluorescence microscope (Olympus, Japan). Apoptotic cells were identified based on nuclear condensation and fragmentation, and the percentage of apoptotic cells *versus* total cells was calculated. At least five randomly selected fields of stained cells were analyzed per sample.