Long non-coding RNA MALAT1 functions as a mediator in cardioprotective effects of fentanyl in myocardial ischemia-reperfusion injury

Zhi-hui Zhao¹, Wei Hao², Qing-tao Meng¹, Xiao-bing Du², Shao-qing Lei¹ and Zhong-yuan Xia¹*

¹ Department of Anesthesiology, Renmin Hospital of Wuhan University, Hubei Zhang Road, Wu chang District No. 99 Jie Fang Road 238, 430060, Wuhan, China
² Department of Anesthesiology, Inner Mongolia Autonomous Region People’s Hospital, Zhao Wu Da Road, No.20, Sai Han District, 010017, Huhhot, Inner Mongolia Autonomous Region, China

Abstract

Long non-coding (lncRNA) MALAT1 can be increased by hypoxia or ischemic limbs. Also, downregulation of MALAT1 contributes to reduction of cardiomyocyte apoptosis. However, the functional involvement of MALAT1 in myocardial ischemia-reperfusion (I/R) injury has not been defined. This study investigated the functional involvement of lncRNA-MALAT1 in cardioprotective effects of fentanyl. HL-1, a cardiac muscle cell line from the AT-1 mouse atrial cardiomyocyte tumor lineage was pre-treated with fentanyl and generated cell model of hypoxia-reoxygenation (H/R). Relative expression of MALAT1, miR-145, and Bnip3 mRNA in cells was determined by quantitative real-time PCR. Cardiomyocyte H/R injury was indicated by lactate dehydrogenase (LDH) release and cell apoptosis. The results showed that fentanyl abrogates expression of responsive gene for H/R and induces downregulation of MALAT1 and Bnip3 and upregulation of miR-145. We found that miR-145/Bnip3 pathway was negatively regulated by MALAT1 in H/R-HL-1 cell with or without fentanyl treatment. Moreover, both MALAT1 overexpression and miR-145 knockdown reverse cardioprotective effects of fentanyl, as indicated by increase in LDH release and cell apoptosis. The reversal effect of MALAT1 for fentanyl is confirmed in cardiac ischemia/reperfusion (I/R) mice. In summary, lncRNA-MALAT1 is sensitive to H/R injury and abrogates cardioprotective effects of Fentanyl by negatively regulating miR-145/Bnip3 pathway.

Keywords: Bnip3; fentanyl; ischemia reperfusion; MALAT1; miR-145

Introduction

Ischemic-reperfusion (I/R) injury is a common pathological process in clinical anesthesia. Methods for controlling and reducing the perioperative I/R injury are always concerned by medical practitioner. Presently, ischemic precondition is regarded as the most effective endogenous cardio-protection mechanism. The use of ischemic precondition reduced myocardial necrosis and myocardial infarction area (Anvari et al., 2012). Therefore, drugs are increasing investigated to simulate process of ischemic precondition. Opioids, a kind of drug that frequently utilized in the management of acute moderate-to-severe postoperative pain, immediately after surgery have been experimental used for protecting I/R injury. Also, exogenous administration of selective μ-opioid agonists produces a reduction of infarct size, when given acutely in patients. Fentanyl is such a potent μ-opioid receptor agonist that has been shown to reduce infarct size in rats via a δ-opioid-dependent mechanism (Lessa and Tibirica, 2006; Wu et al., 2012). Myocardial protection induced by fentanyl has been well recognized in many studies (Murphy et al., 2006; Lu et al., 2014; Xu et al., 2015). For example, fentanyl attenuates myocardial injury caused by high-dose adrenaline (da Luz et al., 2015). Nevertheless, the mechanism underlying cardio-protective effects of fentanyl remains unclear.

Recently, increasing evidence suggests that a significant part of noncoding transcripts are functionally active as physiological and pathological regulation molecules. These noncoding RNAs include microRNAs (miRs, about 20–22 nt)
and long noncoding RNAs (lncRNAs, >200 nt). Accumulated studies introduced the miRs and lncRNAs in cardiovascular diseases. Several lncRNAs or miRs are stimulated and functional involved in acute myocardial infarction (eg, ANRIL, KCNQ1OT1, and MALAT1 or miR-499, and miR-214) (Vausort et al., 2014; Janssen et al., 2016; Xinet al., 2016), mitochondrial function, and apoptosis of cardiomyocytes (eg, CHRF or miR-145, and miR-22) (Li et al., 2012; Wang et al., 2014a,b; Yang et al., 2016). Although most lncRNAs have not been defined in myocardial I/R injury, recent studies increasingly focus on the possible contribution of lncRNAs to this progress (Liu et al., 2014, 2015). Our present study was to determine the role of lncRNA-MALAT1 in cardio-protective effects of fentanyl against myocardial I/R injury. MALAT1 is imprinted in early cardiac development and also was significantly upregulated in patients compared with healthy controls. Previous studies have showed that MALAT1 was increased in ischemic patients, as well as in hypoxia-cultured cells and ischemic limbs (Michalik et al., 2014). However, it is uncertain of its involvement and functional mechanism in myocardial I/R injury.

Some studies have found a new interacting mechanism of which lncRNAs could interact with miRs. Interesting, MALAT1 has a direct interaction with miR-145 (Lu et al., 2016). Known to us, miR-145 is an oxidative stress responding miRNA and plays an important role in protecting cardiomyocytes from apoptosis (Li et al., 2012; Cha et al., 2013). Moreover, researchers found that miR-145 targets and regulates Bnip3 expression, a sensor for myocardial I/R injury (Diwan et al., 2007; Kubli et al., 2008).

Therefore, this study established in vitro myocardial cell model of hypoxia/reoxygenation and investigated that the involvement of MALAT1, as well as its regulatory role in miR-145/Bnip3 signals in cardioprotective effects of fentanyl against myocardial I/R injury.

**Materials and methods**

**Cell culture**

Mouse cardiomyocyte line of HL-1 was cultured in Claycomb medium (Sigma–Aldrich) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/mL streptomycin, 0.1 mM norepinephrine, and 2 mM L-glutamine. For experimental protocols, cells were divided into three groups: control, cells with no treatment; H/R, cells were subjected to 10 h of hypoxia with 95% N₂ + 5% CO₂, and subsequent 2 h of reoxygenation with 95% O₂ + 5% CO₂; H/R + fentanyl cells were pretreated with 50 ng/mL for 2 h and subsequent H/R.

![Figure 1](image-url)

**Figure 1** Determination of changes of gene phenotype induced by Fentanyl in H/R mice myocardial cell line HL-1 Cells were pretreated with fentanyl for 2 h and experienced H/R. Relative expression of (A) MALAT1 and (B) miR-145 was determined by quantitative real-time PCR. (C) Determination of expression level of Bnip3 mRNA and protein by quantitative real-time PCR and western blot, respectively. **P < 0.01 compared with control; ## P < 0.01 compared with H/R.
Quantitative real-time PCR

Quantitative real-time PCR (RT-qPCR) was performed, to determine the relative expression of MALAT1, Bnip3 mRNA, and miR-145. RNA was isolated using the TRIzol® Plus RNA Purification Kit (Ambion). After quantified by spectrophotometric method, a mount of 1 μg RNA was converted to cDNA using reverse transcriptase. The cDNA products were assayed for gene expression by SYBR Green I-based RT-qPCR, using the SYBR Premix Ex Taq II (TAKARA) with the relevant primers in 7500 Real-Time PCR System (Life Technologies). The expression of GAPDH serves as the internal reference for MALAT1 and Bnip3 mRNA; the expression of U6 is the internal reference for miR-146.

Western blot

Cells were collected and were cracked with RIPA Lysis Buffer (Beyotime, Beijing). Total proteins in cellular extracts were purified by centrifugalization and equal amount of them was then separated by SDS–PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis). The proteins of gel were transferred to PVDF membrane. After blocked with 5% nonfat milk (diluted in Tris-buffered saline, TBS), proteins in membrane were experienced incubation with anti-Bnip3 (Abcam, Cambridge, UK, diluted 1:250 in TBS) and anti-β-Actin antibodies overnight at 4°C, washing with TBS containing 0.1% Tween-20 and incubation with anti-rabbit immunoglobulin G (IgG)-horseradish peroxidase conjugate. Proteins finally were visualized using enhanced chemiluminescence (Beyotime, Beijing) in E-Gel® Imager (Thermo Fisher). The gene expression of β-Actin is the internal reference for Bnip3 protein.

Transfection

siRNAs were transfected to HL-1 cells, using Lipofectamine RNAiMAX reagent (Life Technologies). For each siRNA transfection, HL-1 cells were seeded per well in 48-well Plates 12–16 h before transfection. Small interfering RNA that against MALAT1 (si-MALAT1) or miR-145 (miR-145 inhibitor), or the scrambled negative control (si-control or negative control) were used for transfection. Episomal pcDNA plasmid (only pcDNA vector or pcDNA containing MALAT1) were transfected into HL-1 cells using Lipofectamine 2000 reagent (Life Technologies) to overexpress MALAT1 overexpression. After
48 or 24 h transfection, cells were harvested for RT-qPCR analysis or TUNEL staining.

Detection of lactate dehydrogenase level
Lactate dehydrogenase (LDH) functions as marker for cardiomyocyte injury. Medium or serum LDH level was examined by ELISA kit.

Determination of apoptosis by TUNEL staining
After 24 h or 48 h transfection, cells were treated with 50 ng/mL fentanyl for 2 h and subsequent H/R. Cells were collected and fixed with 4% methyl aldehyde. TUNEL staining was performed with In Situ Cell Death Detection Kit according to manufacturers’ protocols. The positive cells were numbered on 10 random fields of vision and averaged.

Animals and ischemia/reperfusion surgery
Male C57BL/6 mice (weight at 25–35 g) in this experiments were obtained from Laboratory Animal Center of Wuhan University and were raised in the alternation of day and night for 12 h with free water intake. Feed room temperature

Figure 3 MALAT1 overexpression abrogates effectiveness of fentanyl on miR-145 and Bnip3 expression in H/R-HL-1 cell. Cells were pre-transfected with pcDNA-MALAT1 before exposed to fentanyl and H/R for subsequent gene expressional analysis of (A) miR-145 and (B) Bnip3 mRNA and protein. Cells were pre-transfected with si-MALAT1 before exposed to H/R for subsequent gene expressional analysis of (C) miR-145, and (D) Bnip3 mRNA and protein. **P < 0.01 compared with control; ***P < 0.01 compared with H/R or H/R + si-control; &P < 0.01 compared with H/R + pcDNA.
was kept at 18–25°C. All protocols were proved by Ethics Committee of Renmin Hospital of Wuhan University.

For experiments, animals were randomly divided into five groups: sham, cardiac I/R model, fentanyl, fentanyl + Ad-GFP, and fentanyl + Ad-MALAT1. Cardial I/R surgery was performed as described (Liu et al., 2014) with minor changes. In brief, animals were anesthetized by chloral hydrate with a dose of 30 mg/kg. Thoracotomy was performed on the left side in the fourth inter-costal space. After removing the pericardium, the left anterior descending artery (LAD) at the inferior edge of the left atrium of animals was ligatured with an 8-0 prolene suture. After 45 min of ischemia, the left anterior coronary artery was released and perfused. Adenoviral vectors overexpressing MALAT1 (Ad-MALAT1) (200 nM), within tuberculin syringe was inserted in the second left intercostals space of animal with the help of 27 gauge needle and when pulsatile blood appeared in the needle hub, Ad-MALAT1 was injected with a continuous and slow movement. After 48 h injection, Fentanyl with 10 mg/kg dose was administrated via intravenous injection before 5 min of reperfusion. The expression of MALAT1 in heart tissue was confirmed by quantitative real-time PCR. Serum was collected for LDH level detection at 4 h post reperfusion.

Statistical analysis

Data are presented as mean ± SEM. Statistical analysis were performed with SPSS (16.0 version) and done with Student’s t-test or one-way ANOVA. A P-value less than 0.05 are considered significant.
Results

Fentanyl induced changes of gene phenotype in H/R mice myocardial cell

To identify the involved molecules in myocardium protective action of fentanyl in I/R injury, we measured the expression of MALAT1, miR-145, and Bnip3 in HL-1 cell after 10 h hypoxia and 2 h reoxygenation (H/R), in presence or absence of fentanyl. Figure 1A shows that H/R induced 2.9-fold increase of MALAT1 level, whereas this elevation was partly abolished (1.5-fold decrease) by fentanyl pretreatment. In the contrary, expression level of miR-145 was downregulated by H/R treatment and mostly recovered after fentanyl treatment (Figure 1B). Also, we determined the expression of myocardial apoptosis-related Bnip3 gene in HL-1 cell in Figure 1C, in which Bnip3 mRNA and protein were upregulated responding to H/R treatment and downregulated by fentanyl.

MALAT1 regulates miR-145/Bnip3 expression

We next examined the regulatory role of MALAT1 on miR-145 and Bnip3 expression, by determining expression of miR-145 and Bnip3 in pcDNA-MALAT1- or si-MALAT1-tranfected HL-1 cells. We observed the downregulation of miR-145 and upregulation of Bnip3 mRNA, and protein in HL-1 cell with MALAT1 overexpression compared with that transfected with pcDNA (Figure 2A). Meanwhile, MALAT1 overexpression had no effect on autophagy-specific genes expression including Beclin-1 and LC3-I/II (Figure 2A). The opposite trend of expression of miR-145 was emerged in HL-1 cell with MALAT1 knockdown (Figure 2B). These data suggested the being regulated of miR-145/Bnip3 by MALAT1.

MALAT1 overexpression abrogates effectiveness of fentanyl on miR-145 and Bnip3 expression in H/R-HL-1 cell

To study the MALAT1/miR-145/Bnip3 signaling pathway, constituting the mechanism by which fentanyl protects HL-1 cell from H/R injury, we investigated the effect of MALAT1 overexpression on expression of miR-145/Bnip3 in fentanyl-treated H/R-HL-1 cell. The results showed that MALAT1 attenuated increase of miR-145 expression induced by fentanyl, compared with H/R + fentanyl + pcDNA cell (Figure 3A). Meanwhile, MALAT1 overexpression promoted the expression of Bnip3 mRNA and protein in comparison to pcDNA in fentanyl-treated H/R-HL-1 cells (Figure 3B). In cells with si-MALAT1 transfection, MALAT1 knockdown reversed the biological action of H/R, and induced upregulation of miR-145 and Bnip3 mRNA and protein (Figures 3C and 3D).

MALAT1 overexpression attenuated myocardium protective action of fentanyl

Observing that MALAT1 regulates miR-145/Bnip3 signals in H/R-HL-1 cell in presence of fentanyl, we next evaluated the role of MALAT1 overexpression on myocardium protective action of fentanyl. Cell injury was indicated by LDH release and cell apoptosis. Data showed that LDH release and percentage of cell apoptosis were substantially enhanced by H/R treatment and significantly reduced by fentanyl (Figures 4A and 4B) confirming the myocardium protective action of fentanyl. While the cardio-protection action of fentanyl was abolished by MALAT1 overexpression, indicating by increase in LDH release and cell apoptosis compared with pcDNA (Figures 4A and 4B).

Knock-down of miR-145 attenuated myocardium protective action of fentanyl

To identify the role of miR-145 in myocardium protective action of fentanyl, we examined LDH release and cell apoptosis in H/R-HL-1 cell with miR-145 specific siRNA (miR-145 inhibitor) after fentanyl treatment. The result showed the same trend of LDH release and cell apoptosis as in HL-1 cell with MALAT1 overexpression, in which miR-145 knockdown abrogated effectiveness of fentanyl and contributed to LDH release and cell apoptosis (Figures 5A and 5B).
Determination of role of MALAT1 overexpression in myocardium protective action of fentanyl in cardiac I/R mice

Considering the reversal role of MALAT1 overexpression in myocardium protective action of fentanyl in HL-1 cell, we determined that role in the in vitro mouse cardiac I/R model via animal co-injected with fentanyl and Ad-MALAT1. We found that Ad-MALAT1 injection reversed inhibitory action of fentanyl and induced serum LDH release indicating the harmful impact of MALAT1 in cardio-protection of fentanyl (Figure 6).

Discussion

Accumulated evidences suggested that lncRNAs function as the responder for stimulus from drugs and crucially regulate disease progresses by association with its downstream signaling pathway (Tang et al., 2013; Lin et al., 2015). Our present study defined lncRNA-MALAT1 as a key regulator in mediating cardio-protective effects of fentanyl against I/R injury.

Studies showed that fentanyl is such a certain drug for relieving myocardial I/R injury by reducing infarct size in animal I/R injury model (Gross et al., 2006). Although this myocardial preservation by fentanyl has been put forward as early as 1980s and verified in experimental animals (Kato and Foex, 2000; Kato et al., 2000; Lessa and Tibirica 2006; Xu et al., 2015), the understanding of functional mechanism of fentanyl still limits. Maybe the limitation of understanding functional mechanism of fentanyl restricts its application in myocardial preservation.

Myocardial I/R injury was often accompanied by changes in gene expression (Jones et al., 2003; Lorenzen et al., 2013). Data from increasing number of studies have indicated that noncoding RNAs are associated with important regulatory functions in the heart (Mercer and Mattick, 2013), also they constitute the molecular basis for myocardial I/R injury. The present study showed that lncRNA-MALAT1 was stimulated and upregulated by H/R, whereas it was attenuated by fentanyl pretreatment before H/R procedure. The upregulation of MALAT1 in response to H/R is accordance with previous studies (Michalik et al., 2014). These data indicated that MALAT1 has a potential role in mediating cardio-protective effects of fentanyl.

The regulatory role of MALAT1 in cellular physiology depends on a variety of mechanisms as other lncRNAs (Hou et al., 2016). We thus detected the downstream molecule of MALAT1. miR-145, a known cardiomyocytes injury related molecule, has been demonstrated to be sponged by MALAT1 in cervical cancer (Lu et al., 2016). Our data suggested that miR-145 was downregulated by H/R treatment and its expressional level was mostly recovered by fentanyl. These data suggested miR-145 possibly being as a responder and regulated by MALAT1. This was confirmed by observation that miR-145 level was negatively responding to MALAT1 in MALAT1-overexpression or knockdown HL-1 cell. Also, the target Bnip3 for miR-145 (Li et al., 2012) negatively expressed along with abnormal expression of miR-145.

Moreover, miR-145/Bnip3 pathway responding to MALAT1 in cardio-protective effects of fentanyl was observed in H/R-HL-1 cell. This cardio-protective effect of fentanyl was incubated by decrease in LDH release and percentage of cell apoptosis in comparison to H/R. The myocardial cell apoptosis is an important link in reperfusion injury. Inhibition of apoptosis related signal transduction pathways is a benefit for prevention and cure of myocardial I/R injury. miR-145/Bnip3 is such a cardiomyocyte injury- and apoptosis-related signaling pathway in cultured cardiomyocytes under oxidative stress (Li et al., 2012). Especially, the sensor and pivotal role of Bnip3 for myocardial ischemia and reperfusion has been examined in many studies (Hamacher-Brady et al., 2007; Kubli et al., 2008). Hence, these data further confirm the involvement of MALAT1 in cardioprotective effect of fentanyl against I/R injury. The critical role of MALAT1 in cardio-protective effect of fentanyl was also observed in cardiac I/R mice. MALAT1 overexpression reversed inhibitory action on serum LDH release by fentanyl.

In summary, we initiated an exploratory analysis on the expression of lncRNA-MALAT1 in process of cardio-protective effect of fentanyl in myocardial ischemia reperfusion injury and determined its downstream mechanism of miR-145/Bnip3.

Conflict of interest

The authors have no conflicts of interest to declare.

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