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Long non-coding RNA nuclear-enriched abundant transcript 1 inhibition blunts myocardial ischemia reperfusion injury via autophagic flux arrest and apoptosis in streptozotocin-induced diabetic rats

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HIGHLIGHTS

- We investigated effects of lncRNA-Nuclear-enriched abundant transcript (Neat1) on myocardial ischemia reperfusion injury in diabetic rats.
- Neat1 was highly expressed in ischemia reperfusion-treated diabetic rat myocardial tissues. Overexpression of Neat1 promoted production of LDH, inhibited SOD content and cardiomyocyte viability. Neat1 overexpression promoted production of serum myocardial enzymes and increased infarct size.
- I/R treatment caused more injuries in diabetic rats compared with normal rats. Elevated Neat1 expression aggravates myocardial I/R injury via activation of apoptosis and autophagy.

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ABSTRACT

Background and aims: This study aimed to investigate the effects of long non-coding RNA (lncRNA)-nuclear-enriched abundant transcript (Neat1) on myocardial ischemia reperfusion injury in diabetic rats *ex vivo* and *in vivo*.
Methods: Screening for LncRNA Neat1 expression was performed in rat myocardial tissues using microarray analysis and verified by qRT-PCR. Cell viability of rat cardiomyocytes was analyzed by MTT assay. Levels of autophagy-related proteins Atg7, Atg5, LC3-II/LC3-I and p62 were determined by Western blot assay. Left ventricular end diastolic diameter (LVEDD), left ventricular end systolic diameter (LVESD), left ventricular ejection fraction (LVEF) and fractioning shortening were obtained by transthoracic echocardiography. Left ventricular end systolic pressure (LVESP), left ventricular end diastolic pressure (LVEDP), maximum rate of increase or decrease of left ventricular pressure (± dp/dtmax) and heart rate were obtained by computer algorithms and an interactive videographics programme. Myocardial infarct size was determined by Evans blue and triphenyltetrazolium chloride (TTC) staining. Myocardial apoptotic index was analyzed by TUNEL assay and immunohistochemical staining. Autophagic flux was examined by evaluating fluorescent LC3 puncta.
Results: Neat1 was highly expressed in ischemia reperfusion-treated diabetic rat myocardial tissues. Overexpression of Neat1 promoted the production of lactate dehydrogenase, inhibited superoxide dismutase content and cardiomyocyte viability. Neat1 overexpression also promoted the production of serum myocardial enzymes, including creatine kinase and creatine kinase-MB, and increased infarct size. By promoting myocardial apoptosis and autophagy, Neat1 aggravated myocardial ischemia reperfusion (I/R) injury in diabetic rats. Neat1 promoted cardiomyocyte autophagy by up-regulating Foxo1 expression to increase hypoxia-reoxygenation injury.
Conclusions: I/R treatment caused more injuries in diabetic rats compared with normal rats. Elevated Neat1 expression aggravates myocardial ischemia reperfusion injury via activation of apoptosis and autophagy in diabetic rats. Foxo1 is one of the molecular mechanisms underlying Neat1-induced autophagy.

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1. Introduction

Ischemic heart disease (IHD) is a threat for human health. The key to treat IHD is to restore blood supply to ischemic myocardium as soon as possible [1]. However, restoration of blood is always inevitably accompanied by further cardiac injury, widely acknowledged as ischemia/reperfusion (I/R) injury [2]. I/R injury is a myocardial injury caused by coronary blood flow restoration, which causes mounting deaths in the world. Clinically, many studies have strongly demonstrated an increased susceptibility to cardiac I/R injury in patients with diabetes. Diabetes, one of the most common risk factors for cardiovascular disease, is a high-risk factor for high mortality independent of cardiovascular disease [1]. However, mechanisms leading to exacerbated post-myocardial ischemia (MI) remodeling and poor outcome in diabetic patients are incompletely understood. Therefore, to explore regulation of I/R injury under the diabetic status is important to find a new therapeutic strategy for prevention of ischemic heart disease.

Autophagy always occurs in normal cardiac myocardium. Nevertheless, it is increased in hearts affected by myocardial hypertrophy and ischemia [3]. Autophagy has been demonstrated to support the adaptive response of the heart, thus protecting it from hemodynamic overload and acute ischemic death. However, cell death induced by autophagy also occurs following MI/R [4]. Recent studies suggest that impaired autophagic flux is critical in reperfusion injury [5]. It is well recognized that diabetic patients endure greater ischemic heart disease-associated morbidity compared to nondiabetics, with poorer outcome even after successful reperfusion. However, whether and how diabetes may alter cardiac autophagic flux in response to MI/R, thus contributing to accelerated cardiomyocyte injury, has surprisingly not been deeply investigated.

Long noncoding RNAs (LncRNAs) are endogenous regulatory RNAs molecules ranging in length from 200 nt to 100 kb, which represent a great majority of noncoding RNAs [6,7]. When lncRNAs were firstly identified, they were not taken seriously with respect to biological functions [8] because of their low preservation, low expression level and high tissue specificity [9]. More recently, a number of lncRNAs have been shown to have significant and diverse functions in the development and progression of various diseases [10]. It has been found that lncRNAs play pathological and crucial functions in cardiovascular diseases [11]. For instance, lncRNA autophagy promoting factor (APF) could regulate myocardial infarction (MI) [12]. Down-regulation of linRNA-KCNQ1OT1 may defend against myocardial I/R injury following acute myocardial infarction by regulating AdipoR1 [13]. Nuclear-enriched abundant transcript 1 (Neat1) is a non-protein-coding RNA and has been reported to function as a main component involved in the formation and maintenance of par speckles, nuclear bodies associated with the retention in the nucleus of specific mRNAs [14–16]. The mouse *Neat1* gene has two isoforms, 3.2 kb Neat1_1 and 20 kb Neat1_2 [17]. Neat1 has been shown to protect cells from early apoptosis, suggesting its potential regulatory effect in lethal diseases [18]. Nevertheless, effects of lncRNA-Neat1 on myocardial I/R injury have not been adequately studied in diabetes.

In the present study, we predicted new biomarkers by microarray analysis in a diabetic I/R rat model. Biological functions of the selected lncRNA-Neat1 in myocardial ischemia reperfusion injury in diabetic rats were investigated by *ex vivo* and *in vivo* experiments.

2. Materials and methods

2.1. Microarray analysis

The microarray analysis was performed at the Shanghai Outdu biotechnology corporation (Shanghai, China). Significant Analysis of Microarray (SAM) software was used to analyze normal and diabetic rat myocardial tissue samples (n = 3) for differentially expressed lncRNAs in diabetic and normal rats. The total RNA of myocardial tissue of rats was extracted and the purity and integrity of RNA were detected. After hybridization, the different intensity fluorescence of lncRNA was obtained by chip hybridization, and the fluorescence intensity values were obtained by image scanning. The differently expressed lncRNAs were selected according to the criterion of $p < 0.05$ and fold change value > 2.

2.2. Experimental animals

All practice on rats conforms to the National Institutes of Health Guidelines on the Use of Laboratory Animals and was approved by the West China Hospital. A total of 90 healthy adult male Sprague-Dawley (SD) rats at 8–10 weeks of age, purchased from Cavens Laboratory Animal Ltd. (Changzhou, Jiangsu, China), were used to establish myocardial ischemia reperfusion (MI/R) injury model. Streptozotocin (STZ) was used to inducted diabetic rats. 1–3 days old SD rats were also purchased to establish hypoxia/re-oxygenation (H/R) model of cardiomyocytes. The feeding conditions of the rats were no specific pathogen levels. Rats were fed with a standard laboratory animal chow, with free access to tap water, and housed in a temperature- and humidity-controlled room with a 12/12 h light-dark cycle.

2.3. Induction of diabetes

A total of 60 rats were handled and adapted to their new environment with a basal diet for 14 d prior to 42 d study. All rats were fed *ad libitum* on basal diet and water. All rats in the diabetic group received streptozotocin (STZ) injection at a dose of 65 mg/kg as previously reported [19]. Normal rats received an equal volume of citrate buffer. Three days post-STZ injection, tail vein blood glucose samples were collected and measured to ensure that glucose levels were greater than 15 mM. All rats were housed 8 weeks after vehicle or STZ injection.

2.4. Neonatal rat cardiomyocytes (NCMs) isolation and culture

Neonatal rats were sacrificed and the hearts were quickly retrieved and minced into small pieces in ice-cold (4 °C) Hanks' balanced salt solution, and the hearts were removed under sterile conditions to harvest cardiomyocytes. Cardiomyocytes were then digested by 0.1% trypsin (Sigma-Aldrich, St. Louis, MO, USA) for 15 min at 37 °C, and centrifuged (850 rpm, 5 min) at room temperature. The supernatant was discarded. Heart-tissue pellet was re-suspended in a 10 cm tissue-culture dish containing M199 medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal calf serum (FCS, Thermo Fisher Scientific), 1 × nonessential amino acid (Thermo Fisher Scientific), 5 mM d-glucose (Thermo Fisher Scientific) and penicillin/streptomycin (100 U/mL/100 μg/mL, Thermo Fisher Scientific). The dish was kept in a bio-incubator for 2 h at 37 °C, with 5% circulating CO_2 to enrich cardiomyocyte culture. Non-attaching cardiomyocytes were collected, centrifuged and re-plated in a new 35 mm tissue-culture dish pre-treated with 0.1% gelatin (Thermo Fisher Scientific), and then maintained in the bio-incubator at 37 °C with 5% circulating CO_2. Cells were randomized into three groups: (i) Control group (culture medium containing 5 mM d-glucose); (ii) Osmotic control group (culture medium containing 5 mM d-glucose and 28 mM mannitol); (iii) High glucose group (culture medium containing 33 mM d-glucose).