LncRNA NONRATT021972 siRNA attenuates P2X7 receptor expression and inflammatory cytokine production induced by combined high glucose and free fatty acids in PC12 cells

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Abstract Diabetic neuropathy (DNP) is a frequent chronic complication of diabetes mellitus with potentially life-threatening outcomes. High glucose and elevated free fatty acids (FFAs) have been recently recognized as major causes of nervous system damage in diabetes. Our previous study has indicated extracellular stimuli, such as high glucose and/or FFA stress, may activate the p38 mitogen-activated protein kinase (MAPK) signaling pathway and induce a p38 MAPK-dependent sensitization of the P2X7 receptor and release of inflammatory factors in PC12 cells, while the mechanisms underlying remain to be elucidated. Long noncoding RNAs (lncRNAs) play important roles in diverse biological processes, including activation of a series of pathway signalings. Here, we showed combined high D-glucose and FFAs (HGHF) induced an increment of lncRNA-NONRATT021972 (NONCODE ID, nc021972) in PC12 cells. Nc021972 small interference RNA (siRNA) alleviated HGHF-induced activation of p38 MAPK, expression of the P2X7 receptor, and $[Ca^{2+}]_i$ increment upon P2X7 receptor activation. Further experiments showed that there existed a crosstalk between nc021972 and the p38 MAPK signaling pathway. Inhibition of p38 MAPK signaling decreased nc021972-induced expression of the P2X7 receptor and $[Ca^{2+}]_i$ increment upon P2X7 receptor activation. Also, nc021972 siRNA inhibited HGHF-induced PC12 release of TNF- α and IL-6 and rescued decreased cell viability mediated by the P2X7 receptor. Therefore, inhibition of nc021972 may

serve as a novel therapeutic strategy for diabetes complicated with nervous inflammatory diseases.

Keywords Diabetic neuropathy · High glucose · High fatty acids · P2X7 receptor · Long noncoding RNA · PC12 cell

Introduction

Type 2 diabetes mellitus (T2DM) and prediabetes are characterized by both hyperglycemia and dyslipidemia [1, 2]. Diabetic neuropathy (DNP), such as cardiac autonomic neuropathy (CAN), is a relatively common and often devastating complication of T2DM. Autonomic innervation regulates heart rate and overall cardiac output [3, 4]. There is sympathetic neuroaxonal dystrophy in T2DM [5]. Elevated serum levels of glucose and high free fatty acids (FFAs) result in sympathetic neuron dysfunction [5, 6]. Therefore, high glucose (HG) and high FFAs (HF) may contribute to the functional changes of the sympathetic nerve system in diabetes. A model of high glucose and FFAs has been used widely in cultured cells to mimic the metabolic derangements that occur in T2DM [7, 8]. The PC12 cell line induced by nerve growth factor (NGF) is a well-established model for studying the model of receptor function in sympathetic neurons [6, 9, 10].

The sympathetic ganglia and their postsynaptic nerves release adenine triphosphate (ATP) [11–15]. Extracellular ATP can activate P2 receptors [14, 16]. P2 receptors are divided into P2X and P2Y receptors. Abundant evidence suggests that inflammation may be a causative factor in T2DM. Low-grade inflammation plays a crucial role in the pathogenesis of T2DM. Inflammatory condition in T2DM may lead to intracellular ATP release into extracellular environment [17]. In

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addition, disruption of cellular membrane caused by the high plasma glucose and FFAs [18] can contribute to the accumulation of extracellular ATP [19]. Evidence has indicated that the P2X7 receptor was involved in the process of inflammatory response and release of inflammatory mediators [20, 21]. Our previous studies also showed that the P2X7 receptor played a major role in the transmission of inflammatory signals and contributed to the pathological process of sympathetic injury [6, 10, 22–25].

It is estimated that only 2 % of the mammalian genome is composed of genes that encode proteins, while the bulk is transcribed as noncoding RNA [26, 27]. A large number of long noncoding RNAs (lncRNAs) are expressed in the nervous system [26, 27]. At present, only a little of lncRNAs have been characterized thoroughly; most lncRNA studies are still at a relatively early stage. lncRNAs are involved in the fundamental cellular processes including regulating gene expression at epigenetics, transcription, and post-transcription in cell homeostasis [28, 29]. Stress and environmental stimuli can regulate the expression of lncRNAs [30]. lncRNAs participate in the action in both normal development and disease state [31]. The roles of lncRNAs in the function of sympathetic nerves remain poorly characterized. NONRATT021972 (NONCODE ID, nc021972), a lncRNA (<http://www.noncode.org>) [32], was detected to be up-regulated in the diabetic rat superior cervical ganglion in our preliminary study. This finding suggests nc021972 may be involved in the pathophysiological processes related to the sympathetic neuron under the setting of diabetes. Our previous studies have shown that the P2X7 receptor was implicated in the pathological injury of sympathetic nerves [6, 10, 22–25]. The aim of the study was to examine the effects of nc021972 small interference RNA (siRNA) on the P2X7 receptor and its possible role in neuroinflammation induced by elevated glucose and FFAs in PC12 cells.

Materials and methods

Materials

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Hyclone (Logan, UT, USA). BioSource Cytoscreen Immunoassay Kit for IL-6 and TNF- α production was purchased from BioSource International (Camarillo, CA, USA). Fluo-3 acetoxymethyl ester (fluo-3 AM) and cell counting kit-8 (CCK-8) were purchased from Beyotime Institute of Biotechnology (Shanghai, China). Nc021972-siRNA was purchased from RiboBio Co., Ltd (Guangzhou, China). Lipofectamine 2000 (lipo2000) and pcDNA3 were obtained from Invitrogen (Carlsbad, CA, USA). Trizol Total RNA Reagent was purchased from Beijing Tiangen Biotech (Beijing, China) and complementary

DNA (cDNA) synthesis was carried out using the RevertAid™ H Minus First Strand cDNA Synthesis Kit which was obtained from Fermentas (Burlington, Ontario, Canada). Restriction endonucleases *Bam*HI and *Xho*I and T4 DNA ligase were purchased from Promega Corporation (Beijing, China). The real-time PCR primers of the P2X7 receptor, nc021972, and β -actin were obtained from Sangon Biotech. (Shanghai, China). Protein extraction and quantitation kits were purchased from Bio-Rad Inc. (CA, USA). The rabbit anti-P2X7 (corresponding to amino acid residues 576–595 of the rat P2X7 receptor, C-terminus) was bought from Alomone Laboratories (Jerusalem, Israel). The rabbit anti-p38 and anti-p-p38 were bought from Cell Signaling Technology, Inc. (Beverly, MA, USA). Horseradish peroxidase-conjugated secondary antibodies were purchased from Beijing Zhongshan Biotech Co. Ltd (Beijing, China). Enhanced chemiluminescence (ECL) reagents were obtained from Beyotime (Shanghai, China). Other reagents, such as D-glucose, oleate, palmitate, SB-203580, and 3'-O-(4-benzoyl)benzoyl-ATP (BzATP), were all purchased from Sigma (Sigma-Aldrich, St. Louis, MO) unless specifically noted. SB-203580 was dissolved in dimethyl sulfoxide (DMSO) which was taken as a solvent control in this study. The PC12 cell line was originally obtained from American Type Culture Collection (ATCC, Manassas, VA, USA).

Cell culture

The NGF-induced PC12 cells were maintained in high-glucose DMEM containing 10 % FBS, 5 % horse serum, 100 U/mL penicillin, and 100 mg/mL streptomycin sulfate at 37 °C in a humidified atmosphere containing 5 % CO₂. As already reported, the glucose concentration in DMEM was 25 mM and was called the control condition. To produce hyperglycemia, DMEM was supplemented with 75 mM D-glucose [33]. The cells were grouped into control (25 mM D-glucose), HG 50 mM (50 mM D-glucose), and HG 75 mM (75 mM D-glucose). FFAs were a mixture of oleate and palmitate, 2:1 (w/w) [34]. For HF experiment, the cells were grouped into control (1 % BSA), HF 0.25 mM, HF 0.5 mM, and HF 1.0 mM [6]. For HGHF experiment, PC12 cells were grouped into cultured with control (25 mM D-glucose and 1 % BSA), isotonic control (25 mM D-glucose, 50 mM mannitol, and 1 % BSA), HG 75 mM (75 mM D-glucose), HF 0.75 mM (0.75 mM FFAs), and HGHF (75 mM D-glucose and 0.75 mM FFAs). PC12 cells of D-glucose and/or FFA groups were cultured with concomitant lowering of the serum concentration in the medium to 2 % in order to keep the cells in the quiescent state. The media were replaced every 24 h during these periods. Other test agents, such as p38 mitogen-activated protein kinase (MAPK) inhibitor, were added during the culture periods as specified. FFAs and other test agents were not

included in all buffer solutions of the experiments in order to avoid possible acute effects.

Cell viability assay

Cell viability was evaluated using the CCK-8 assay according to the manufacturer's instructions. Briefly, after the indicated treatments, 10 μ L of the CCK-8 solution was added to the culture medium and the mixture was incubated for an additional 3 h. The absorbance was determined at 450 nm using an automatic microplate reader (PerkinElmer, Waltham, MA, USA).

Measurement of IL-6 and TNF- α production

IL-6 and TNF- α were measured with a BioSource Cytoscreen Immunoassay kit according to the manufacturer's instructions.

Measurement of $[Ca^{2+}]_i$

The cells were incubated under the indicated conditions. $[Ca^{2+}]_i$ was measured with fluo-3 AM as described previously [35]. Briefly, PC12 cells were loaded with fluo-3 AM. The final concentration of fluo-3 AM was 2 μ M/L. Then, cells were washed with PBS three times and cultured for another 25 min to ensure fluo-3 AM was converted into fluo-3 in cells according to the manufacturer's instructions. The fluo-3-generated fluorescence was monitored on an automatic microplate reader (PerkinElmer, Waltham, MA, USA) with emission wavelength of 530 nm at 480-nm excitation wavelengths. After measuring the basal fluorescence, 100 μ M BzATP was added to the suspension to observe the fluorescence. The fluorescence was an indicator of $[Ca^{2+}]_i$. The alteration value of fluorescence after BzATP stimuli was shown as the difference value (*D* value) between the peak value of fluorescence and the initial value of fluorescence.

siRNA transfection

Three different siRNAs targeting specific sequences of nc021972 and a negative control scrambled siRNA (not homologous to any gene, NC-siRNA) were synthesized by RiboBio Co., Ltd. The specific sequences of three siRNA duplexes were NO.001 (5'-GAATGTTGGTCATATCAAA-3'), NO.002 (5'-GAACCGTACTGCTCCTAAT-3'), and NO.003 (5'-GACTATGAAGTGTGTAATT-3'). The optimal nc021972-siRNA was selected based on the results of real-time PCR (data not shown). The transfections of nc021972-siRNA were performed with lipo2000 according to the manufacturer's instructions. The fluorescence of cy3-NCsi transferred in the cells, analyzed via a TE-300 Nikon (Nikon, Tokyo, Japan) fluorescence microscope, was applied to show

the transfection efficiency of nc021972 siRNA. P2X7 (NM_019256) siRNA was obtained and the siRNA target sequence was described in our previous study [6].

Isolation and cloning of nc021972 cDNA

Total messenger RNA (mRNA) was extracted from the PC12 cells using the Trizol Total RNA Reagent and subjected to cDNA synthesis using the RevertAid™ H Minus First Strand cDNA Synthesis Kit. The specific primer set, forward (5'-CGGGATCCAGGCCTGCTGAAAATGACTGAGTATAAAC-3') and reverse (5'-CCGCTCGAGTTCATAGTACCATAACTATTTTATTACATTAC-3'), and Taq DNA polymerase were used to amplify the total length of nc021972. The underlined parts of the primer sequences represent restriction enzyme sites *Bam*HI and *Xho*I, respectively. The enzyme-digested PCR products were cloned into the *Bam*HI:*Xho*I site of vector pcDNA3. The recombinant plasmid was named pcDNA3-nc021972.

Plasmid transfection

The PC12 cells were transfected with 2 μ g of the pcDNA3 and pcDNA3-nc021972 plasmids using lipo2000 according to the manufacturer's protocol. Stable clones expressing recombinant nc021972 were established in the presence of 500 μ g/mL of G418. Then, the expression of nc021972 was evaluated by real-time PCR.

Real-time PCR

Total RNA was isolated from PC12 cells using the Trizol Total RNA Reagent. cDNA synthesis was performed with 2 μ g total RNA using the RevertAid™ H Minus First Strand cDNA Synthesis Kit. The primers were designed with Primer Express 3.0 software (Applied Biosystems), and the sequences were as follows: nc021972, forward 5'-TTAGGAGCAGTACGGTTCA-3' and reverse 5'-GGAGTACGTGCTGTGAAG-3'; P2X7, forward 5'-CTTCGGCGTGCGTTTTG-3' and reverse 5'-AGGACAGGGTGGATCCAATG-3'; and β -actin, forward 5'-TAAAGACCTCTATGCCAACACAGT-3' and reverse 5'-CACGATGGAGGGGCCGGACTCATC-3'. Quantitative PCR was performed using the SYBR® Green MasterMix in an ABI PRISM® 7500 Sequence Detection System (Applied Biosystems Inc., Foster City, CA). The quantification of gene expression was performed using the $\Delta\Delta$ CT calculation with CT as the threshold cycle. The relative levels of target genes, normalized to the sample with the lowest CT, were given as $2^{-\Delta\Delta CT}$ [36].

Western blotting analysis

The cells were lysed in lysis buffer at 4 °C for 30 min. The supernatant was taken, and the protein concentration was determined using the Bradford protein assay system (Bio-Rad). A total of 20 µg protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and was transferred to polyvinyl difluoride membranes (Millipore, Bedford, MA) by electroblotting. After blocking with 5 % nonfat dry milk, the blots were incubated with primary antibodies (diluted 1:1000) and developed with appropriate horse-radish peroxidase-conjugated secondary antibodies (diluted 1:5000). Then, using the ECL kit, chemiluminescent signals were collected on autoradiography film. The quantity of band intensity was carried out using Image Pro-Plus software. Protein expression levels were represented as densitometric ratios of the targeted protein to β -actin.

Statistical analysis

All experiments were performed in triplicate and the data were presented as means $\pm$ SEM. The differences between the sample means were compared using analysis of variance. All analyses were performed using SPSS for Windows, version 11.5 (SPSS Inc., Chicago, IL). $p < 0.05$ was considered statistically significant.

Results

Nc021972 was up-regulated in PC12 cells treated with HG and HF

To facilitate our deep insight into a possible role of nc021972, a PC12 cell line model with up-regulation of nc021972 expression was established in vitro. Both high D-glucose and high FFAs are commonly added in extracellular environment of the neuron to simulate neuronal damage in diabetes. PC12 cells were cultured with control (25 mM D-glucose) and different high concentrations of D-glucose (50 and 75 mM) for 5 days. As demonstrated in Fig. 1, the expression of nc021972 was elevated following 75 mM D-glucose treatment for 3 days ($p < 0.01$) (Fig. 1a). After PC12 cells were treated with increasing doses of high FFAs (0.25–1.0 mM) for 5 days, a significant increment of nc021972 expression was detected in PC12 cells cultured with 0.75 mM for 3 days ($p < 0.01$) (Fig. 1b). Furthermore, we observed whether there existed a synergism of 75 mM D-glucose and 0.75 mM FFAs in inducing nc021972 expression after treatment of cells for 3 days. Nc021972 expression prompted by HGHF was higher than that evoked by high D-glucose or high FFAs alone by 1.91- and 1.93-fold, respectively ($p < 0.05$) (Fig. 1c). Therefore, in the remaining experiment, combined treatment of PC12 cells

Fig. 1 Nc021972 was up-regulated in PC12 cells treated with high D-glucose and high FFAs. **a** The expression of nc021972 determined by quantitative real-time PCR in PC12 cells treated with control and different high concentrations of D-glucose (HG 50 mM and HG 75 mM) for 5 days. The experiment was done in triplicate and repeated three times. *One asterisk* $p < 0.05$ vs control at 1 day, *two number signs* $p < 0.01$ vs control at 3 days, *two ampersands* $p < 0.01$ vs 75 mM at 1 day. **b** The expression of nc021972 determined by quantitative real-time PCR in PC12 cells treated with control and different high concentrations of FFAs (HF 0.25 mM, HF 0.75 mM, and HF 1 mM) for 5 days. The experiment was done in triplicate and repeated three times. *One asterisk* $p < 0.05$ vs control at 1 day, *two number signs* $p < 0.01$ vs control at 3 days, *two ampersands* $p < 0.01$ vs HG 75 mM at 1 day. **c** The expression of nc021972 determined by quantitative real-time PCR in PC12 cells cultured with HG 75 mM and/or HF 0.75 mM for 3 days. The experiment was done in triplicate and repeated three times. *Two asterisks* $p < 0.01$ vs control, *one number sign* $p < 0.05$ vs HG 75 mM group, *one ampersand* $p < 0.05$ vs HF 0.75 mM group

with 75 mM D-glucose and 0.75 mM FFAs for 3 days was adopted to achieve maximized nc021972 increment in PC12 cells.

Silencing efficiency of nc021972 induced in PC12 cell lines cultured with HGHF

To achieve an efficient nc021972 silencing, the interfering efficiency of the siRNA was examined in in vitro transfection

of PC12 cells according to the manufacturer's instructions. PC12 cells were cultured with control (25 mM D-glucose and 1 % BSA), HGHF, or HGHF treated with nc021972-siRNA (HGHF+nc021972-siRNA-1, HGHF+nc021972-siRNA-2, and HGHF+nc021972-siRNA-3), NC-siRNA, or lipo2000. At a final concentration of 50 nM, the maximal silencing efficiency of siRNA NO.001, which was determined by real-time PCR, was 19.80 % at 24 h, 46.56 % at 48 h, and 73.43 % at 72 h after transfection in PC12 cells cultured with HGHF (Fig. 2a). The transfection efficiency of nc021972 siRNA-1 at 72 h attached about 95 %, as indicated by the fluorescence of cy3-NCsi transferred in the cells (Fig. 2b). Thus, siRNA NO.001 showed optimal effect compared to that of NC-siRNA, and thus was an ideal siRNA targeting to nc021972 in PC12 cells. The concentration of 50 nM and experimental condition of 72 h after transfection were good choices for the next experiments in PC12 cells.

Silencing nc021972 suppressed the activation of the P2X7 receptor evoked by HGHF in PC12 cells

To investigate the effects of the nc021972 siRNA on the activation of the P2X7 receptor evoked by HGHF, PC12 cells cultured with control, HGHF, or HGHF treated with nc021972-siRNA, NC-siRNA, or lipo2000, respectively, for 72 h. The expression of P2X7 receptor mRNA and protein was tested. Exposure of cells to HGHF elevated the expression of P2X7 receptor mRNA ($p < 0.05$) (Fig. 3a) and protein

($p < 0.01$) (Fig. 3b). NC-siRNA or lipo2000 did not alter the enhanced expression of P2X7 receptor mRNA and protein in PC12 cells ($p > 0.05$) (Fig. 3a, b). However, nc021972-siRNA suppressed the increased expression of P2X7 receptor mRNA and protein in PC12 cells treated with HGHF ($p < 0.05$) (Fig. 3a, b). Also, the Ca^{2+} signals in response to BzATP in PC12 cells were measured (Fig. 3c, d). The ability of BzATP to elevate $[\text{Ca}^{2+}]_i$ was markedly increased in cells precultured with HGHF ($p < 0.01$) (Fig. 3c, d). The enhanced BzATP-induced Ca^{2+} signals evoked by HGHF could be hampered by nc021972-siRNA ($p < 0.01$), but were not changed by NC-siRNA ($p > 0.05$) (Fig. 3d).

Silencing nc021972 prevented the activation of the P2X7 receptor induced by HGHF via p38 MAPK signaling in PC12 cells

In our previous study, we have indicated that the activation of p38 MAPK pathway signaling was implicated in P2X7 increment in PC12 cells treated with high FFAs [6]. We speculated that p38 MAPK pathway signaling may be also activated in PC12 cells treated with HGHF. Results derived from western blot analysis showed HGHF induced an increase of p38 phosphorylation ($p < 0.01$) (Fig. 4a, b) and P2X7 receptor ($p < 0.01$) (Fig. 3b); such increment of p38 phosphorylation (Fig. 4a, b) and P2X7 receptor (Fig. 3b) was inhibited by nc021972 knockdown ($p < 0.01$). These results suggest that

Fig. 2 Silencing efficiency of nc021972 induced in PC12 cell lines cultured with HGHF. **a** Silencing efficiency in nc021972 at 24, 48, and 72 h after transfection with nc021972 siRNA by real-time PCR. **b** Transfer efficiency of nc021972 siRNA at 72 h as shown by the fluorescence of cy3-NCsi transferred in the cells. Two asterisks $p < 0.01$ vs control, one number sign $p < 0.05$, two number signs $p < 0.01$ vs HGHF

Fig. 3 Silencing nc021972 suppressed the activation of the P2X7 receptor evoked by HGHF in PC12 cells. **a** mRNA expression of the P2X7 receptor determined by real-time PCR. The protein level of the P2X7 receptor was tested by Western blotting analysis. Representative images of Western blotting (top) are indicated, and the corresponding relative protein levels of the P2X7 receptor were quantified by densitometry (bottom) (**b**). Representative images of Ca^{2+} signals promoted by BzATP in PC12 cells are shown (**c**). After basal fluorescence

had been measured, BzATP was added to the suspension to observe the fluorescence. Then, the BzATP-induced Ca^{2+} signal was detected until it dropped to the basal fluorescence. The histogram (**d**) shows the difference value (*D* value) between the peak value of fluo-3 fluorescence and the initial value of fluo-3 fluorescence in each group. The experiment was done in triplicate and repeated three times. One asterisk $p < 0.05$ vs control, one number sign $p < 0.05$ and two number signs $p < 0.01$ vs HGHF

nc021972 knockdown leads to the inactivation of p38 MAPK signaling and subsequent P2X7 receptor.

To further examine whether the p38 MAPK pathway was involved in nc021972-induced activation of the P2X7 receptor, nc021972 was transfected into PC12 cells. As described in Fig. 4c, the expression of nc021972 was significantly increased after the transfection of nc021972 ($p < 0.01$). The activity of the P2X7 receptor, as demonstrated by its expression and Ca^{2+} signals promoted by BzATP, was measured (Fig. 4d–f). We found that pretreatment of PC12 cells with SB-203580 (10 $\mu\text{mol/L}$), a p38 MAPK pathway inhibitor, strongly blocked the effect of nc021972-induced P2X7 receptor expression and Ca^{2+} signals promoted by BzATP in PC12 cells ($p < 0.01$), whereas DMSO has no effect on the above changes mediated by nc021972 ($p > 0.05$) (Fig. 4d–f).

Inhibition of the P2X7 receptor by P2X7 siRNA also hampered the enhanced Ca^{2+} signals stimulated by BzATP in nc021972-transfected PC12 cells ($p < 0.01$) (Fig. 4e, f).

Silencing nc021972 prevented P2X7 receptor-mediated PC12 neuroinflammation and cell viability induced by HGHF in PC12 cells

To investigate the role of nc021972 siRNA in PC12 neuroinflammation and cell viability, nc021972 siRNA was transfected into PC12 cells. IL-6 and TNF- α release was recorded. HGHF and nc021972-siRNA was found to have no effect on basal IL-6 and TNF- α release ($p > 0.05$) (Fig. 5a, b). HGHF enhanced BzATP-stimulated IL-6 release in PC12 cells ($p < 0.01$), which was suppressed by nc021972-siRNA

Fig. 4 Silencing nc021972 prevented the activation of the P2X7 receptor induced by HGHF via p38 MAPK signaling in PC12 cells. The protein level of phosphorylation of p38 was tested by Western blotting analysis. Representative images of Western blotting (**a**). The relative protein level of p-p38 was quantified by densitometry (**b**). *Two asterisks* $p < 0.01$ vs control, *two number signs* $p < 0.01$ vs HGHF. Overexpression of nc021972 (pcDNA3-nc021972) was induced by transfection of nc021972 into PC12 cells pretreated with SB-203580 or P2X7 receptor siRNA (P2X7si), for 72 h. The levels of nc021972 were tested by real-time PCR analysis (**c**). Representative images of Western blotting (**top**) are indicated, and the corresponding relative protein levels of P2X7

receptor were quantified by densitometry (**bottom**) (**d**). Representative images of Ca^{2+} signals promoted by BzATP in PC12 cells are shown (**e**). After basal fluorescence had been measured, BzATP was added to the suspension to observe the fluorescence. Then, the BzATP-induced Ca^{2+} signal was detected until it dropped to the basal fluorescence. The histogram (**f**) shows the difference value (*D* value) between the peak value of fluo-3 fluorescence and the initial value of fluo-3 fluorescence in each group. The experiment was done in triplicate and repeated three times. *Two asterisks* $p < 0.01$ vs pcDNA3, *two number signs* $p < 0.01$ vs pcDNA3-nc021972

Fig. 5 Silencing nc021972 prevented P2X7 receptor-mediated PC12 neuroinflammation and cell viability induced by HGHF in PC12 cells. Basal and BzATP-induced IL-6 (**a**) and TNF- α (**b**) release and cell viability of PC12 cells (**c**) were recorded after transfection of nc021972 siRNA. The experiment was done in triplicate and repeated three times. One asterisk $p < 0.05$ and two asterisks $p < 0.01$ vs control, one number sign $p < 0.05$ and two number signs $p < 0.01$ vs HGHF. Overexpression of

nc021972 (pcDNA3-nc021972) was induced by transfection of nc021972 into PC12 cells pretreated with SB-203580 or P2X7 receptor siRNA (P2X7si), for 72 h. Basal and BzATP-evoked IL-6 (**d**) and TNF- α (**e**) release in PC12 cells and cell viability (**f**) were recorded. The experiment was done in triplicate and repeated three times. One asterisk $p < 0.05$ and two asterisks $p < 0.01$ vs pcDNA3, one number sign $p < 0.05$ and two number signs $p < 0.01$ vs pcDNA3-nc021972

($p < 0.01$) but not altered by NC-siRNA or lipo2000 ($p > 0.05$) (Fig. 5a). Also, the TNF- α release stimulated by BzATP was enhanced in PC12 cells treated with HGHF ($p < 0.01$); this can be inhibited by nc021972-siRNA ($p < 0.01$) while not impeded by NC-siRNA or lipo2000 ($p > 0.05$) (Fig. 5b). No significant difference of BzATP-induced IL-6 and TNF- α release was found between HGHF+NC-siRNA and HGHF+lipo2000 ($p > 0.05$) (Fig. 5b). Additionally, the cell viability was decreased after HGHF treatment ($p < 0.05$), while it was improved when PC12 cells were transfected with nc021972 siRNA plus HGHF treatment ($p < 0.05$) (Fig. 5c).

To further confirm the effect of nc021972 on PC12 neuroinflammation and cell viability and make sure of P2X7 receptor involvement, nc021972 was transfected into PC12 cells to activate the P2X7 receptor. BzATP-evoked IL-6 and TNF- α release was increased after transfection of nc021972 ($p < 0.01$). Inhibition of the P2X7 receptor by P2X7 siRNA hampered IL-6 and TNF- α release stimulated by BzATP in nc021972-transfected PC12 cells ($p < 0.01$) (Fig. 5d, e). Also, the decreased cell viability in PC12 cells transfected with nc021972 could be rescued by pretreated PC12 cells with P2X7 siRNA ($p < 0.05$) (Fig. 5f). These results suggest that the regulation of nc021972 on neuroinflammation and cell viability is mediated by the P2X7 receptor in PC12 cells.

Discussion

Compelling evidence on the importance of inflammation in the pathogenesis of DNP has been accumulated over the last decades [4]. However, there is no effective therapy for inflammation or for DNP. lncRNAs, typically defined as transcripts longer than 200 nucleotides, are recently regarded as a potential therapeutic target for inflammatory relevant diseases, including diabetes and DNP [26–38]. However, the functional question of massive lncRNAs in DNP has not been characterized [26, 27, 30]. In this study, we found that HGHF could induce overexpression of lncRNA-nc021972 in PC12 cells. Thus, nc021972 may participate in the pathologic process of PC12 neuronal damage induced by HGHF. Furthermore, nc021972 was found to activate the p38 MAPK signaling pathway and sensitize the P2X7 receptor, which may participate in IL-6 and TNF release and cell viability of PC12 cells under the setting of HGHF.

High glucose and FFAs in T2DM are associated with malfunction of the nervous system [5, 6, 39]. At sites of high glucose and FFAs, significant increase of extracellular ATP may possibly respond to cell inflammation [17, 18]. The massive extracellular ATP might be one component of the “cytotoxic milieu” of the neuron [40]. Continued activation of the

P2X7 receptor by ATP may result in the release of pro-inflammatory molecules such as IL-6, which exaggerates neuronal damage in synergy with extracellular ATP [6, 25]. Evidence has shown the impact of high glucose and/or FFAs on P2X7-mediated PC12 neuroinflammation in vivo and in vitro [6, 17, 25]. Our earlier results have indicated a phenomenon of IL-6 release in PC12 cells cultured in a high-FFA condition and an involved mechanism of p38 MAPK-dependent sensitization of the P2X7 receptor [6]. Consistently, we also found HGHF could induce p38 MAPK signaling in the present study. The p38 MAPK signaling pathway is usually activated by many extracellular stimuli, including high glucose and/or FFA stress [41], while the mechanisms underlying are largely unknown. Therefore, we aimed to explore the mechanism by which high FFAs and/or glucose induced p38 MAPK signaling and P2X7 receptor activation.

Recently, the role of lncRNAs in hyperglycemia and/or high FFA-induced continuous cellular stress and aberrant p38 MAPK activity has received much attention [42]. Here, we found that HGHF could induce nc021972 expression and phosphorylation of p38 in PC12 cells. Nc021972 knockdown could significantly inhibit HGHF-induced levels of phosphorylated p38 MAPK. These data suggest activation of the p38 MAPK pathway may contribute to the effect of nc021972 on PC12 cellular response to HGHF. To further confirm this, we overexpressed nc021972 in PC12 cells pretreated with SB-203580, a specific inhibitor of the p38 MAPK pathway, and analyzed whether SB-203580 could reduce the P2X7 receptor expression induced by nc021972. Our data showed that pharmacological inhibition of the p38 MAPK pathway by SB-203580 specifically blocked nc021972-induced enhancement of P2X7 receptor expression and augmentation of BzATP-evoked Ca^{2+} signals in PC12 cells. These experiments further supported that p38 MAPK signaling might be involved in the activation of the P2X7 receptor mediated by nc021972. Taken together, our findings indicated that there existed a crosstalk between nc021972 and p38 MAPK signaling. In the setting of HGHF, nc021972-regulated p38 MAPK signaling would affect P2X7 receptor expression in PC12 cells.

The nucleotide receptor P2X7 is recently regarded as a potential therapeutic target for inflammatory relevant diseases such as diabetes [17, 21, 22]. As a ligand-gated cation channel, the P2X7 receptor can be activated by extracellular ATP. Overstimulation of the P2X7 receptor by massive amounts of extracellular ATP may induce uncontrolled Ca^{2+} influx, and activate the release of mature cytokines like IL-6 and TNF- α , which in turn could induce neurotoxicity and cell death [21, 22]. Therefore, target on the P2X7 receptor raised the possibility of inhibiting inflammation and thus may be one of the potential therapeutic strategies for DNP. In this study, nc021972 siRNA treatment was shown to inhibit HGHF-induced activation of the P2X7 receptor and subsequent IL-6

and TNF- α release, and rescued the decreased viability of PC12 cells mediated by the P2X7 receptor. These findings suggest that nc021972 siRNA could relieve neuronal-like PC12 cells from neuroinflammation induced by HGHF. Inhibition of nc021972 may serve as a novel therapeutic strategy for DNP.

In summary, our results indicated the involvement of lncRNA-nc021972 in HGHF-induced neuroinflammation of PC12 cells. Nc021972 was significantly up-regulated in in vitro diabetic models. Its silence significantly alleviated HGHF-induced activation of the P2X7 receptor and subsequent TNF- α and IL-6 release. This finding provides a novel explanation for the universal role of the emergence of lncRNAs against inflammation, which may be helpful for deepening our understanding of the related mechanism involved in the pathogenesis of DNP. In view of the complex nature of lncRNA regulation, further investigation delineating the detailed mechanism underlying the effect of nc021972 on DNP is warranted.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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