

# Sarsasapogenin ameliorates diabetes-associated memory impairment and neuroinflammation through inhibiting thrombin/PAR-1 pathway

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## Research

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# Abstract

**Background:** Sarsasapogenin (Sar), a natural steroidal compound, shows neuroprotective, promoting cognition, anti-inflammatory, anti-thrombosis effects, etc. However, whether Sar can ameliorate diabetes-associated cognitive impairment and whether it is related to the anti-inflammatory and anti-thrombosis effects of Sar, it remains unknown.

**Methods:** Streptozotocin-induced diabetic rats were treated orally with Sar (20 and 60 mg/kg), and normal rats was orally administrated of Sar (60 mg/kg). Cognitive tests were performed using Morris water maze. Nucleotide-binding domain and leucine-rich repeat containing protein 1 (NLRP1) inflammasome (NLRP1, cleaved caspase 1, IL-1 $\beta$ , and IL-18), as well as thrombin and its receptor protease-activated receptor 1 (PAR-1) pathway and advanced glycation endproducts (AGEs) and its receptor RAGE axis were examined in the brain of diabetic rats. Meanwhile high glucose-cultured human SH-SY5Y cells were used to further investigate the effects of Sar (0.2, 1, 5  $\mu$ M) on central neurons and deeply explore the mechanism.

**Results:** Sar markedly increased numbers of crossing platform and percentage of time spent in the target quadrant in Morris water maze tests in diabetic rats, accompanied by inhibitions of NLRP1 inflammasome, PAR-1 upregulation, and AGEs/RAGE axis in cerebral cortex. Moreover, Sar mitigated neuronal damages, NLRP1 inflammasome activation, and PAR-1 upregulation in high glucose-cultured SH-SY5Y cells, and the effects were similar to those of the PAR-1 inhibition with a selective PAR-1 antagonist vorapaxar. But Sar also did not affect thrombin activity in the brain of diabetic rats and high glucose-cultured SH-SY5Y cells. Further studies indicated that the key molecules of NLRP1 inflammasome and the phosphorylated NF-kappaB p65 were remarkably decreased in SH-SY5Y cells cultured with high glucose after PAR-1 knockdown by using F2R (the gene symbol of PAR-1) shRNA, and these effects were confirmed by using Sar addition in such condition.

**Conclusions:** These findings demonstrated that Sar could improve memory impairment caused by diabetes, which was achieved through suppressing neuroinflammation from the activated NLRP1 inflammasome and NF-kappaB mediated by thrombin/PAR-1 activation in brain. Moreover, Sar was proved to be a pleiotropic neuroprotective agent and memory enhancer.

## Background

Diabetes-associated cognitive decline (DACD), one of the most common diabetic complications in central nervous system, is increasingly focused. Although the pathogenesis is complex, evidences verify that cognitive impairment in diabetes is related to angiopathy [1, 2] and neuroinflammation [3, 4]. However, the specific mechanism is not well clarified.

Thrombin, a serine protease generated by the cleavage of prothrombin, is an essential component of the coagulation cascade. Thrombin plays important roles in the regulation of numerous physiological and pathological processes, such as vascular tone, inflammation, atherogenesis, carcinogenesis, and

neurodegeneration. An early research finds that intracerebroventricular infusion of thrombin demonstrates significant cognitive impairments and neuropathology in rats [5]. Neural extracellular matrix proteases including thrombin contribute to the aberrations of synaptic plasticity and learning in major neuropsychiatric disorders [6]. Further studies indicate that thrombin is an inflammatory mediator of cerebral blood vessels or an inflammatory neurotoxin that alters synaptic transmission and synaptic plasticity [7, 8], and cognitive impairment [9]. Protease-activated receptors (PARs) mediate the toxic effects of thrombin on the central nervous system diseases [10, 11]. PAR-1, a G protein-coupled receptor, is a high-affinity receptor for thrombin in brain. PAR-1 deficiency has been testified to be neuroprotective effects against neuronal damages and neurologic deficits in cerebral hypoxia/ischemia [12, 13]. PAR-1 antagonism ameliorates the clinical symptoms of experimental autoimmune encephalomyelitis via inhibiting breakdown of blood-brain barrier, which is also confirmed in neurons, astrocytes, and vascular endothelial cells cultured with thrombin [14]. Thus, over-activation of thrombin/PAR-1 pathway contributes to the neurological deficit and cognitive dysfunction.

Prolonged high glucose promotes thrombin generation and prothrombotic fibrin clot phenotype. Evidences demonstrate that diabetic patients are in a hypercoagulable state, and overproduction of thrombin is an important reason [15, 16]. A recent research demonstrates that brain pericyte-thrombin interaction plays a key role in causing blood-brain barrier dysfunction in obesity-associated diabetes in mice [17]. Nevertheless, whether over-activated thrombin/PAR-1 pathway contributes to cognitive impairment caused by diabetes, it is not clear. Nucleotide binding domain and leucine-rich repeat containing protein 1 (NLRP1) can recruit apoptosis-associated speck-like protein and assembles to inflammasome, which activates caspase-1 (i.e. cleaved caspase-1) and leads to the production of inflammatory cytokines mainly interleukin-1 $\beta$  (IL-1 $\beta$ ) and IL-18, in multiple types of central nervous injury. Adamczak et al. study indicates that activation of the NLRP1 inflammasome is involved in neuron apoptosis and cognitive impairment [18]. Meng et al. report that the NLRP1 inflammasome is activated in high glucose-cultured cortical neurons and cerebral cortices of diabetic rats [19]. Further, NLRP1 inflammasome activation occurs in the frontal cortex and hippocampus of male mice exposed to chronic glucocorticoids, sequentially induces neurodegeneration [20]. However, whether activation of the NLRP1 inflammasome was involved in the pathogenesis of DACD, there is no report. Moreover, inflammatory markers are associated with hemostatic markers and thrombus formation in diabetic microvascular complications [21]. Whether the proinflammatory effect of thrombin/PAR-1 signaling was associated with activated NLRP1 inflammasome, it is still unknown. Actually, early reports indicate that thrombin/PAR-1 pathway leads to the activation of NF- $\kappa$ B, a key nuclear transcription factor of inflammatory mediators [22, 23]. These reports suggested a possible interaction between thrombin/PAR-1 pathway and inflammatory markers. In the meantime, seeking and finding of compounds with such properties are also very important.

Natural product sarsasapogenin, a major steroidal sapogenin of the timosaponins, is purified from the Chinese Materia Medica Rhizoma Anemarrhenae. Timosaponin AI, AIII, and BII can all be transformed into sarsasapogenin through deglycosylation to exert the bioactivities. Plenty of evidences show that timosaponins can ameliorate the learning and memory abilities or dementia by the anti-inflammatory

efficacy in a variety of animal models [24–26], including sarsasapogenin [27]. Our previous study proved that total steroidal saponins from *Rhizoma Anemarrhenae* improved DACD in rats through inhibition of the interaction between amyloid-beta peptides and neuroinflammation in brain [28]. On the other hand, an early report evaluates the effects of six steroidal saponins isolated from *Anemarrhenae Rhizome* on platelet aggregation and hemolysis in human blood, finding that only timosaponin A-III appears a strong effect on hemolysis [29]. Lu et al. prove that timosaponin BII also possesses significant antiplatelet and anticoagulation effects, and contributes to its neuroprotective effect against damage following cerebral ischaemia [30]. Moreover, a mass of researches demonstrate that timosaponins have strong anti-inflammatory properties, involving the decreases in nuclear translocation of NF- $\kappa$ B, expression levels of IL-1 $\beta$ , tumor necrosis factor  $\alpha$ , IL-6, cyclooxygenase 2, and inducible nitric oxide synthase [31–33]. Moreover, the *in vitro* and *in vivo* anti-inflammatory effects of sarsasapogenin were more potent than timosaponin AIII [32]. Additionally, our recent study indicated that sarsasapogenin could attenuate diabetic nephropathy in rats through inhibiting NLRP3 inflammasome and AGEs/RAGE axis [34]. Thus, we think that sarsasapogenin may improve DACD likely by anti-inflammatory efficacy as well as thrombosis inhibition and anticoagulation mediated by thrombin/PAR-1 signaling, but the molecular mechanisms are not clear.

In short, the aims of the present study are: firstly, whether sarsasapogenin can attenuate DACD in diabetic rats; secondly, whether thrombin/PAR-1 pathway is activated in the brain of diabetic rats and SH-SY5Y cells exposed to chronic high glucose; thirdly, whether activations of NLRP1 inflammasome and NF- $\kappa$ B mediate the proinflammatory effects of activated thrombin/PAR-1 pathway; finally, whether suppression of the neuroinflammation regulated by thrombin/PAR-1 signaling in brain contributes to the beneficial effects of sarsasapogenin on DACD in rats.

## Methods

### Animals

Nine-week-old Male Sprague Dawley rats were bred in the Experimental Animal Room of Jiangsu Key Laboratory of New Drug Research and Clinical Pharmacy, Xuzhou Medical University. All rats were housed under controlled room of humidity (50%  $\pm$  10%) and temperature (24  $\pm$  1 °C) in a normal rhythms of everyday life (12 h day/night cycle) with free access to water and rodent chow. Animal experiments were approved by the Animal Ethics Committee of Xuzhou Medical University. All experiments were conformed to Guidelines for Ethical Conduct in the Care and Use of Animals, and the stress to the animals was minimized.

### Design of the animal experiments

The rats (about 10 weeks of age) with absolute diet for 12 h more were intraperitoneally subjected to a single dose of streptozotocin (STZ, 60 mg/kg), freshly dissolved in 0.1 mol/L sodium citrate buffer at pH 4.5 [35]. Age-matched normal rats received an injection of sodium citrate buffer alone. Development of diabetes was confirmed by fasting blood glucose (FBG) level using a reagent kit of glucose detection

(Jiancheng Bioengineering Institute, Nanjing, China). The rats with FBG levels higher than 13.9 mmol/L (250 mg/dL) were considered to be diabetic rats on day 5 after STZ injection [35]. Then, the diabetic rats were randomly divided into three groups with 10 animals each group, i.e., diabetic rats, diabetic rats treated with two doses of sarsasapogenin (Sar, 20 and 60 mg/kg, p.o.). Sarsasapogenin (purity > 98%, Beijing Medicass Biotechnologies, Co. Ltd., China) was suspended in 1% (w/v) sodium carboxymethylcellulose. Meanwhile, normal rats and normal rats with 60 mg/kg of Sar (both n = 10) were designed. Uncontrolled diabetic rats and age-matched normal rats both received sodium carboxymethylcellulose solution only.

After treatment for eight weeks, animals were performed for learning and memory tests in Morris water maze for five consecutive days. On the following day, the animals were sacrificed under isoflurane anesthesia, and the blood was collected by femoral vein bleeding. Then the whole brain was rapidly removed, and two sides of the hippocampus and the cerebral cortex were isolated, storing at -80 °C until the biochemical assays.

### Morris water maze test

The Morris water maze tasks were employed for assessing the learning and memory ability according to our previous reports [4, 28]. A place navigation test was performed wherein the extent of learning was assessed for four consecutive days, and the escape latency (the time to reach the platform with seconds) was measured. A spatial probe test was performed wherein the extent of memory was assessed on day 5. Both the times of crossing the former platform and the percentage of time spent in the former platform quadrant were recorded.

### Culture and treatments of SH-SY5Y cells

The SH-SY5Y cell line was purchased from Guangzhou Jennio Biotech Co., Ltd., China. The cells were cultured in Dulbecco's modified Eagle medium with Ham's F12 medium (DMEM/F12) containing 10% fetal bovine serum. DMEM/F12 culture medium and fetal bovine serum were purchased from Hyclone (Logan, UT, USA). After incubation for 24 h under normal conditions (medium containing 17.5 mmol/L glucose, 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin, 5% CO<sub>2</sub>, 37 °C), and synchronization for 12 h, SH-SY5Y cells were divided into the following groups: normal glucose group (NG, 17.5 mmol/L glucose), high glucose group (HG, 70 mmol/L glucose), low, middle, and high concentrations of Sar group (HG+Sar, 70 mmol/L glucose + 0.2, 1, 5 µmol/L Sar, respectively), and PAR-1 inhibitor group (HG+Vor, 70 mmol/L glucose + 0.1 µmol/L vorapaxar). Sar (SSP21002, purity > 98%, Beijing Medicass Biotechnologies, Co. Ltd., China) was dissolved in dimethylsulfoxide and made into stock solution (25 mmol/L) for use. Vorapaxar (Synonyms: SCH 530348, purity > 99.1%) purchased from Medchem Expression Company (HY-10119, St. New Jersey, USA) was diluted with dimethylsulfoxide and made into stock solution for use. After treatment with the above different agents for 48 h, the cells were harvested for indices analysis. The culture time was selected according to the changes of PAR-1 protein by immunofluorescence in SH-SY5Y cultured with 70 mmol/L glucose for 24, 48, and 72 h, respectively.

## Assay of cell viability

Cell viability was used for cytotoxicity assessment with a CCK-8 kit (Dongren Chemical Technology Co., Ltd., Shanghai, China) as previous report [36]. Briefly, cell suspension (100  $\mu$ l/well,  $1.0 \times 10^6$ /ml) was preincubated in a 96-well plate for 24-48 h at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>. After the cells were incubated in different groups for 48 h, 10  $\mu$ l of the CCK-8 solution was added to each well of the plate and incubated for 2-4 h in incubator. After 10  $\mu$ l of 1% (w/v) SDS was added to each well in dark at room temperature, the absorbance was determined at 450 nm using a microplate reader. The net absorbance of cells cultured with normal glucose was considered as 100% cell viability.

## Assay of lactate dehydrogenase (LDH) release

On the day of LDH assay, the assay reagents (Beyotime Biotechnology, Nantong, China) were prepared according to the manufacturer's protocol [37]. The cells at passage 11-13 were used to determine LDH release. One milliliter of cells at a density of  $5 \times 10^5$  cells/mL (RPMI containing 10% FBS) were seeded in each well in a 96-well plate and grown for 48 h. The cells were washed with PBS three times and performed with different treatments. One hour before the scheduled detection time point, the cell culture plate was taken out from the incubator, and the reagent provided by the kit was added to the "control well with maximum enzyme activity". After adding the reagents of LDH release, mix repeatedly by pipetting several times and continue to incubate in the incubator. After the predetermined time was reached, the culture plate was centrifuged for 5 min in a multi-well plate centrifuger at 400 g. The supernatant (120  $\mu$ L) of each well was taken and added to the corresponding well in a new 96-well plate, 60  $\mu$ L of test solution was added into each well, fully mixed, incubated at room temperature for 30 min in the dark, and then the absorbance was then measured at 490 nm. The measured absorbance of each group should be subtracted from the background blank control well absorbance, and cytotoxicity or mortality (%) = (sample absorbance - control absorbance) / (absorbance of cell maximum enzyme activity - control absorbance).

## Assay of thrombin activity

Thrombin activity was assessed by a fluorometric assay based on the cleavage rate of the biosynthetic thrombin substrate Boc-Asp (OBzl)-Pro-Arg-AMC according to previous study [38]. Protein concentration in the supernatant of cell or tissue homogenates was determined by the BCA method. The buffer solution (pH = 8.8) contains Tris/HCL 1.21 g, CaCl<sub>2</sub> 22.2 mg, NaCl 1.755 g, and BSA 0.2 g in 200 mL deionized water. The 77 mg Boc-Asp (OBzl)-Pro-Arg-AMC fluorogenic substrate (Nanjing Peptide Industry Biotechnology Co., Ltd, China) was dissolved in 10 mL of dimethylsulfoxide, mixed well in dark, and stored at -20°C in avoiding light until use, and 1.0 mg bestatin (Selleckchem, China) in 1 mL dimethylsulfoxide for storage at -20°C until use. According to the total protein amount of 60  $\mu$ g, the enzymatic sample was added to the reaction system with a final volume 100  $\mu$ L including bestatin and the fluorescent substrate in a black 96-well microplate, and the control groups were designed. After fully mixed, the reaction was carried out in an oven at 37 °C for 50 min, then the optical density value was

immediately measured with a fluorescence spectrophotometer at Ex/Em=360 nm/465 nm. The RFU values of all the groups were recorded.

### **IL-18 and IL-1 $\beta$ assay by ELISA**

IL-18 and IL-1 $\beta$  levels were measured by using corresponding human IL-18 and IL-1 $\beta$  ELISA kits (Wuhan Boster Bio-technology Co., Ltd., China) according to the manufacturer's instructions.

### **Assay of AGEs levels**

AGEs assay in tissues was performed according to our previous report [39]. Simply, the brain tissue was homogenized and centrifuged, and the residual pellets were collected and repeatedly washed with different reagents in order. The final pellets suspended in 1.0 mL of HEPES buffer containing 290 U of type I collagenase (Sigma-Aldrich Company, USA) and antiseptics were mixed and shaken for 24 h at 37 °C. After centrifugation, the supernatant was collected for the determination of fluorescence intensity at Ex/Em=370 nm/440 nm. AGEs levels in cerebral cortex were expressed as alteration of the enzyme activity of type I collagenase (U/ mg protein).

### **Western blot assay**

Protein concentration of the sample was determined using a BCA protein assay kit (Thermo Scientific, Rockford, IL, USA). The protein samples were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA). The membrane was blocked with 2% milk powder solution for 60 min. Primary antibodies including anti-NLRP1 (Abcam Company, USA, 1:1000), anti-cleaved-caspase-1 (Bioworld Company, USA, 1:1000), anti-PAR-1 (Sigma-Aldrich Company, USA, 1:1000), anti-RAGE (Abcam Company, UK, 1:1000), anti-NF- $\kappa$ B p65 antibody (Affinity Biosciences, UAS, 1:1000), and anti-NF- $\kappa$ B p-p65 (Affinity Biosciences, UAS, 1:1000) were incubated overnight at 4°C, followed by IRDye purified goat anti rabbit IgG(H+L) secondary antibodies (Li-Cor Inc., Lincoln, NE), respectively. Immunoreactive blots were detected using Infrared Imaging System (Gene Company Limited, Hong Kong, China), and the signal densities on the blots were measured with Image J software; meanwhile, they were normalized using rabbit anti- $\beta$ -actin antibody (Bioworld Technology, St. Louis, USA) or rabbit anti-GAPDH antibody (ABclonal Biotechnology Co., Ltd., USA) as an internal control.

### **Immunofluorescence assay**

Immunofluorescence assay was performed according to the report [40]. Cells plated on coverslips were fixed in ice cold 4% paraformaldehyde for 15 min at -20°C, permeabilized with 0.1% TritonX-100 in PBS for 10 min, treated with blocking medium (1% bovine serum albumin in PBS) for 30 min, and then incubated overnight at 4°C with anti-PAR-1 antibody (Cat No.Cleaved-Ser42, Sigma-Aldrich Company, USA), anti-NF- $\kappa$ B p65 antibody, or anti-NF- $\kappa$ B p-p65 (Affinity Biosciences, UAS). Immune-reacted primary antibody was detected following for 1 h incubation in dark place at 37°C with a secondary antibody Dylight 594 Affinipure donkey anti-rabbit IgG (H+L) (Earthox, Millbrae, CA, USA). The cells were further

stained with DAPI (Vector, Burlingame, CA, USA) for 2 min in dark place at room temperature and washed, and then were mounted onto microscope slides in mounting medium. Observations were carried out by using an Olympus BX43F fluorescence microscope (Tokyo, Japan).

### Real-time qPCR assay

Total RNA was isolated from tissues or cells using TRIzol Reagent (Invitrogen, USA). According to the cDNA synthesis kit (#RR037 A, Takara, Japan), the mRNA was reverse-transcribed into single-stranded cDNA. The Roche 480 LightCycler® system with SYBR Green dye binding to PCR product was used to quantify target mRNA accumulation via fluorescence PCR using human or rat  $\beta$ -actin as a reference. The assay genes were rat PAR-1 (forward 5'-GCCATCGCTGTGTTTGTCTT -3', reverse 5'-CAGGAACCGGTCAATGCTTA -3') and  $\beta$ -actin (forward 5'- CCCATCTATGAGGGTTACGC -3', reverse 5'-TTAATGTCACGCACGATTTTC -3') as well as human PAR-1 (forward 5'- CGCAGAGCCCGGGACAATGG -3', reverse 5'-CGGTGCGGGCAGACAACA -3') and  $\beta$ -actin (forward 5'-TGACGTGGACATCCGCAAAG -3', reverse 5'- CTGGAAGGTGGACAGCGAGG -3'). Lower Ct value indicated higher amounts of PCR products. For the amplification reaction in each well, a Ct was observed in the exponential phase of amplification, and the quantification of relative expression levels was achieved using standard curves for both target and endogenous control samples. Relative transcript abundance of a gene is expressed as  $2^{-\Delta\Delta Ct}$  values ( $\Delta Ct = Ct_{\text{target}} - Ct_{\text{reference}}$ ).

### Statistical analysis

All statistical analyses were carried out using GraphPad Prism 7.0 software. One-way ANOVA with Dunnet's post hoc test was performed for analysis, or an unpaired, two-tailed Student's t test for statistical analysis, wherever applicable. Data were represented as mean  $\pm$  S.E.M.  $P < 0.05$  was considered statistically significant.

## Results

### Sar ameliorated the memory ability of diabetic rats

The learning and memory ability was assessed by using Morris water maze test (Fig. 1). In the place navigation test, mean escape latency ( $F_{(4,157)} = 20.28, P < 0.01$ ) of the diabetic rats was constantly significantly (all  $P < 0.01$ ) longer than that of the normal rats during four-day trainings (Fig. 1A). Chronic Sar treatment decreased mean escape latency of the diabetic rats, and the low dose group showed a significant (both  $P < 0.05$ ) decrease on the first and second day of training, but the high dose group did not exhibit significant decrease throughout the training days (Fig. 1A). In the probe trial, the numbers of crossing the former platform ( $F_{(4,43)} = 3.895, P < 0.01$ ) and the percentage of time spent in the target quadrant ( $F_{(4,35)} = 6.330, P < 0.01$ ) were both markedly reduced in diabetic rats, while high dose of Sar significantly elevated the crossing times ( $P < 0.05$ ) and the time percentage ( $P < 0.01$ ) of diabetic rats (Fig. 1B, C). Moreover, low dose of Sar significantly elevated the time percentage ( $P < 0.01$ ) but

moderately increased the crossing times in diabetic rats (Fig. 1C, B). In addition, Sar does not affect learning and memory ability of the normal rats (Fig. 1A-C). These results demonstrated that Sar could ameliorate the memory impairment caused by diabetes.

According to our previous report [34], Sar did not ameliorate the elevated blood glucose and the reduced body weight of streptozotocin-induced diabetic rats, and Sar also had no effects on both blood glucose and body weight of the normal rats.

Sar suppressed NLRP1 inflammasome activation and PAR-1 up-regulation in brain of diabetic rats

To explore the reasons that Sar ameliorated memory impairment of the diabetic rats, changes of the NLRP1 inflammasome and thrombin/PAR-1 signaling were examined in brain. We found that the NLRP1 inflammasome was activated in the hippocampus of the diabetic rats, evidenced by significant increases in NLRP1 expression ( $F_{(3,16)} = 6.849$ ,  $P < 0.01$ ) as well as IL-1 $\beta$  ( $F_{(4,40)} = 7.797$ ,  $P < 0.01$ ) and IL-18 ( $F_{(4,42)} = 13.71$ ,  $P < 0.01$ ) levels (Fig. 2). Nevertheless, treatment with Sar markedly suppressed the activation of the NLRP1 inflammasome in the hippocampus of the diabetic rats (Fig. 2). Moreover, the NLRP1 inflammasome was also activated in the cerebral cortex of the diabetic rats through significant increases in NLRP1 expression ( $F_{(3,16)} = 14.27$ ,  $P < 0.01$ ) and levels of IL-1 $\beta$  ( $F_{(4,44)} = 147.8$ ,  $P < 0.01$ ) and IL-18 ( $F_{(4,44)} = 61.76$ ,  $P < 0.01$ ) (Fig. 3), whereas Sar treatment similarly restrained the activated NLRP1 inflammasome (Fig. 3). Additionally, Sar did not influence IL-1 $\beta$  and IL-18 levels in both brain regions of the normal rats (Fig. 2, 3).

To explore the relationship of NLRP1 inflammasome and thrombin/PAR-1 signaling in the brain of diabetic rats, thrombin activity and protein expression of thrombin receptor PAR-1 were assessed. From Fig. 4A, we can see thrombin activity ( $F_{(3,16)} = 0.238$ ,  $P = 0.869$ ) was so low in the cerebral cortex of rats that it was not obvious difference among normal, DM, and Sar treatment groups, but thrombin receptor PAR-1 was significantly up-regulated in both mRNA levels ( $F_{(3,16)} = 8.303$ ,  $P < 0.01$ ) and protein expression ( $F_{(3,16)} = 7.07$ ,  $P < 0.01$ ) in the cerebral cortex of diabetic rats (Fig. 4B, C). High dose of Sar treatment markedly decreased the mRNA levels ( $P < 0.05$ ) and protein expression ( $P < 0.01$ ) of PAR-1 in the cerebral cortex of diabetic rats (Fig. 4B, C), suggesting an inhibitory effect of Sar on activated PAR-1 signaling in brain of diabetic condition.

Sar suppressed AGEs/RAGE axis in cerebral cortex of diabetic rats

The effects of Sar on AGEs/RAGE interaction were also examined. AGEs levels ( $F_{(4,44)} = 33.26$ ,  $P < 0.01$ ) and RAGE expression ( $F_{(3,12)} = 6.453$ ,  $P < 0.01$ ) were both markedly increased in the cerebral cortex of diabetic rats, while treatments with low and high doses of Sar significantly ( $P < 0.05$  or  $P < 0.01$ ) decreased not only AGEs levels but also RAGE expression in the cerebral cortex of diabetic rats (Fig. 5A, B). These indicated that Sar could suppress AGEs/RAGE axis in the brain of diabetic rats.

Sar attenuated HG-induced neuronal damages in SH-SY5Y cells

To fully examine the effects of Sar on the central neurons in diabetic condition, HG-cultured SH-SY5Y cells were used. In order to find the applicable cell culture protocol, PAR-1 expression was selected to be observed in SH-SY5Y cells cultured with high glucose for different time. We found that 70 mmol/L glucose notably increased the protein expression of PAR-1 in SH-SY5Y cells after culture for both 48 h and 72 h by immunofluorescence analysis (Fig. S1). So the in vitro experiment protocol SH-SY5Y cells cultured with 70 mmol/L glucose for 48 h was adopted. Then, neuronal damages as well as changes of thrombin/PAR-1 signaling and the proinflammatory pathways were further investigated with the above experiment protocol.

The concentration of Sar was screened by using cell viability, the results were put in the supplement materials (Fig. S2). It was found that HG markedly decreased cell viability ( $F_{(5,12)} = 9.123$ ,  $P < 0.01$ ) and increased LDH release ( $F_{(5,18)} = 36.32$ ,  $P < 0.01$ ) in SH-SY5Y cells after culture for 48 h, compared with normal glucose (Fig. 6A, B), whereas co-treatment of HG and neither of the three concentrations of Sar (0.2, 1, 5  $\mu\text{M}$ ) or Vor (a selective antagonist of PAR-1) dramatically (each  $P < 0.01$ ) attenuated the neuronal damages of SH-SY5Y cells caused by high glucose culture (Fig. 6A, B). From these results we drew a conclusion that Sar like PAR-1 inhibitor protected against high glucose-induced neuronal injury.

Sar inhibited HG-induced PAR-1 up-regulation and NLRP1 inflammasome activation in SH-SY5Y cells

In the cell experiments, we further investigated the alterations of thrombin/PAR-1 signaling and NLRP1 inflammasome in the central neurons in hyperglycemic condition. We found that thrombin activity was very low in the central neurons, and the enzymatic activity ( $F_{(4,10)} = 0.299$ ,  $P = 0.872$ ) was still not changed among NG, HG, and Sar treatment groups (Fig. 7A). But thrombin receptor PAR-1 was remarkably up-regulated both in mRNA levels ( $F_{(4,10)} = 21.69$ ,  $P < 0.01$ ) and protein expression ( $F_{(5,24)} = 20.0$ ,  $P < 0.01$ ) in HG-cultured SH-SY5Y cells, compared with those in NG group (Fig. 7B-D). Three concentrations of Sar (0.2, 1, 5  $\mu\text{M}$ ) all declined the mRNA levels and protein expression of PAR-1 (all  $P < 0.01$ ) in HG-cultured SH-SY5Y cells (Fig. 7B-D). Moreover, middle concentration of Sar had a similar effect on PAR-1 protein expression to that of a selective antagonist of PAR-1 vorapaxar (Fig. 7C, D), indicating a strong inhibitory efficacy of Sar on PAR-1.

NLRP1 inflammasome was activated by HG culture in central neurons through the elevated NLRP1 ( $F_{(5,18)} = 3.985$ ,  $P < 0.05$ ) and cleaved-caspase 1 ( $F_{(5,24)} = 5.625$ ,  $P < 0.01$ ) expressions as well as IL-1 $\beta$  ( $F_{(5,24)} = 4.976$ ,  $P < 0.01$ ) and IL-18 ( $F_{(5,18)} = 22.38$ ,  $P < 0.01$ ) levels in SH-SY5Y cells (Fig. 8A-D). However, three concentrations of Sar (0.2, 1, 5  $\mu\text{M}$ ) all decreased NLRP1, cleaved-caspase 1, IL-18, and IL-1 $\beta$  levels ( $P < 0.05$  or  $P < 0.01$ ) in HG-cultured SH-SY5Y cells (Fig. 8A-D). Furthermore, blockage of PAR-1 by vorapaxar reversed activation of the NLRP1 inflammasome in HG-cultured SH-SY5Y cells, and high concentration of Sar had a similar effect to a selective antagonist of PAR-1 vorapaxar on NLRP1 inflammasome (Fig. 8A-D).

NLRP1 inflammasome and NF- $\kappa\text{B}$  mediated the proinflammatory efficacy of PAR-1 up-regulation in HG-cultured SH-SY5Y cells

In order to clarify the proinflammatory effect of up-regulated PAR-1 resulted from activation of the NLRP1 inflammasome and NF- $\kappa$ B signaling, key molecules of the NLRP1 inflammasome and the activated form of NF- $\kappa$ B were examined in HG-cultured SH-SY5Y cells after PAR-1 knockdown by using short hairpin RNA (shRNA). The interference efficiency of F2R shRNA (F2R as the gene symbol of PAR-1) was conformed in SH-SY5Y cells (Fig. S3). We found that NLRP1 ( $F_{(4,30)} = 19.10, P < 0.01$ ), IL-1 $\beta$  ( $F_{(4,30)} = 61.64, P < 0.01$ ), and IL-18 ( $F_{(4,20)} = 92.02, P < 0.01$ ) levels were dramatically decreased in SH-SY5Y cells with F2R shRNA after HG culture, compared to those in SH-SY5Y cells with negative control shRNA after HG culture (Fig. 9A-C). This effect was further confirmed by using HG co-treatment with high concentration of Sar (HG + Sar-5) in the same condition of PAR-1 knockdown (Fig. 9A-C). Moreover, the relationship between PAR-1 signaling and NF- $\kappa$ B activation [23] was also testified in the current study. Phosph-NF- $\kappa$ B p65 ( $F_{(4,30)} = 9.712, P < 0.01$ ) level was markedly declined in HG-cultured SH-SY5Y cells with PAR-1 knockdown (Fig. 10A, C), but NF- $\kappa$ B p65 ( $F_{(4,30)} = 1.115, P = 0.368$ ) level was not changed (Fig. 10A, B). Similarly, these were further confirmed by using Sar addition (Fig. 10A-C). The results demonstrated that activation of the NLRP1 inflammasome and NF- $\kappa$ B contributed to the proinflammatory effect of enhanced PAR-1 signaling in central neurons in high glucose condition.

## Discussion

Multiple risk factors are associated with DACD, while angiopathy together with neuroinflammation may be the archcriminal of diabetes-related cognitive deficit [1, 2]. Our findings indicated that up-regulation of thrombin receptor PAR-1, a key molecule in cerebrovascular diseases and neuroinflammation, contributed to cognitive impairment in the diabetic condition, and NLRP1 inflammasome and NF- $\kappa$ B were the executioners. Importantly, we found that a natural product Sar could improve DACD in rats, and Sar was a suitable compound blocking PAR-1 signaling, showing both cerebral angiopathy and neuroinflammation inhibition in brain in diabetic state.

Activation of thrombin/PAR-1 pathway in brain results in diabetes-associated cognitive impairment. Mhatre et al. report that intracerebroventricular infusion of thrombin causes significant cognitive impairment in rats [5], and thrombin contributes to synaptic plasticity and learning ability damages in major neuropsychiatric disorders [6]. Our study demonstrated that learning and memory ability were markedly declined in diabetic rats; however, thrombin activity was not increased in the cerebral cortex of diabetic rats and high glucose-cultured SH-SY5Y cells, whereas thrombin receptor PAR-1 was remarkably up-regulated in both models. Meanwhile, a selective PAR-1 inhibitor reversed the up-regulation of PAR-1 and attenuated the neuronal damages in SH-SY5Y cells caused by high glucose. Semenikhina et al. report that inhibition of PAR-1 ameliorates behavioral deficits and restores hippocampal synaptic plasticity in a rat model of status epilepticus [41], and PAR-1 deficiency protects against neuronal damage and neurologic deficits after unilateral cerebral hypoxia/ischemia in mice [13]. These results indicate that activation of thrombin/PAR-1 pathway in brain likely through PAR-1 up-regulation triggers the cognitive behavioral deficits in diabetes condition.

Nevertheless, a steroidal compound Sar relieved the memory impairment caused by diabetes in rats and neuron injury of SH-SY5Y cells caused by chronic high glucose culture. Hu et al. find that Sar improves memory by elevating the low muscarinic acetylcholine receptor density in the brain of a memory-deficit rat model [27]. Recently, our team found that Sar showed neuroprotective effects by suppressing the overproduction of neurotoxic amyloid-beta peptides in high glucose-cultured HT-22 cells [42]. These studies demonstrate a specific effect of Sar on cognitive defect in rodents. Furthermore, Sar down-regulated the mRNA and protein levels of PAR-1 in the brain of diabetic rats and high glucose-cultured SH-SY5Y cells, and the effect of Sar on PAR-1 protein was the same to that of a PAR-1 inhibitor vorapaxar. Some researches show that the antiplatelet and anticoagulation effects of Sar glucosides may contribute to their neuroprotective effects against damage following cerebral ischaemia damage [29, 30], although PAR-1 is not mentioned to be involved in these effects. Together, the ameliorative effects of Sar on DACD might be related to the inhibition of thrombin/PAR-1 pathway in brain in diabetic state.

Neuroinflammation plays a vital role in the pathogenesis of DACD, and inflammatory mediators are associated with hemostatic markers and thrombus formation in diabetic microvascular complications [21]. So in the current study pro-inflammatory signaling was also examined besides thrombin/PAR-1 pathway in the brain of rats with DACD. NLRP1 inflammasome activation is involved in neuron apoptosis and cognitive impairment in brain-injured patients (Adamczak et al., 2012), and Meng et al. report that NLRP1 inflammasome is activated in high glucose-cultured cortical neurons and cerebral cortices of diabetic rats, subsequently results in neuron dysfunction and apoptosis (Meng et al., 2014). In the present study, cognitive impairment was observed in couple with the activation of NLRP1 inflammasome in the hippocampus and cerebral cortex of diabetic rats, while Sar treatment attenuated the memory impairment and suppressed NLRP1 inflammasome activation in both brain regions. Meanwhile, Sar mitigated the neuronal damages and the activated NLRP1 inflammasome in high glucose-cultured SH-SY5Y cells, and the effects of Sar were similar to those of the PAR-1 inhibition. A recent study in our team indicated that Sar inhibited activation of the NLRP3 inflammasome in the kidney of diabetic rats [34]. From the above reports, we could deduce that inhibition of NLRP1 inflammasome activation in brain was probably one of the reasons for Sar protecting against the memory impairment and the central neuron injury caused by prolonged hyperglycemia.

The pro-inflammatory efficacy of the activated thrombin/PAR-1 pathway may result from activations of NLRP1 inflammasome and NF- $\kappa$ B signaling in high glucose condition. Lee et al. report that IL-1 $\beta$  enhances granzyme B-mediated neurotoxicity by increasing PAR-1 expression [43]. In our findings, key molecules of the NLRP1 inflammasome were markedly decreased in SH-SY5Y cells cultured with high glucose after PAR-1 knockdown by using F2R shRNA. On the other hand, an early report indicates that stimulation of PAR-1 leads to the activation of NF- $\kappa$ B and promotes the survival of prostate cancer cells [23]. In the current study, we found that the phosphorylated NF- $\kappa$ B p65 but not NF- $\kappa$ B p65 was significantly declined in SH-SY5Y cells cultured with high glucose after PAR-1 knockdown by two different methods (Fig. 10A and Fig. 10B, C). Thus, our results demonstrated that the activated NLRP1 inflammasome and NF- $\kappa$ B signaling mediated the pro-inflammatory effects of thrombin/PAR-1 pathway

in brain in prolonged hyperglycemic state, and subsequently caused diabetes-associated cognitive impairment.

Enhancement of AGEs/RAGE interaction may lead to the up-regulation of PAR-1 caused by chronic high glucose. Ishibashi et al. report that AGEs potentiate the citrated plasma-induced oxidative and inflammatory reactions in endothelial cells via up-regulating PAR-1 expression [44]. Our results showed that PAR-1 was up-regulated in the brain of diabetic rats, accompanied by the elevated AGEs levels and RAGE expression. These studies suggested that high glucose stimulated PAR-1 up-regulation was maybe due to the activated AGEs/RAGE axis in hyperglycemic state. In the present study, Sar suppressed AGEs/RAGE axis in the brain of diabetic rats, and our previous report exhibited that Sar mitigated AGEs/RAGE interaction in the renal cortex of diabetic rats [34]. Taken together, Sar down-regulated PAR-1 possibly via inhibiting AGEs/RAGE axis in the brain of diabetic rats.

## Conclusion

This study indicated that sarsasapogenin ameliorated diabetes-associated memory impairment and neuroinflammation in diabetic rats, which was related to the inhibition of thrombin/PAR-1 pathway in brain. Moreover, the activated NLRP1 inflammasome and NF- $\kappa$ B signaling mediated the neuroinflammation from thrombin/PAR-1 activation in diabetic state. Additionally, high glucose stimulated PAR-1 up-regulation was possibly due to the activated AGEs/RAGE axis. Finally, sarsasapogenin was a pleiotropic neuroprotective agent and memory enhancer.

## Abbreviations

AGEs: advanced glycation endproducts; DACD: diabetes-associated cognitive decline; ELISA: enzyme-linked immunosorbent assay; FBG: fasting blood glucose; HG: high glucose; IL: interleukin; LDH: lactate dehydrogenase; NLRP1: Nucleotide-binding domain and leucine-rich repeat containing protein 1; PAR-1: protease-activated receptor 1; qPCR: real-time fluorescence quantitative PCR; RAGE: receptor for advanced glycation endproducts; Sar: sarsasapogenin; shRNA: short hairpin RNA; Vor: vorapaxar

## Declarations

### Conflict of interest

The authors declare no conflict of interest.

### Acknowledgments

Not applicable

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### **Availability of data and materials**

All data supporting the conclusions of this article are included within the article.

### **Authors' contributions**

This work includes significant contributions from all authors. YW Liu conceived the study and contributed to its experimental design. L Kong, Y Liu, YM Zhang, Y Li, ZZ Tang, and X Zhu carried out the laboratory experiments and analyzed the data. L Kong and Y Liu wrote the manuscript. YW Liu and TF Ma contributed to editing the manuscript. All authors read and approved the final manuscript.

### **Ethics approval**

All procedures were approved by the Animal Ethics Committee, Xuzhou Medical University, China, and complied with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

### **Consent for publication**

Not applicable.

### **Competing interests**

The authors declare that they have no competing interests

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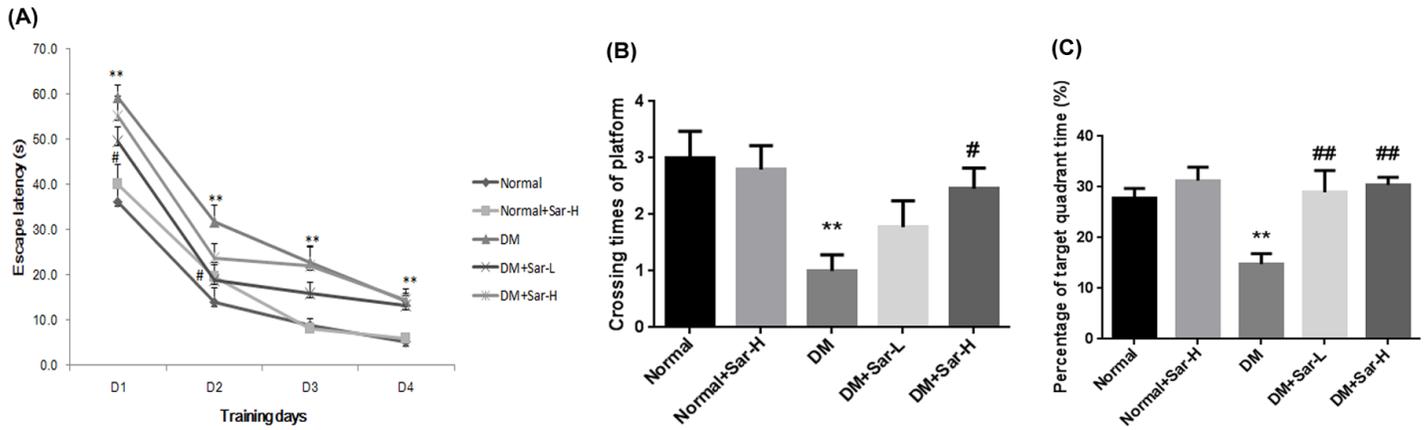
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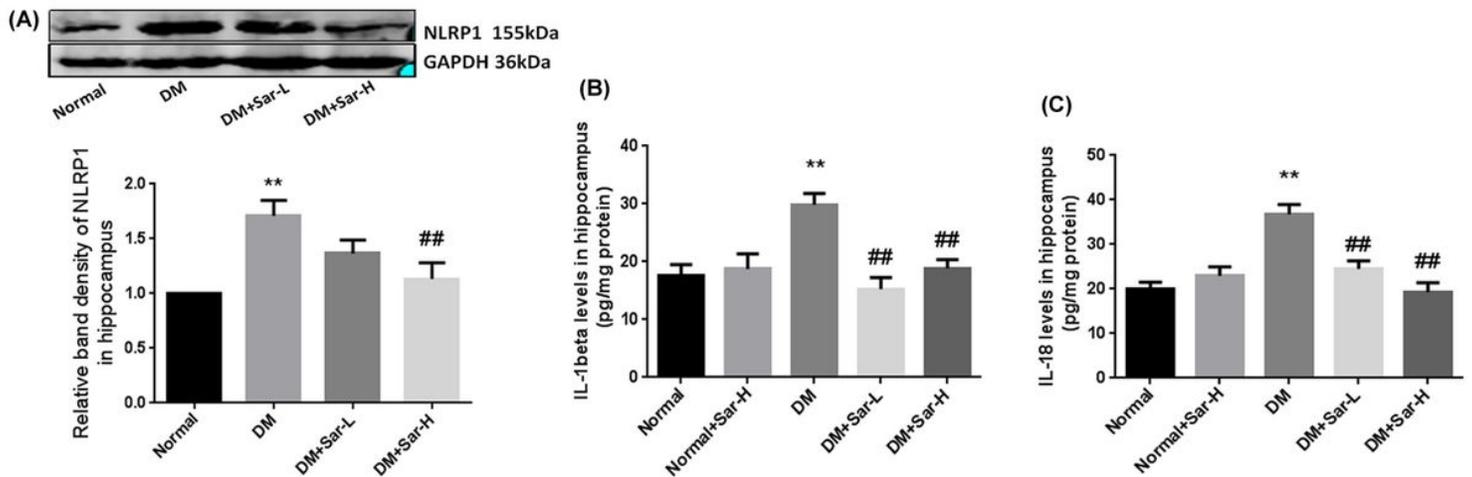
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## Figures



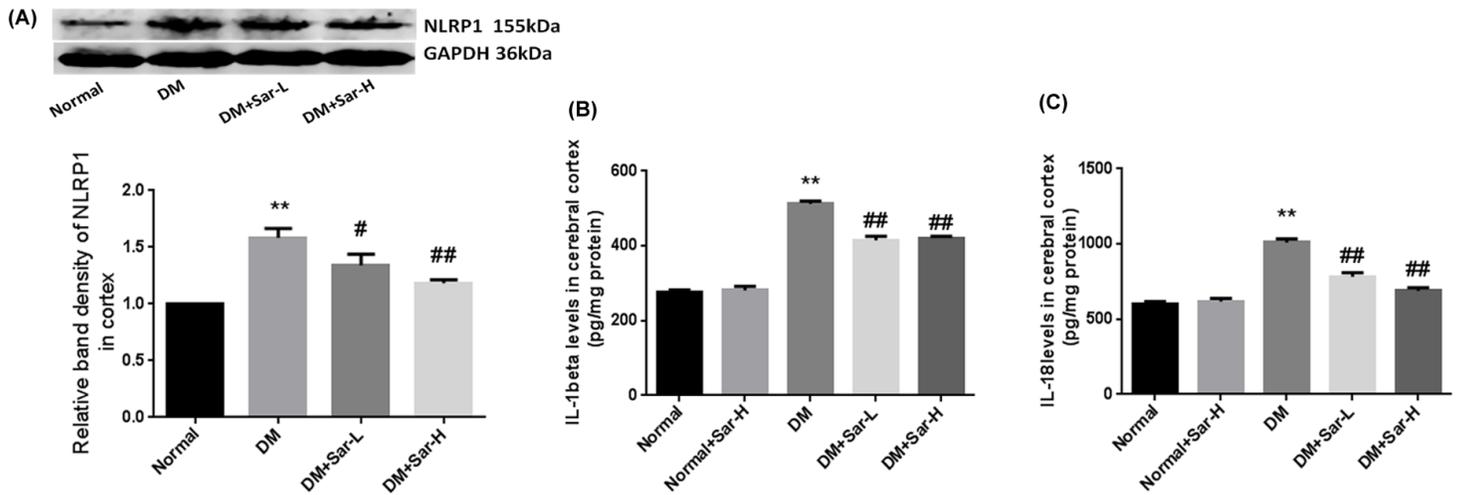
**Figure 1**

Effects of Sar on DACD in rats in Morris water maze test, (A) escape latency, (B) the percentage of time spent in the target quadrant, and (C) the numbers of crossing platform. The groups were in turn normal, normal treated with high dose of Sar (Normal+Sar-H), STZ-induced diabetes (DM), diabetes treated with Sar by low dose (DM+Sar-L) and high dose (DM+Sar-H). Mean  $\pm$  S.E.M, n=7-10. \*\* P < 0.01, vs. Normal; # P < 0.05, ## P < 0.01, vs. DM.



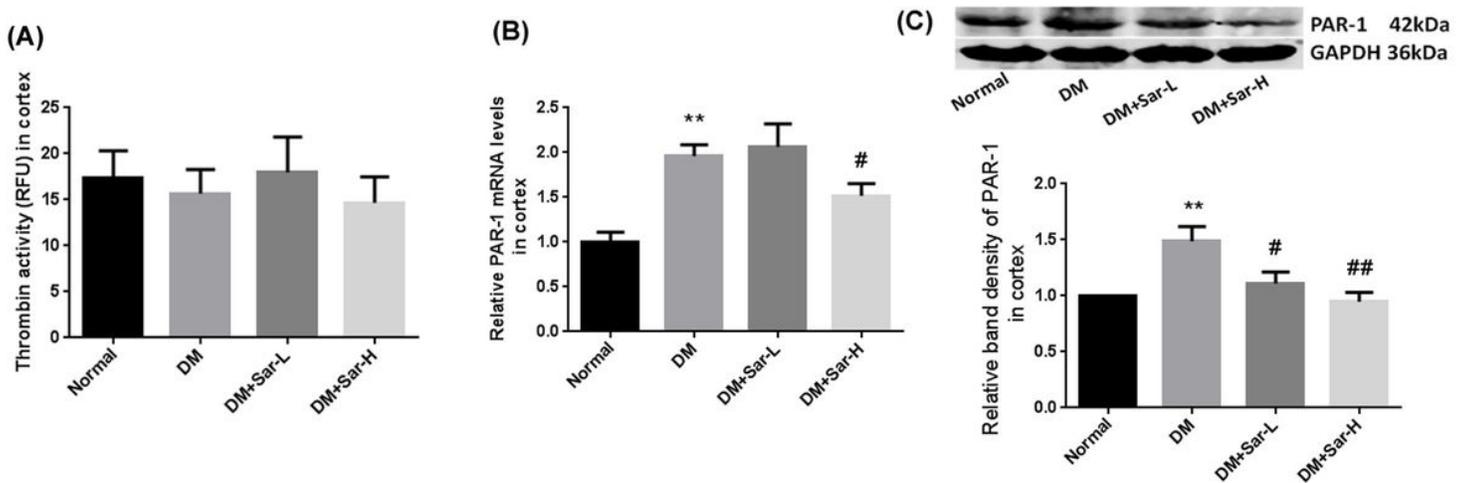
**Figure 2**

Effects of Sar on NLRP1 inflammasome in the hippocampus of STZ-induced diabetic rats, (A) NLRP1 expression, (B) IL-1 $\beta$  levels, and (C) IL-18 levels. The groups include normal, normal treated with high dose of Sar (Normal+Sar-H), STZ-induced diabetes (DM), diabetes treated with Sar by low dose (DM+Sar-L) and high dose (DM+Sar-H). Mean  $\pm$  S.E.M, n=5 (NLRP1), n=8-10 (IL-1 $\beta$  and IL-18). \*\* P < 0.01, vs. Normal; ## P < 0.01, vs. DM.



**Figure 3**

Effects of Sar on NLRP1 inflammasome in the cerebral cortex of STZ-induced diabetic rats, (A) NLRP1 expression, (B) IL-1 $\beta$  levels, and (C) IL-18 levels. The groups include normal, normal treated with high dose of Sar (Normal+Sar-H), STZ-induced diabetes (DM), diabetes treated with Sar by low dose (DM+Sar-L) and high dose (DM+Sar-H). Mean  $\pm$  S.E.M, n=5 (NLRP1), n=9-10 (IL-1 $\beta$  and IL-18). \*\* P < 0.01, vs. Normal; # P < 0.05, ## P < 0.01, vs. DM.



**Figure 4**

Effects of Sar on thrombin/PAR-1 pathway in the cerebral cortex of STZ-induced diabetic rats, (A) thrombin activity, (B) PAR-1 mRNA levels, and (C) PAR-1 expression. The groups were in sequence normal, STZ-induced diabetes (DM), diabetes treated with Sar by low dose (DM+Sar-L) and high dose (DM+Sar-H). Mean  $\pm$  S.E.M, n=5. \*\* P < 0.01, vs. Normal; # P < 0.05, ## P < 0.01, vs. DM.

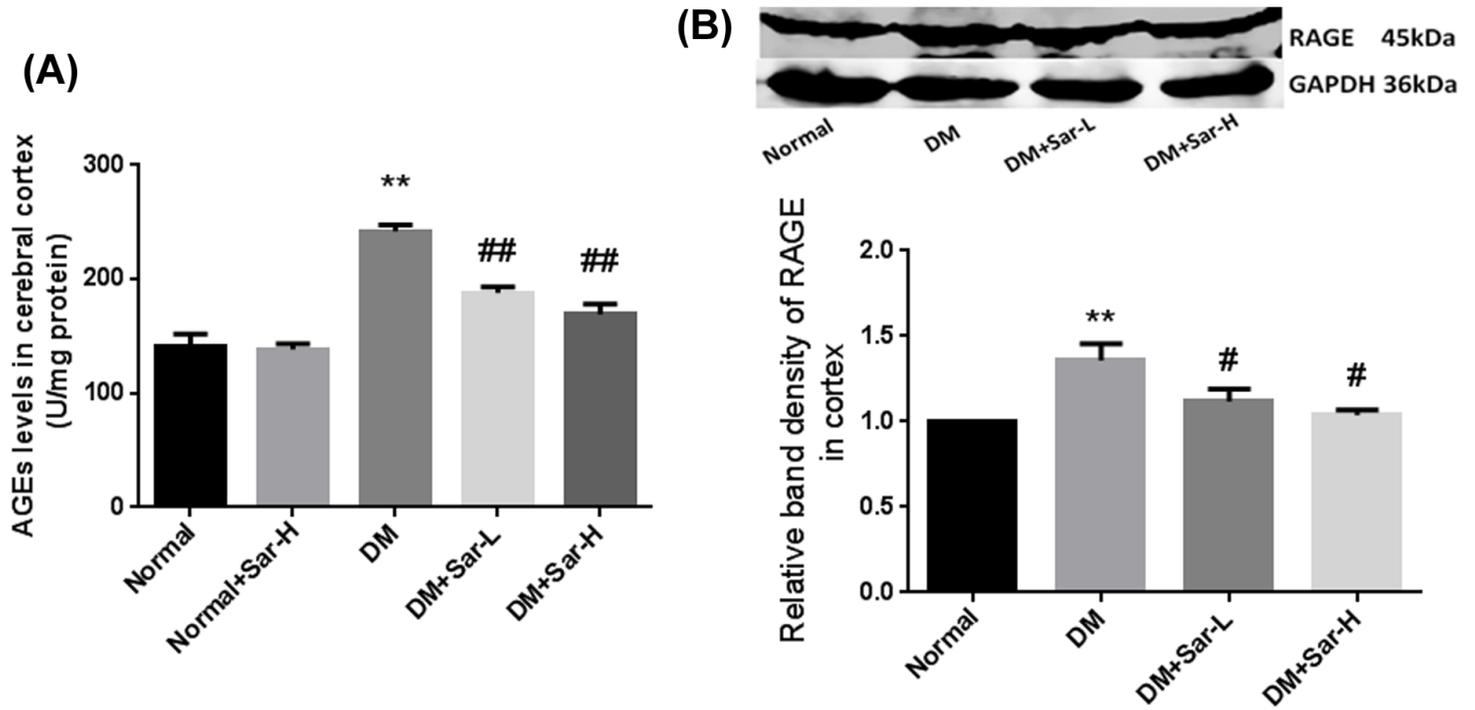


Figure 5

Effects of Sar on AGEs/RAGE axis in the cerebral cortex of STZ-induced diabetic rats, (A) AGEs levels and (B) RAGE expression. The groups include normal, normal treated with high dose of Sar (Normal+Sar-H), STZ-induced diabetes (DM), diabetes treated with Sar by low dose (DM+Sar-L) and high dose (DM+Sar-H). Mean  $\pm$  S.E.M, n=9-10 (AGEs), n=4 (RAGE). \*\* P < 0.01, vs. Normal; # P < 0.05, vs. DM.

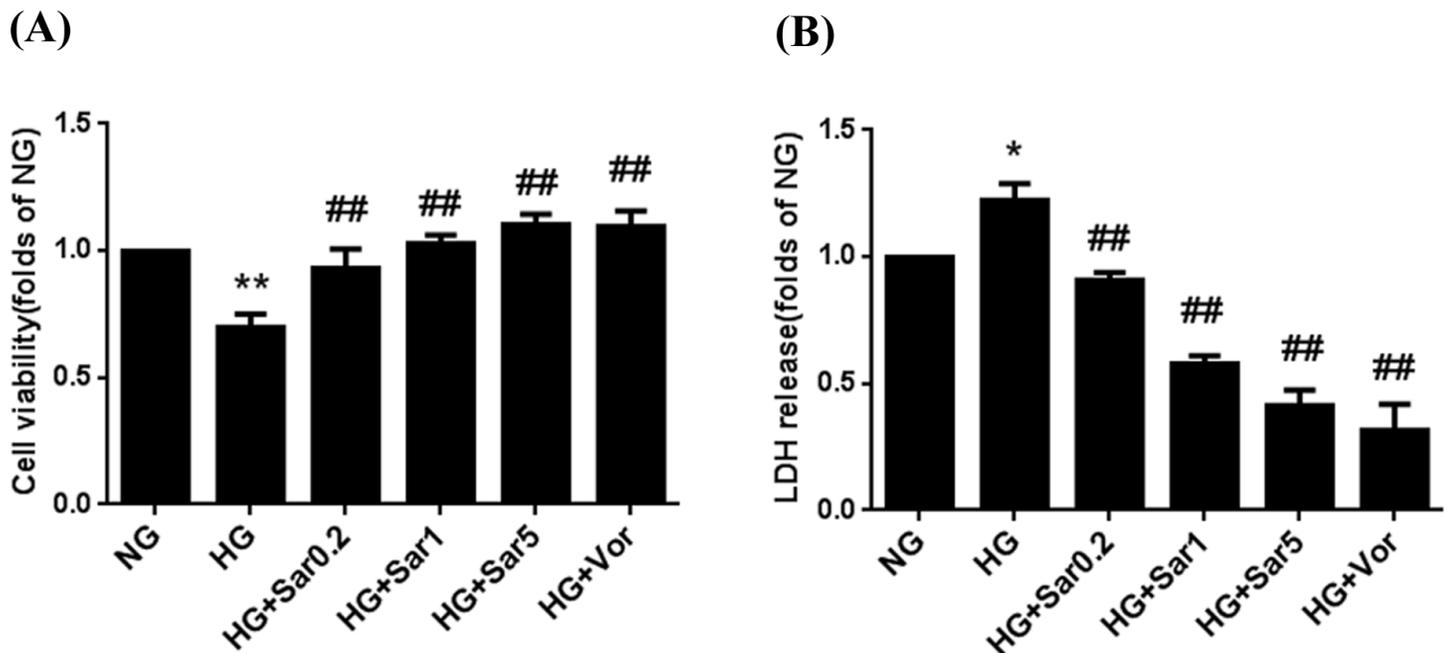
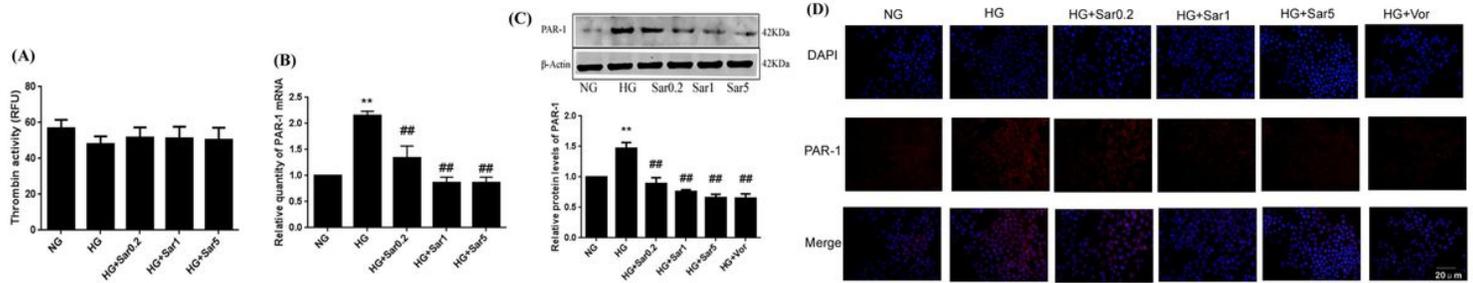


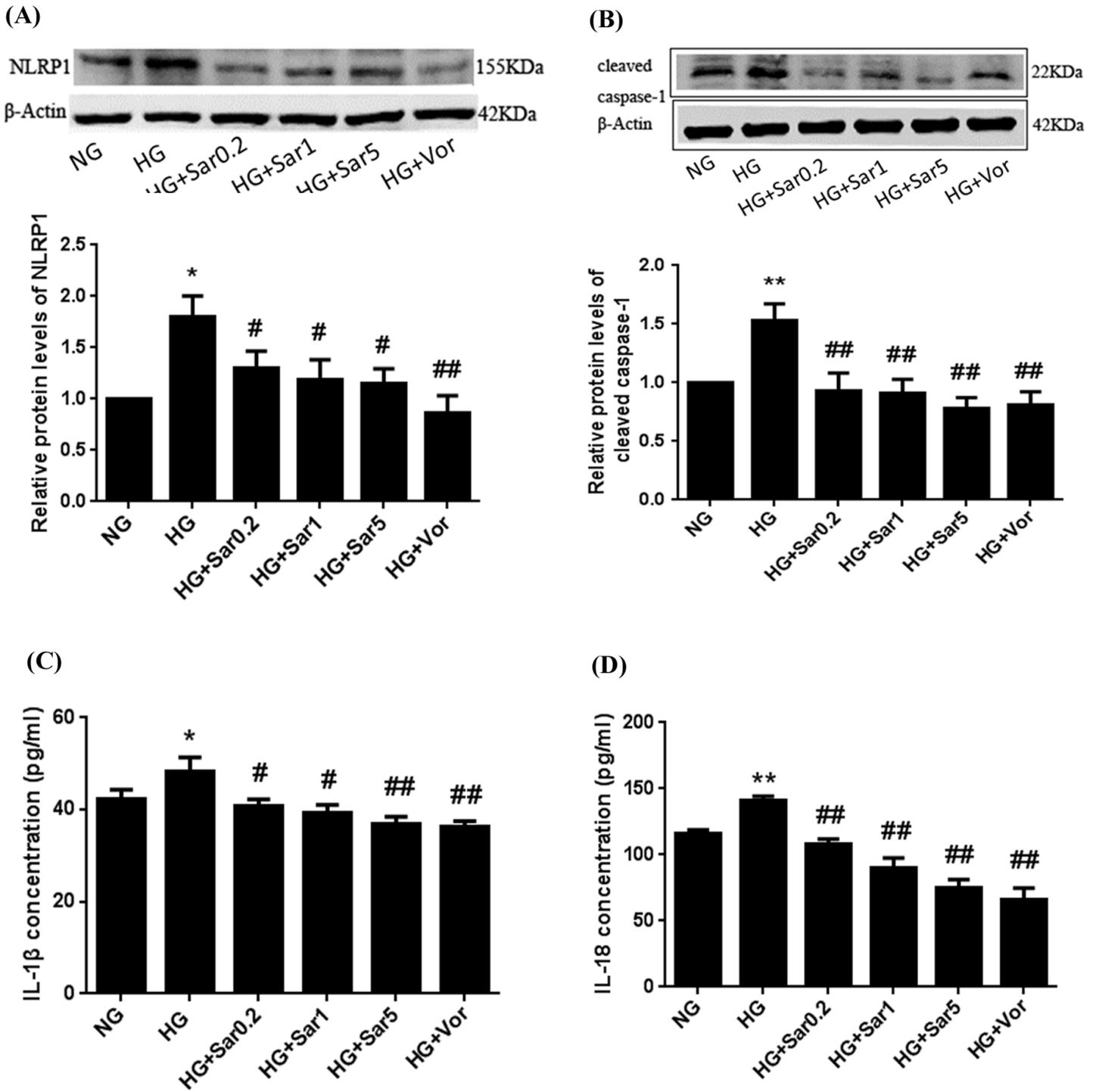
Figure 6

Effects of Sar on neuronal injury in SH-SY5Y cultured with high glucose, (A) cell viability and (B) LDH release. NG, HG, HG+Sar0.2, HG+Sar1, HG+Sar5, and HG+Vor: cells treated with 17.5 mmol/L glucose, 70 mmol/L glucose, 70 mmol/L glucose plus 0.2, 1, 5  $\mu\text{mol/L}$  Sar, or 1.0  $\mu\text{mol/L}$  vorapaxar (Vor, a selective PAR-1 antagonist), respectively. Mean  $\pm$  S.E.M, n=3 (cell viability), n=4 (LDH). \*P < 0.05, \*\*P < 0.01, compared with NG; ## P < 0.01, compared with HG.



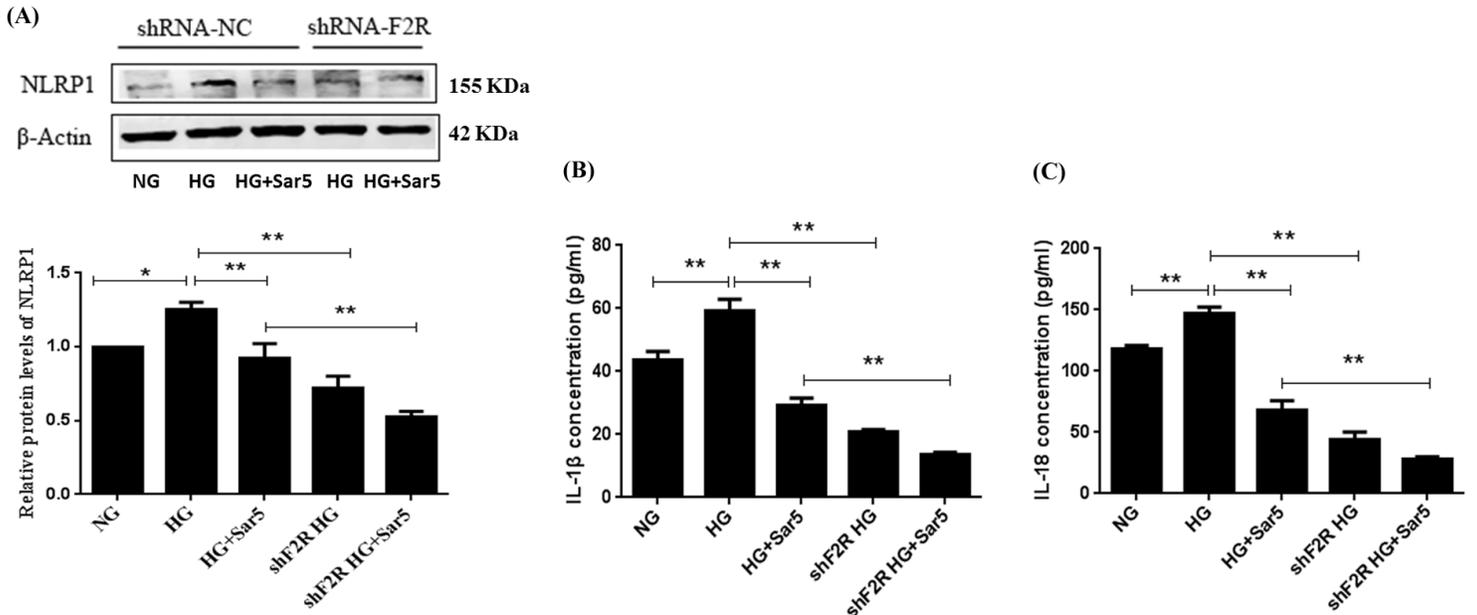
**Figure 7**

Effects of Sar on thrombin/PAR-1 pathway in SH-SY5Y cultured with high glucose, (A) thrombin activity, PAR-1 mRNA levels (B), and PAR-1 expression by Western blot (C) and immunofluorescence (D). NG, HG, HG+Sar0.2, HG+Sar1, HG+Sar5, and HG+Vor: cells treated with 17.5 mmol/L glucose, 70 mmol/L glucose, 70 mmol/L glucose plus 0.2, 1, 5  $\mu\text{mol/L}$  Sar, or 1.0  $\mu\text{mol/L}$  vorapaxar (Vor, a selective PAR-1 antagonist), respectively. Mean  $\pm$  S.E.M, n=3 (thrombin and PAR-1 mRNA), n=5 (PAR-1 expression). \*\* P < 0.01, compared with NG; ## P < 0.01, compared with HG. Scale bar: 20  $\mu\text{m}$ .



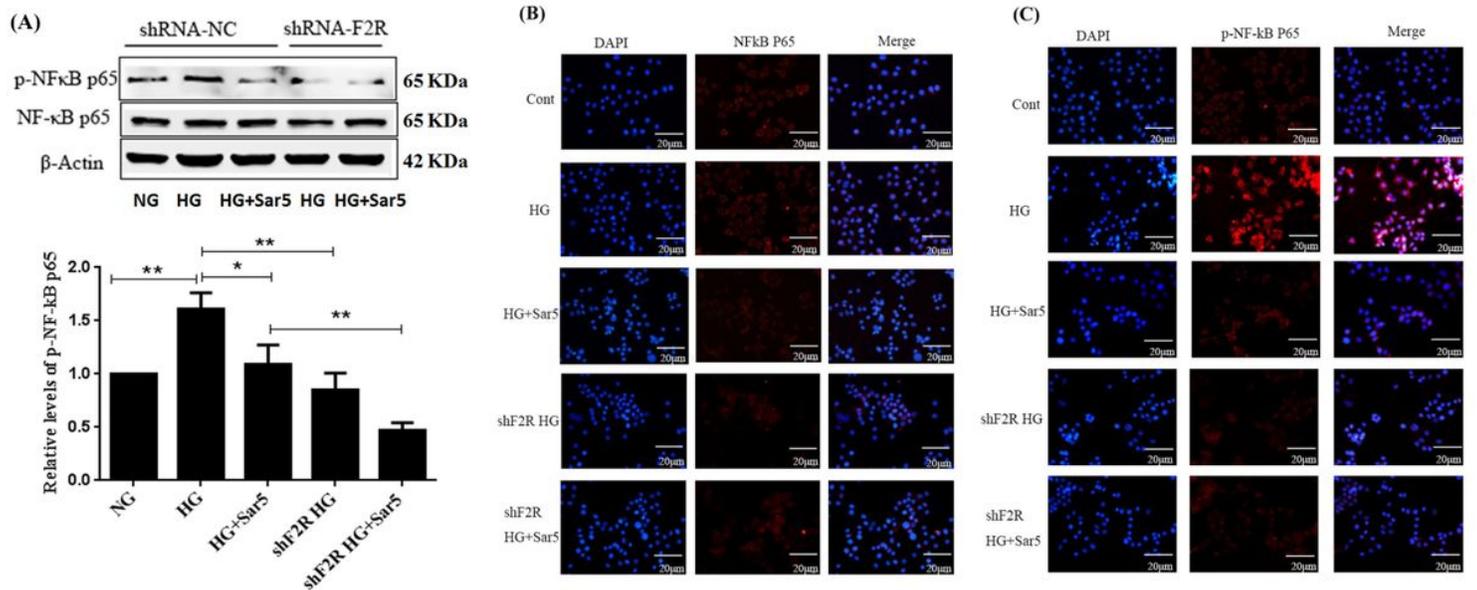
**Figure 8**

Effects of Sar on NLRP1 inflammasome in SH-SY5Y cultured with high glucose, (A) NLRP1 expression, (B) cleaved-caspase 1 expression, IL-1 $\beta$  levels (C), and (D) IL-18 levels. NG, HG, HG+Sar0.2, HG+Sar1, HG+Sar5, and HG+Vor: cells treated with 17.5 mmol/L glucose, 70 mmol/L glucose, 70 mmol/L glucose plus 0.2, 1, 5  $\mu$ mol/L Sar, or 1.0  $\mu$ mol/L vorapaxar (Vor, a selective PAR-1 antagonist), respectively. Mean  $\pm$  S.E.M, n=4 (NLRP1 and IL-18), n=5 (cleaved-caspase 1 and IL-1 $\beta$ ). \* P < 0.05, \*\* P < 0.01, compared with NG; #P < 0.05, ##P < 0.01, compared with HG.



**Figure 9**

Effects of PAR-1 knockdown on NLRP1 inflammasome, (A) NLRP1 expression, (B) IL-1β levels, and (C) IL-18 levels in SH-SY5Y cells treated with high glucose. HG, HG+Sar5, shF2R HG, shF2R HG+Sar5 represent cells infected with lentivirus containing F2R negative control or F2R shRNA (F2R, the gene symbol of PAR-1) subsequently cultured with HG or HG plus 5 μmol/L Sar, respectively. Mean ± S.E.M, n=7 (NLRP1 and IL-1β), n=5 (IL-18). \*P < 0.05, \*\*P < 0.01.



**Figure 10**

Effects of PAR-1 knockdown on p-NF-κB p65 by Western blot (A) as well as NF-κB p65 (B) and p-NF-κB p65 (C) by immunofluorescence in SH-SY5Y cells treated with high glucose (HG). HG, HG+Sar5, shF2R HG, shF2R HG+Sar5 represent cells infected with lentivirus containing F2R negative control or F2R shRNA

(F2R, the gene symbol of PAR-1) subsequently cultured with HG or HG plus 5  $\mu\text{mol/L}$  Sar, respectively. Mean  $\pm$  S.E.M, n=7. \*P < 0.05, \*\*P < 0.01. Scale bar: 20  $\mu\text{m}$ .

## Supplementary Files

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