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ORIGINAL PAPER

High Glucose Enhances Isoflurane-Induced Neurotoxicity by Regulating TRPC-Dependent Calcium Influx

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Abstract Isoflurane is a commonly used inhalational anesthetic that can induce neurotoxicity via elevating cytosolic calcium (Ca^{2+}). High glucose regulates the expression of a family of non-selective cation channels termed transient receptor potential canonical (TRPC) channels that may contribute to Ca^{2+} influx. In the present study, we investigated whether high glucose enhances isoflurane-induced neurotoxicity by regulating TRPC-dependent Ca^{2+} influx. First, we evaluated toxic damage in mice primary cultured hippocampal neurons and human neuroblastoma cells (SH-SY5Y cells) after hyperglycemia and isoflurane exposure. Next, we investigated cytosolic Ca^{2+} concentrations, TRPC mRNA expression levels and tested the effect of the TRPC channel blocker SKF96365 on cytosolic Ca^{2+} levels in cells treated with high glucose or/and isoflurane. Finally, we employed knocked down TRPC6 to demonstrate the role of TRPC in high glucose-mediated enhancement of isoflurane-induced neurotoxicity. The results showed that high glucose could enhance isoflurane-induecd toxic damage in primary hippocampal neurons and SH-SY5Y cells. High glucose enhanced the isoflurane-induced increase of cytosolic Ca^{2+} in SH-SY5Y cells. High glucose elevated TRPC mRNA expression, especially that of TRPC6. SKF96365 and knock down of TRPC6 were able to inhibit the high glucose-induced increase of cytosolic Ca^{2+} and decrease isoflurane-induced neurotoxicity in SH-SY5Y cells cultured with high glucose. Our findings indicate that high glucose could elevate TRPC expression, thus increasing Ca^{2+} influx and enhancing isoflurane-induced neurotoxicity.

Keywords Hyperglycemia · Isoflurane · Neurotoxicity · TRPC channels

Abbreviations

ER	Endoplasmic reticulum
LDH	Lactate dehydrogenase
POCD	Postoperative cognitive dysfunction
TRPC	Transient receptor potential canonical channels

Introduction

Diabetic patients have a high incidence of postoperative cognitive dysfunction (POCD), but the reason is unclear [1, 2]. Studies have shown that neurotoxic damage and neurodegeneration are responsible for POCD [3, 4]. Isoflurane and hyperglycemia both induce neurotoxic damage and have been identified as risk factors for cognitive impairment [5–7].

Intracellular calcium (Ca^{2+}) concentration plays a crucial role regulating many fundamental cellular processes such as neurosecretion, electrical signaling integration, neuronal excitability, synaptic plasticity, cell proliferation, and apoptosis [8, 9]. Accumulating evidence suggests that excessive elevation of intracellular Ca^{2+} is responsible for neurotoxic damage and neurodegeneration [10]. Current research has revealed the existence of damaging metabolic pathways downstream of high glucose to which neurons are particularly vulnerable. High glucose enhanced store-operated Ca^{2+} entry and increased expression of its signaling

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proteins, which elevated intracellular Ca^{2+} concentration [11]. Isoflurane, a widely used anesthetic in clinical practice, has been shown to induce apoptosis, inhibit neurogenesis, and cause learning and memory impairing, especially in young and old mice [12, 13]. Recent studies have suggested that general anesthetics, especially isoflurane, may cause cell death by disrupting intracellular Ca^{2+} homeostasis [14–16]. The endoplasmic reticulum (ER) is the main source of cytosolic Ca^{2+} in neurons and plays an important role in intracellular Ca^{2+} homeostasis, protein synthesis, cell survival, and caspase activation [8, 17–19]. There are two types of Ca^{2+}-release channels in the ER: inositol 1,4,5-triphosphate receptors (IP_3R) and ryanodine receptors (RyRs) [20]. Isoflurane has been shown to induce ER stress by activating both types of channels, leading to neurotoxic damage and cognitive impairment [7, 14, 21, 22].

Transient receptor potential canonical channels (TRPCs) are nonselective Ca^{2+}-permeable channels that can be activated by G-protein-coupled receptors and receptor tyrosine kinases [23]. These channels reportedly act as essential cellular sensors in multiple processes during neuronal development, including neural stem cell proliferation and differentiation, neuronal survival, neurite outgrowth, axon path finding, and synaptogenesis [24]. Seven mammalian TRPC proteins (TRPC1-7) have been discovered, but human TRPC2 is encoded by a pseudo gene [25]. Five TRPC subtypes (TRPC1, TRPC3, TRPC4, TRPC5, TRPC6) are more highly associated with central nervous system diseases [26]. TRPC protein can initiate Ca^{2+} entry pathways and are essential in maintaining cytosolic, ER, and mitochondrial Ca^{2+} levels [27]. Whether TRPCs were involved in isoflurane-induced the increase of cytosolic Ca^{2+} level remains unknown. Hyperglycemia can regulate TRPC expression, which increases cytosolic Ca^{2+} concentration and leads to cell damage [28, 29]. Based on the existing evidence, we hypothesize that hyperglycemia increases cytosolic Ca^{2+} level by regulating TRPC-dependent Ca^{2+} entry, which might enhance isoflurane-induced cytosolic Ca^{2+} overload and neurotoxicity.

Human dopaminergic neuroblastoma SH-SY5Y cells possess biological characteristics similar to normal neural cells and are routinely used to research neurotoxic damage and inhalational anesthetic neurotoxicity [30, 31]. In the central nervous system, the hippocampus is a critical region for learning and memory. Thus, assessing hippocampal neurons toxic damage will determine whether it correlates with and isoflurane-induced cognitive dysfunction [7]. In the present study, we employed mice primary cultured hippocampal neurons and SH-SY5Y cells to estimate whether hyperglycemia cloud enhance isoflurane-induced neurotoxicity. Our findings begin to clarify the molecular mechanisms of hyperglycemia-aggravated neurotoxicity in diabetic patients treated with isoflurane.

Materials and Methods

Materials

The SH-SY5Y cell line was purchased from the Shanghai Institutes for Biological Sciences (Shanghai, China). Isoflurane (purity 99.9%) was purchased from Abbott Laboratories (Shanghai, China). Glucose (purity 99.5%) and SKF96365 were purchased from Sigma (St. Louis, MO). Other reagents used included Dulbecco's modified eagle medium (DMEM)/F12 and fetal bovine serum (FBS) from Gibco (Grand Island, NY), Quest Fluo-8 AM ester from AAT Bioquest Inc. (Sunnyvale, CA), LDH cytotoxicity detection kits from Roche (Indianapolis, IN), antibodies against caspase-9 (Cell Signaling Technology, Danvers, MA), anti bodies against TRPC6 (Abcam, Cambridge, UK), and β-actin (KangChen Bio-tech, Shanghai, China) and annexinV-fluorescein isothiocyanate (FITC),propidium iodide (KeyGEN, Nanjing, China). All reagents were obtained from commercial suppliers and were of standard biochemical quality.

Primary Hippocampal Neuron Culture

Newborn C57BL/6 mice, 24 h old, were purchased from the Southern Medical University (Guangzhou, China). Hippocampi were dissected from the brain on ice and minced in sterile ice-cold D-Hanks' with the blood vessels and meninges carefully removed. The tissues were digested with 0.25% trypsin for 15 min at 37 °C and then the digestion procedure was stopped by adding 5 ml FBS (Gibco, USA). The neurons were centrifuged and suspended to a density of 1×10^6/L in DMEM (HyClone, USA) with 10% FBS in it. The different volumes of neuronal suspensions were inoculated in culture flasks and coated with L-poly lysine (Sigma, USA) and cultured in a humidified 5% CO_2 atmosphere at 37 °C. When the neurons adhered, the medium was changed to neurobasal medium (Gibco, USA). The neurons were then plated on poly-D-lysine (Sigma, USA) coated-glass coverslips, 96-well plates or 24-well plates at a density of 5×10^5 cell/ml after determining the cell density using a hemacytometer. The culture neurons were used for in vitro studies at day 7. For determining the toxic damage of high glucose, neurons were treated with 30 or 50 mM glucose for 1, 2, or 4 days. For determining the toxic damage of isoflurane, They were incubated with 1 or 3% isoflurane plus 21% O_2 and 5% CO_2 for 3, 6, or 12 h. For determining the effect of high glucose on isoflurane-induced neurotoxicity, neurons were cultured with 50 mM glucose for 4 days and simultaneously treated with 3% isoflurane for 6 h on the 4th day.

SH-SY5Y Cell Culture

SH-SY5Y cells were maintained at 37 °C in 5% CO_2 in DMEM/F12 medium supplemented with 10% FBS and 1% penicillin/streptomycin. The culture medium was replaced daily during cell growth. Cells were grown in 100-mm dishes and sub-cultured in 6-well (seeding density 5.0×10^5 cells) or 12-well (seeding density 1.0×10^5 cells) dishes. Experiments were conducted when cells reached 85% confluence.

For determining the toxic damage of high glucose, neurons were treated with 30 or 50 mM glucose for 1, 2, or 4 days. For determining the toxic damage of isoflurane, They were incubated with 1 or 3% isoflurane plus 21% O_2 and 5% CO_2 for 3, 6, or 12 h. For determining For determining the effect of high glucose on isoflurane-induced neurotoxicity, cells were cultured with 50 mM glucose for 4 days and simultaneously treated with 3% isoflurane for 6 h on the 4th day.

Isoflurane Concentration of Medium

An anesthesia machine was used to deliver isoflurane to a sealed plastic box in a 37 °C incubator. Actual isoflurane concentration of medium was measured by gas chromatograph as previously described [32]. According to the analysis results, 1% isoflurane was equal to 0.76 mM isoflurane in medium and 3% isoflurane was equal to 2.07 mM isoflurane in medium. Control cells with non-isoflurane treatment were incubated with 5% CO_2 in a 37 °C incubator. We used a Datex infrared gas analyzer (Puritan-Bennett, Tewksbury, MA) to continuously monitor the delivered concentrations of CO_2, O_2, and isoflurane.

Knock-Down of TRPC6

Small interfering RNA (siRNA) was transfected to knock down expression of TRPC6 (Santa CruzBio-technology). SH-SY5Y cells were mixed with TRPC6 siRNA (GCA GCAUCAUUCAUUGCAAGAUUUA) or a control sequence (GCAACUAACUUCGUUAGAAUCGUUA). For each transfection, 4 µl of siRNA duplex which gave final concentration of 80 nM siRNA in 100 µl siRNA transfection medium and 6 µl of siRNA transfection reagent in 100 µl siRNA transfection medium were mixed and incubated for 45 min at room temperature. For each transfection, 0.8 ml siRNA transfection medium was added to each tube, mixed gently, overlaid onto washed cells and incubated for 5–7 h at 37 °C in a CO_2 incubator. After incubation, 1 ml of normal growth medium containing twice the normal serum and antibiotic concentration (2×normal growth medium) was added without removing the transfection mixture. Cells were incubated with siRNA for 48 h before analysis.

Lactate Dehydrogenase (LDH) Release Determination

LDH release (a measure of cell injury) in culture medium was detected with a commercial kit according to the manual instructions as previously described [33]. LDH release from damaged cells was assayed by measuring absorbance at 490 nm.

Measurements of Cytosolic Ca^{2+}

Cytosolic Ca^{2+} ($[Ca^{2+}]i$) was measured with Quest Fluo-8 AM ester. Briefly, a 5 mM stock solution was prepared in high-quality anhydrous DMSO, and a 10 mM working solution was prepared in Hanks and HEPES buffer (HHBS). Cells were incubated with 5 µM Quest Fluo-8 AM ester for 20 min at room temperature and then washed twice in HHBS to remove excess probe. The experiments were analyzed at excitation and emission wavelengths of 490 and 525 nm, respectively. To determine either the free Ca^{2+} concentration in the solution ($[Ca^{2+}]i$) or the dissociation constant (K_d) of a single wave length Ca^{2+} indicator, the following equation was used: $[Ca^{2+}]i = K_d[F - F_{min}]/[F_{max} - F]$. F is the fluorescence of the indicator at experimental Ca^{2+} levels, F_{min} is the fluorescence in the absence of Ca^{2+}, and F_{max} is the fluorescence of the Ca^{2+}-saturated probe. K_d is a measure of the affinity of the probe for Ca^{2+} and is provided in the kit manual. The fluorescence intensities of Fluo-8 in SH-SY5Y cells were recorded using a confocal scanning laser microscope (CSLM) (FV300; Olympus, Tokyo, Japan).

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

TRPC mRNA levels were measured by qRT-PCR. Total RNA was extracted from SH-SY5Y cells using TRIzol (Invitrogen, Carlsbad, CA). cDNA was synthesized from 2 µg total RNA using PrimeScript® RT Master Mix (Takara, Otsu, Japan), and qRT-PCR was performed on a Lightcycler 480 (Applied Biosystems, Foster City, CA) using the SYBR Green Master Mix Kit (Takara). Relative amounts of TRPC mRNA subtypeswere quantified using the $2^{-\Delta\Delta C_T}$ method [34]. qRT-PCR was performed on an ABI Prism 7500 sequence detector (Applied Biosystems). The primers were human TRPC subtypes and β-actin. TRPC1-forward: 5′-GGACTGTGTAGGCATCTTCTG-3′, reverse: 5′-CAA TGACAGCTCCCACAAAG-3′;TRPC3-forward:5′-AGC ACATGCAGCTTCTTTC-3′, reverse: 5′-TCCATGTAA ACTGGGTGGTT-3′; TRPC4-forward: 5′-CGAAGGTAA

TAGCAAGGACAAG-3′, reverse: 5′-GCAGAGCCATTG CTTATGTT-3′; TRPC5-forward: 5′-AGCCTGTTCCAG CTCTCTTC-3′, reverse: 5′-GAGGCGAGTTGTAACTTG TTC-3′; TRPC6-forward: 5′-AATTGAGGATGACGCTGA TGTG-3′, reverse: 5′-GACTCGGCACCAGATTGAAG-3′; and β-actin-forward: 5′-TGGATCAGCAAGCAGGAG TA-3′, reverse: 5′-TCGGCCACATTGTGAACTTT-3′.

Western Blot

Total proteins were harvested from cells with lysis buffer after incubation as described. After centrifugation, protein concentrations were determined with a bicinchoninic acid (BCA) protein assay kit. Equal amounts of protein extracts were separated by 10% SDS-PAGE and transferred to PVDF membranes (Immobilon-P, Millipore, Bedford, MA, USA), and blocked with 5% nonfat dry milk in Tris-buffered saline. They were then immunoblotted with caspase-9 (1:500), TRPC6 (1:500) or β-actin antibody (1:1000), diluted in blocking solution containing 5% nonfat dry milk and 0.1% Tween-20 in Tris-HCl-buffered saline overnight at 4 °C. After they were rinsed, membranes were incubated with horseradish peroxidase-conjugated anti-rabbit immunoglobulin at 1:1000 for 1 h. Finally, Those blots were further incubated with HRP-conjugated secondary antibody, developed in ECL solution, and exposed onto hyperfilm (Amersham Biosciences) for 1–10 min. The optical densities (ODs) of individual bands were quantified using the Chemi-Imager digital imaging system (Alpha Innotech, San Leandro, CA) and Quantity One analysis software (Bio-Rad, Hercules, CA). Cleaved caspase-9 protein expression levels were normalized to corresponding β-actin bands.

Flow Cytometry Apoptosis Assays

Cells were seeded onto 24-well plates at 5×10^5 cells/well in 500 µl culture medium. Cells were rinsed with PBS, harvested, and resuspended in 500 µl binding buffer. To this cell suspension, we added 5 µl annexinV-FITC (a marker of early apoptosis) and 5 µl propidium iodide (a marker of late apoptosis). After a 10-min incubation, cell apoptosis was determined by flow cytometry (BD FACS Calibur, BD Biosciences, Franklin Lakes, NJ).

Statistical Analysis

Data are presented as means ± standard error of the means (SEMs). Comparisons between two groups were performed using independent-sample *t* tests, and multiple comparisons among groups were performed by one-way ANOVA or two-way using SPSS software 13.0 (SPSS Inc., Chicago, IL). All reported *P* values and confidence intervals are Tukey corrected. Statistical significance was set at $P<0.05$.

Results

Hyperglycemia Enhanced Isoflurane-Induced Toxic Damage in Primary Cultured Hippocampal Neurons

Primary hippocampal neurons were treated with 30 mM glucose for 1, 2, or 4 days, compared to control group, high glucose induced LDH production in time-dependent manners (1.77 ± 0.20 vs. 1.00 ± 0.07, 2.78 ± 0.17 vs. 1.00 ± 0.15, 3.26 ± 0.21 vs. 1.00 ± 0.12, $P<0.05$). Primary hippocampal neurons were treated with 50 mM glucose for 1, 2, or 4 days, compared to control group, high glucose induced LDH production in time-dependent manners (2.98 ± 0.19 vs. 1.00 ± 0.07, 4.77 ± 0.27 vs. 1.00 ± 0.15, 5.29 ± 0.20 vs. 1.00 ± 0.12, $P<0.05$). Compared to 30 mM glucose group, 50 mM glucose induced LDH production in concentration-dependent manners (2.98 ± 0.19 vs. 1.77 ± 0.07, 4.77 ± 0.27 vs. 2.78 ± 0.17, 5.29 ± 0.20 vs. 3.26 ± 0.27, $P<0.05$). Notably, the increases of LDH production were significant in cells treated with 30 mM glucose for 4 days.

Primary hippocampal neurons were treated with 1% isoflurane for 3, 6, or 12 h, compared to control group, isoflurane induced LDH production in time-dependent manners (1.72 ± 0.18 vs. 1.00 ± 0.07, 2.24 ± 0.19 vs. 1.00 ± 0.10, 2.39 ± 0.15 vs. 1.00 ± 0.08, $P<0.05$). Primary hippocampal neurons were treated with 3% isoflurane for 3, 6, or 12 h, compared to control group, isoflurane induced LDH production in time-dependent manners (2.74 ± 0.19 vs. 1.00 ± 0.08, 3.14 ± 0.21 vs. 1.00 ± 0.09, 3.99 ± 0.17 vs. 1.00 ± 0.09, $P<0.05$). Compared to 1% isoflurane group, 3% isoflurane induced LDH production in concentration-dependent manners (2.74 ± 0.19 vs. 1.72 ± 0.18, 3.14 ± 0.21 vs. 2.24 ± 0.19, 3.99 ± 0.17 vs. 2.39 ± 0.15, $P<0.05$). We found that the increases of LDH production were significant in cells treated with 3% isoflurane for 6 h.

Primary hippocampal neurons were cultured with or without 30 mM glucose for 4 days before treatment with 3% isoflurane for 6 h. Neurons damage was measured by LDH assays, and apoptotic neurons were detected with cleaved caspase-9 expression. Compared to control group, high glucose and isoflurane caused neurons significant toxic damage (3.72 ± 0.25, 3.02 ± 0.10 vs. 1.00 ± 0.09, $P<0.05$) and apoptosis (2.52 ± 0.25, 2.98 ± 0.20 vs. 1.00 ± 0.09, $P<0.05$). Compared to isoflurane group, high glucose pretreatment significantly enhanced the isoflurane-induced increases of LDH

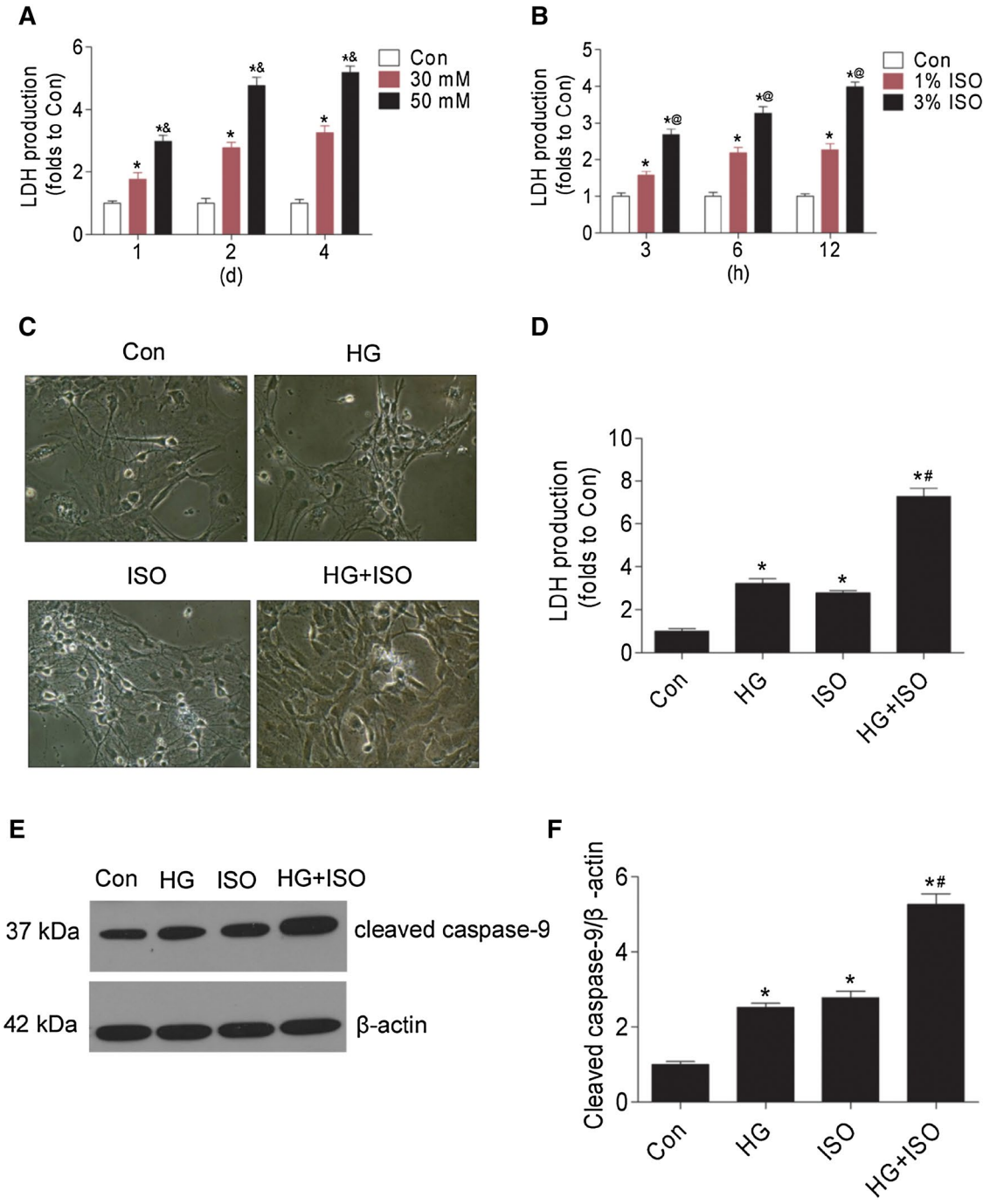

Fig. 1 High glucose enhanced isoflurane-induced toxic damage of primary hippocampal neurons. **a** Hippocampal neurons were treated with 30 or 50 mM glucose for 1, 2, or 4 days. **b** Hippocampal neurons were treated with 1 or 3% ISO for 3, 6, and 12 h; LDH production was measured with an LDH assay. $*P<0.05$ vs. Con, $\&P<0.05$ vs. 30 mM, $@P<0.05$ vs. 1% ISO, one-way ANOVA with Tukey correction. **c** Representative microscopic photographs show the morphology of hippocampal neurons treated with high glucose or/and isoflurane. Sections were pictured at 200× power. **d** LDH production. **e, f** Cleaved caspase-9 protein expression was detected by western blot. *Con* untreated control cells, *HG* cells cultured with 30 mM glucose for 4 days, *ISO* cells treated with 3% ISO for 6 h, *HG+ISO* cells cultured with 30 mM glucose for 4 days then treated with 3% ISO for 6 h. Values are the mean ± SEM of n = 3; $*P<0.05$ vs. Con, $\&P<0.05$ vs. 30 mM, $@P<0.05$ vs. 1% ISO, $\#P<0.05$ vs. ISO, two-way ANOVA and independent-sample *t* tests between two groups with Tukey correction

production (7.89 ± 0.32 vs. 3.02 ± 0.10, $P < 0.05$) and cleaved caspase-9 expression (5.59 ± 0.37 vs. 2.98 ± 0.20, $P < 0.05$) (Fig. 1).

Hyperglycemia Enhances $[Ca^{2+}]i$ Increase and Isoflurane-Induced Damage in SH-SY5Y Cells

SH-SY5Y Cells were treated with 1 or 3% isoflurane for 3, 6, or 12 h before we measured $[Ca^{2+}]i$ concentration and LDH production. Compared to control group, $[Ca^{2+}]i$ concentration (nM) of cells treated with 1% isoflurane for 6 and 12 h was elevated (802.12 ± 72.18 vs. 596.12 ± 51.10, 880.24 ± 85.19 vs. 521.09 ± 49.10, $P < 0.05$), LDH production of cells treated with 1% isoflurane for 12 h was elevated (1.89 ± 0.12 vs. 1.00 ± 0.08, $P < 0.05$). Compared to control group, $[Ca^{2+}]i$ concentration (nM) of cells treated with 3% isoflurane for 3, 6 or 12 h was elevated (882.22 ± 79.11 vs. 552.15 ± 48.06, 1482.52 ± 101.18 vs. 596.12 ± 51.10, 1780.74 ± 125.10 vs. 521.09 ± 49.10, $P < 0.05$), LDH production of cells treated with 3% isoflurane for 3, 6 or 12 h was elevated (1.87 ± 0.10 vs. 1.00 ± 0.09, 3.19 ± 0.21 vs. 1.00 ± 0.06, 7.82 ± 0.37 vs. 1.00 ± 0.09, $P < 0.05$).

Cells were treated with 30 or 50 mM glucose for 1, 2, or 4 days before assaying $[Ca^{2+}]i$ concentration and LDH production. Compared to control group, $[Ca^{2+}]i$ concentration (nM) of cells treated with 30 mM glucose for 2 or 4 days was elevated (892.12 ± 75.10 vs. 576.05 ± 48.10, 1050.44 ± 92.79 vs. 561.11 ± 47.12, $P < 0.05$), LDH production of cells treated with 30 mM glucose for 2 or 4 days was elevated (2.79 ± 0.17 vs. 1.00 ± 0.09, 2.92 ± 0.20 vs. 1.00 ± 0.05, $P < 0.05$). Compared to control group, $[Ca^{2+}]i$ concentration (nM) of cells treated with 50 mM glucose 1, 2 or 4 d was elevated (912.42 ± 89.15 vs. 602.15 ± 48.06, 1282.02 ± 121.58 vs. 576.05 ± 48.10, 1610.24 ± 115.10 vs. 561.11 ± 47.12, $P < 0.05$), LDH production of cells treated with 50 mM glucose for 1, 2 or 4 days was elevated (2.87 ± 0.12 vs. 1.00 ± 0.06, 4.59 ± 0.30 vs. 1.00 ± 0.06, 5.22 ± 0.27 vs. 1.00 ± 0.05, $P < 0.05$) (Fig. 2).

Cells were cultured with or without 50 mM glucose for 4 days before treatment with 3% isoflurane for 6 h. Comapred to control group, either high glucose or isoflurane increased $[Ca^{2+}]i$ concentration (nM) (1601.25 ± 99.48, 1705.61 ± 128.76 vs. 552.56 ± 79.45, $P < 0.05$) and LDH production (3.12 ± 0.42, 3.59 ± 0.21 vs. 1.00 ± 0.23, $P < 0.05$). Compared to isoflurane group, high glucose pretreatment significantly enhanced the isoflurane-induced increases of $[Ca^{2+}]i$ concentration (nM) (3676.54 ± 198.97 vs. 1705.61 ± 128.76, $P < 0.05$) and LDH production (7.96 ± 0.37 vs. 3.59 ± 0.21, $P < 0.05$) (Fig. 3).

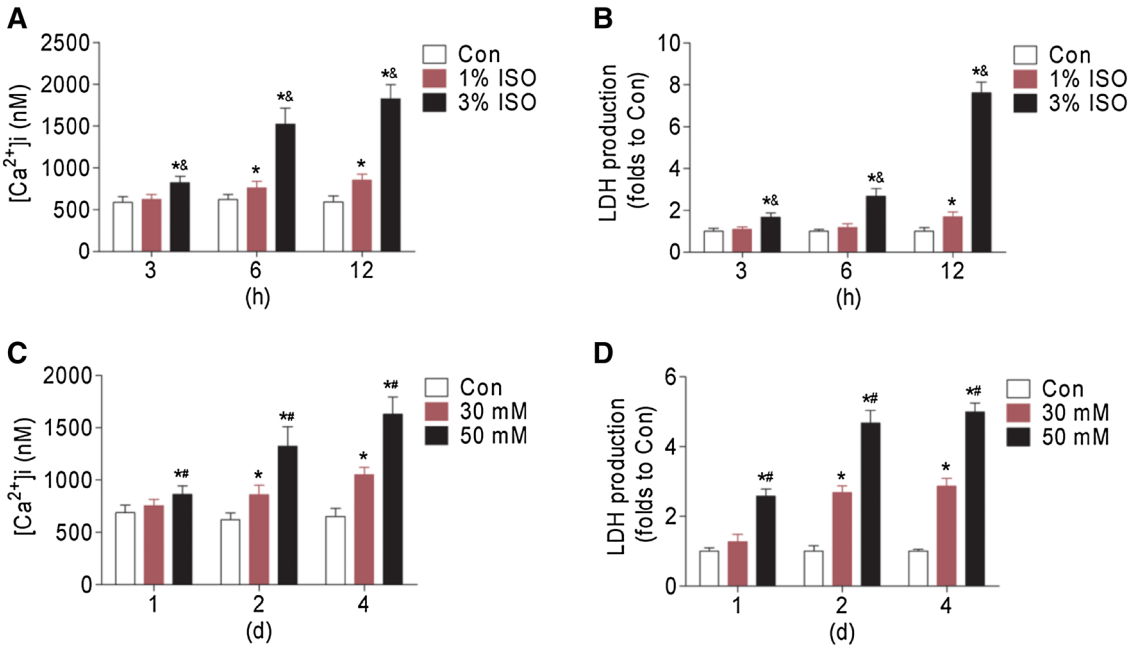

Fig. 2 Isoflurane (ISO) or high glucose treatment led to an increase of cytosolic Ca^{2+} ($[Ca^{2+}]i$) and LDH production. SH-SY5Y cells were either treated with 1 or 3% ISO for 3, 6, and 12 h or cultured with 30 or 50 mM glucose for 1, 2, or 4 days. $[Ca^{2+}]i$ levels were measured by Quest Fluo-8 AM ester flow cytometry. LDH production was measured with an LDH assay. **a, b** ISO-induced $[Ca^{2+}]i$ levels and LDH production. **c, d** Hyperglycemia-induced $[Ca^{2+}]i$ levels and LDH production. Values are the mean ± SEM of n = 6; *$P < 0.05$ vs. untreated control; &$P < 0.05$ vs. 1% ISO; #$P < 0.05$ vs. 30 mM, one-way ANOVA with Tukey correction

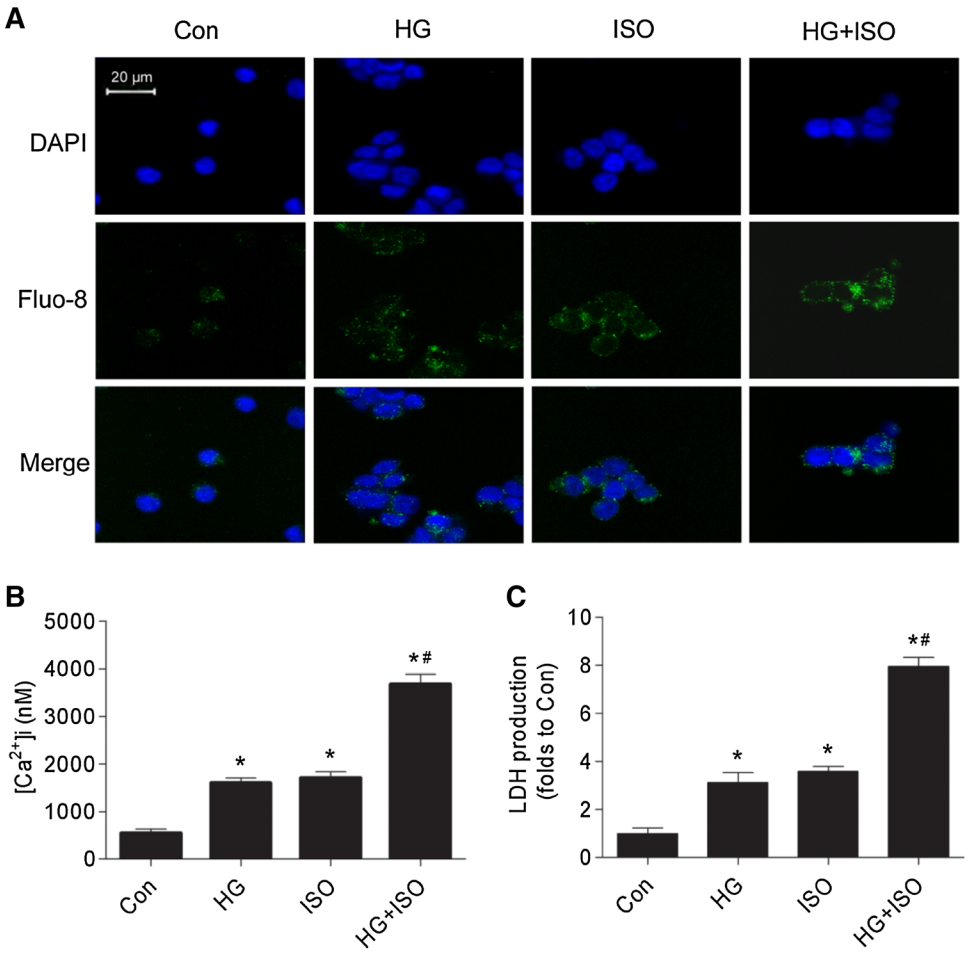

Fig. 3 High glucose enhanced isoflurane (ISO) induced cytosolic Ca^{2+} ($[Ca^{2+}]i$) and LDH production. *Con* untreated control cells, *HG* cells cultured with 50 mM glucose for 4 days, *ISO* cells treated with 3% ISO for 6 h, *HG + ISO* cells cultured with 50 mM glucose for 4 days then treated with 3% ISO for 6 h. **a** Representative confocal scanning laser microscopy images. **b** [Ca2+]i levels measured by Quest Fluo-8 AM ester flow cytometry. **c** LDH production. Values are the mean ± SEM of n = 3; $*P < 0.05$ vs. Con; $\#P < 0.05$ vs. ISO, two-way ANOVA among groups and independent-sample *t* tests between two groups with Tukey correction

SKF96365 Inhibits Hyperglycemia-Mediated Enhancement of Isoflurane-Induced Increase of $[Ca^{2+}]i$ and Neurotoxicity in SH-SY5Y Cells

Cells were cultured with 50 mM glucose for 4 days before TRPC mRNA expression levels were quantified by qRT-PCR. Compared to control group, high glucose could elevate TRPC1, TRPC5, and TRPC6 mRNA levels, with the most significant increase in TRPC6 (1.59 ± 0.09 vs. 1.00 ± 0.20, 2.25 ± 0.30 vs. 1.00 ± 0.13, 7.76 ± 0.85 vs. 1.00 ± 0.22, $P < 0.05$). Cells were treated with 3% isoflurane for 6 h before TRPC mRNA expression levels were quantified by qRT-PCR. Compared to control group, isoflurane could elevate TRPC1, TRPC3, TRPC4, and TRPC5 mRNA levels (2.59 ± 0.08 vs. 1.00 ± 0.18, 6.33 ± 0.16 vs. 1.00 ± 0.15, 5.02 ± 0.20 vs. 1.00 ± 0.12, 2.65 ± 0.17 vs. 1.00 ± 0.10, $P < 0.05$). Compared to cells treated with isoflurane, not TRPC1, TRPC3, TRPC4, TRPC5, only TRPC6 mRNA expression was elevated significantly in cells treated with high glucose and isoflurane (3.26 ± 0.18 vs. 1.16 ± 0.11, $P < 0.05$). To determine the effect of TRPC on the hyperglycemia- or isoflurane-induced increase of $[Ca^{2+}]i$, we treated cells with different concentrations of the nonselective TRPC inhibitor SKF96365. Compared to 50 mM glucose group, 10, 20 or 40 μM SKF96365 could inhibit high glucose-induced increase of $[Ca^{2+}]i$ concentration (nM) (1257.45 ± 98.76, 985.23 ± 104.16, 807.46 ± 88.56 vs. 1678.26 ± 102.39, $P < 0.05$). Compared to isoflurane group, 10, 20 or 40 μM SKF96365 could inhibit isoflurane-induced increase of $[Ca^{2+}]i$ concentration (nM) (1707.25 ± 97.06, 1581.62 ± 99.11, 1387.16 ± 91.51 vs. 1916.35 ± 105.19, $P < 0.05$). The results suggested that high glucose and isoflurane increased $[Ca^{2+}]i$ via TRPC-dependent Ca^{2+} influx (Fig. 4).

After pretreatment with 40 μM SKF96365 for 30 min, cells were cultured with or without 50 mM glucose for 4 days and incubated with 3% isoflurane for 6 h. Compared to cells treated with high glucose and isoflurane group, SKF96365 could inhibit the hyperglycemia-mediated enhancement of the isoflurane-induced increase in $[Ca^{2+}]i$ (nM) (2287.76 ± 107.51 vs. 1396.85 ± 118.19, $P < 0.05$) (Fig. 5a, b).

Cell damage was measured by LDH assays, and apoptotic cells were detected with cleaved caspase-9 expression and flow cytometry. The results showed that SKF96365 could inhibit the high glucose-mediated enhancement

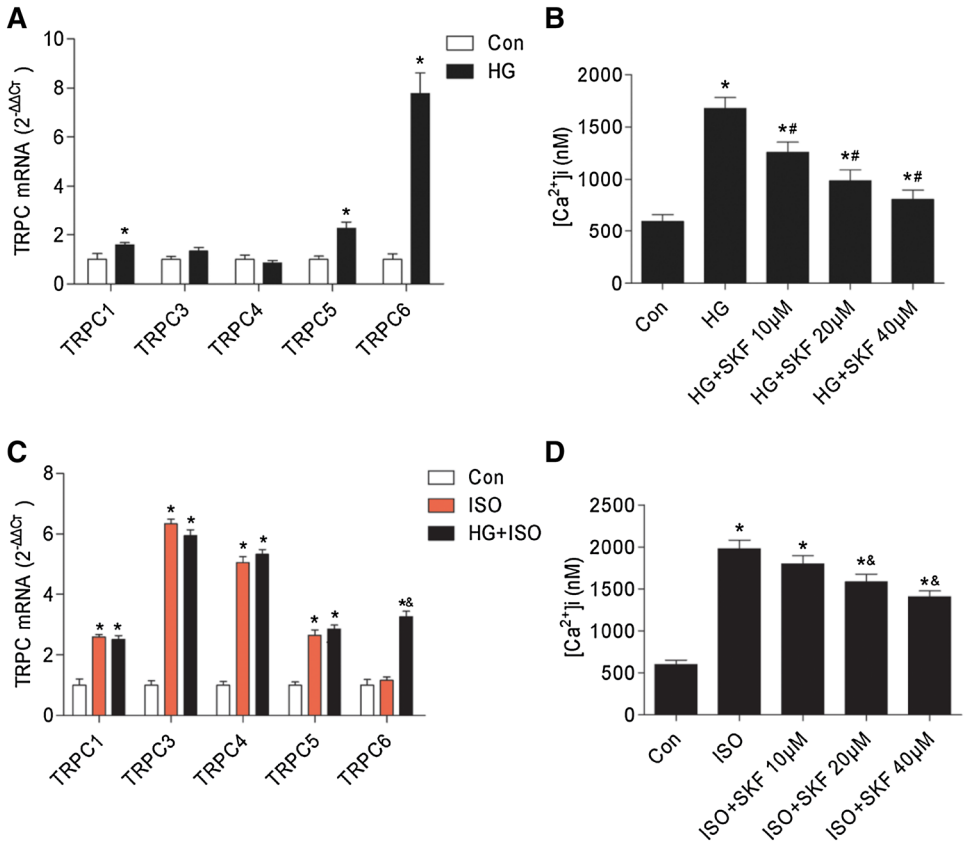

Fig. 4 High glucose and isoflurane increased TRPC mRNA expression, and SKF96365 inhibited the hyperglycemia- or isoflurane-induced increase of [Ca2+]i. **a** SH-SY5Y cells were cultured with 50 mM glucose for 4 days. TRPC mRNA subtypes were quantified by qRT-PCR. **b** SH-SY5Y cells were pretreated with 10, 20, or 40 μM SKF96365 for 30 min and then cultured with 50 mM glucose for 4 days. **c** *ISO* cells treated with 3% ISO for 6 h, *HG*+*ISO* cells treated with 50 mM glucose for 4 days then treated with 3% ISO for 6 h; TRPC mRNA subtypes were quantified by qRT-PCR. **d** SH-SY5Y cells were pretreated with 10, 20, or 40 μM SKF96365 for 30 min and then cultured with 3% ISO for 6 h. [Ca2+]i levels were measured with Quest Fluo-8 AM ester flow cytometry. Values are the mean ± SEM of n = 3; **P* < 0.05 vs. Con; #*P* < 0.05 vs. HG; &*P* < 0.05 vs. ISO, one-way ANOVA with Tukey correction

of isoflurane-induced LDH production (3.56 ± 0.19 vs.6.75 ± 0.29, $P < 0.05$), cleaved caspase-9 expression (1.95 ± 0.12 vs. 2.98 ± 0.20, $P < 0.05$) and the increase of apoptotic cells (%) (28.76 ± 5.12 vs. 49.86 ± 6.42, $P < 0.05$) (Figs. 5c, 6).

Knock-Down of TRPC6 Inhibits Hyperglycemia-Mediated Enhancement of Isoflurane-Induced Increase of $[Ca^{2+}]i$ and Neurotoxicity in SH-SY5Y Cells

SH-SY5Y cells were transfected with TRPC6 siRNA or negative control siRNA. qRT-PCR and western bolting were used to determined mRNA and protein expression of TRPC6. The results showed that mRNA and protein expression of TRPC6 were significantly decreased in cells transfected with siRNA compared to cells transfected with negative control siRNA (0.29 ± 0.07 vs. 1.09 ± 0.10, 0.36 ± 0.09 vs. 0.92 ± 0.11, $P < 0.05$). Compared to control, the mRNA and protein expression of TRPC6 in cells transfected with negative control siRNA were not significantly different. (Fig. 7).

After transfected with TRPC6 siRNA or negative control siRNA, cells were cultured with or without 50 mM glucose for 4 days and incubated with 3% isoflurane for 6 h. Compared to cells treated with high glucose, siRNA could inhibit the hyperglycemia-induced increase of TRPC6 expression (1.18 ± 0.10 vs. 1.98 ± 0.11, $P < 0.05$), the increase of $[Ca^{2+}]i$ (nM) (1009.46 ± 87.50 vs. 1297.05 ± 98.12, $P < 0.05$), LDH production (1.97 ± 0.08 vs.3.05 ± 0.09, $P < 0.05$), cleaved caspase-9 expression (1.15 ± 0.08 vs. 1.88 ± 0.10, $P < 0.05$) and the increase of apoptotic cells (11.06 ± 2.12 vs. 18.86 ± 3.52, $P < 0.05$). Compared to cells treated with high glucose and isoflurane, Knock-down of TRPC6 inhibits could inhibit the hyperglycemia-mediated enhancement of the isoflurane-induced increase of TRPC6 expression (2.01 ± 0.12 vs. 3.35 ± 0.15, $P < 0.05$), the increase of $[Ca^{2+}]i$ (nM) (1879.16 ± 107.42 vs. 2797.07 ± 102.19, $P < 0.05$), LDH production (3.47 ± 0.10 vs. 6.25 ± 0.15, $P < 0.05$), cleaved caspase-9 expression (2.16 ± 0.09 vs. 3.08 ± 0.16, $P < 0.05$) and the increase of apoptotic cells (%) (19.46 ± 5.12 vs. 28.87 ± 4.52, $P < 0.05$). (Figs. 8, 9).

Discussion

There are three main findings of the present study. First, either hyperglycemia or isoflurane increased $[Ca^{2+}]i$ and induced neurotoxic damage. Second, either hyperglycemia or isoflurane increased $[Ca^{2+}]i$ by regulating TRPC-dependent Ca^{2+} entry. Third, blocking or knockdown

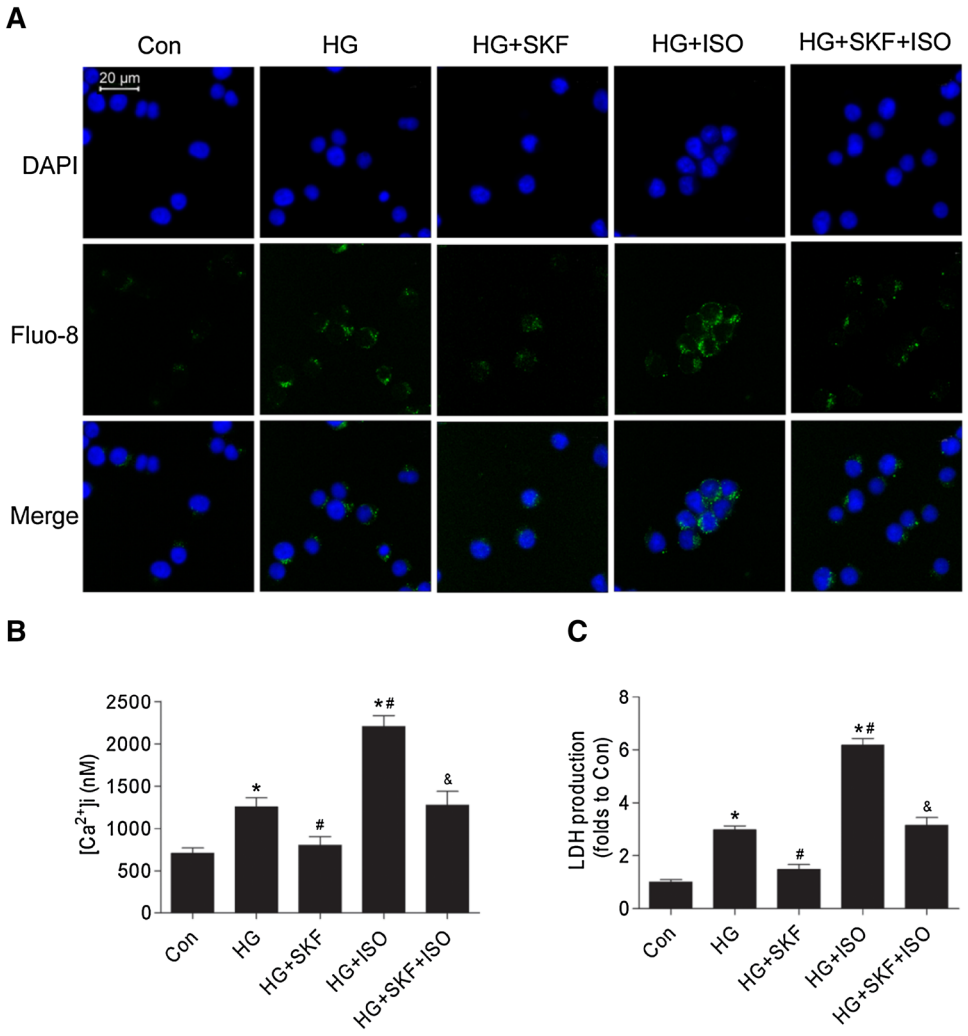

Fig. 5 SKF96365 inhibited the increase of cytosolic Ca^{2+}([Ca^{2+}]i) and damage in SH-SY5Y cells treated with high glucose (HG) and isoflurane. *Con* untreated cells, *HG* cells cultured with 50 mM glucose for 4 days, *HG + SKF* cells pretreated with 40 μM SKF96365 for 30 min and then cultured with 50 mM glucose for 4 days, *HG + ISO* cells cultured with 50 mM glucose for 4 days and then treated with 3% isoflurane (ISO) for 6 h, *HG + SKF + ISO* cells were treated with 3% ISO for 6 h after pretreated with 40 μM SKF96365 for 30 min and treatment with 50 mM glucose for 4 days. **a** Representative confocal scanning laser microscopy image. **b** [Ca2+]i levels measured with Quest Fluo-8 AM ester flow cytometry. **c** LDH production. Values are the mean ± SEM of n = 3; *$P < 0.05$ vs. Con; #$P < 0.05$ vs. HG; &$P < 0.05$ vs. HG + ISO, one-way ANOVA with Tukey correction

TRPC6 inhibited the hyperglycemia-mediated enhancement of isoflurane-induced cytosolic Ca^{2+} overload and toxic damage. Collectively, our findings indicate that hyperglycemia enhances isoflurane-induced neurotoxicity by affecting TRPC-dependent Ca^{2+} influx.

Diabetes is associated with damage of the central nervous system, being linked with development of cognitive and memory impairments [35]. An extensive body of literature supports the link between hyperglycemia and neurotoxicity for central nervous system damage [36–38]. Hyperglycemia induces cell toxic damage by altering Ca^{2+} homoeostasis [39]. A previous study reported that isoflurane can reversibly increase intracellular Ca^{2+} ([Ca^{2+}]i) in isolated hippocampal neurons [40]. This increase in [Ca^{2+}]i is primarily caused by isoflurane-induced ER stress via IP_3R or RyR activation [6, 7, 14]. Longer exposure to isoflurane producing extensive and prolonged dysfunction of Ca^{2+} homoeostasis may inhibit protein synthesis, ultimately inducing cytotoxicity. An elevated cytosolic Ca^{2+} level can induce apoptosis by causing an overload of mitochondrial Ca^{2+}, resulting in mitochondrial membrane potential collapse and subsequent release of cytochrome C into the cytosolic space, activation of caspase-9 and -3, and subsequent apoptosis. At the same time, it can activate apoptotic-related enzymes such as calpain [41, 42]. Research demonstrated diabetic patients have a high incidence of POCD [1].A previous study reported that isoflurane itself could impact glucose regulation and trigger a hyperglycemic response because it impairs glucose clearance and increases glucose production [43]. The effect of hyperglycemia on isoflurane-induced neurotoxicity and disruption of Ca^{2+} homoeostasis remains unknown. In the present study, we first treated primary hippocampal neurons with isoflurane and glucose to determine the effect of high glucose on anesthetic's neurotoxic damage. The results showed that hyperglycemia could enhance isoflurane-induced neurotoxicity. Next, we treated SH-SY5Y cells with 1 or 3% isoflurane for 3, 6, or 12 h to determine the anesthetic's effect on [Ca^{2+}]i and neurotoxic damage. The results showed that isoflurane increased [Ca^{2+}]i and induced neurotoxic damage in a time- and

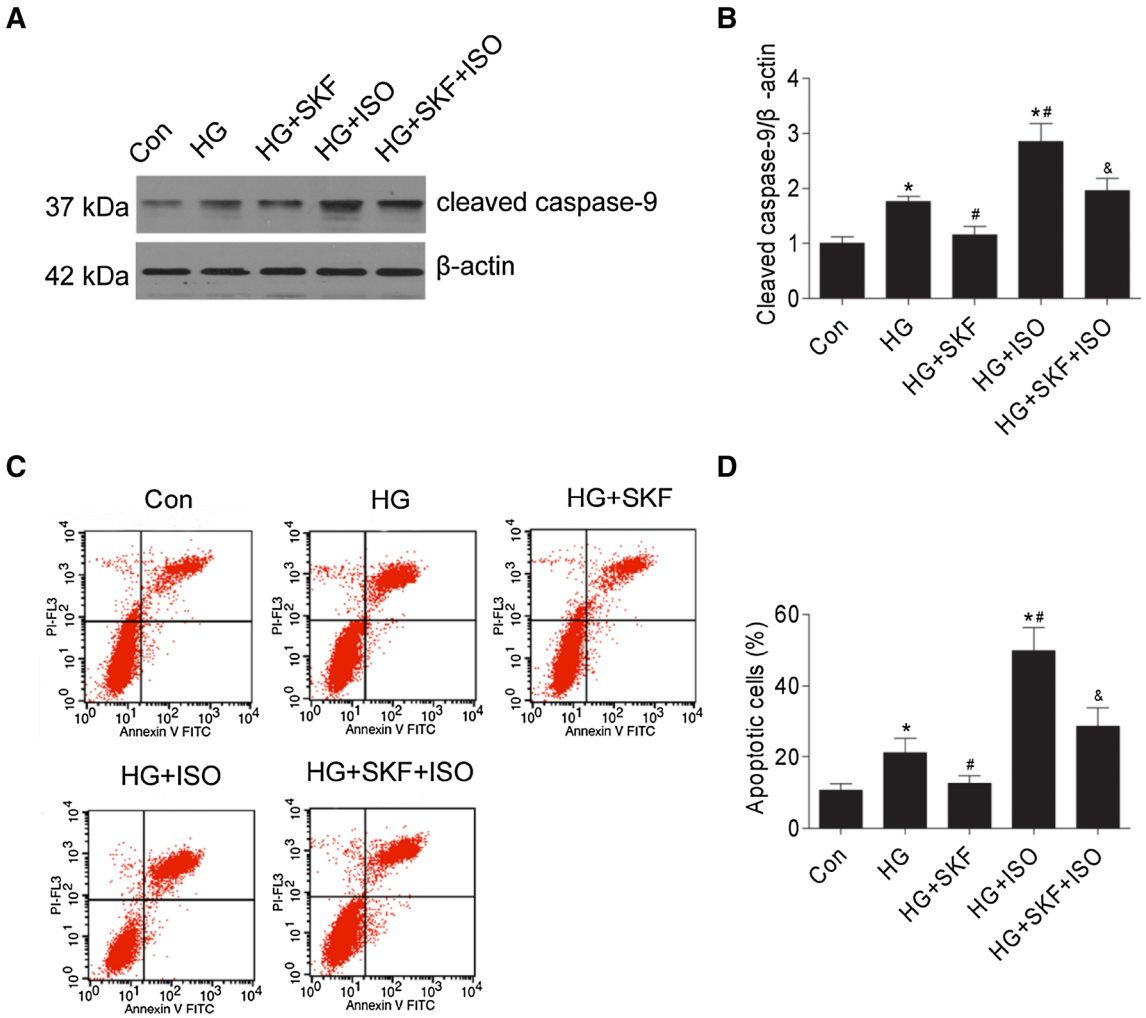

Fig. 6 SKF96365 inhibited isoflurane (ISO-) induced neurotoxicity in SH-SY5Y cells cultured with high glucose. *Con* untreated cells, *HG* cells cultured with 50 mM glucose for 4 days, *HG+SKF* cells pretreated with SKF96365 for 30 min and then cultured with 50 mM glucose for 4 days, *HG+ISO* cells treated with 50 mM glucose for 4 days and then treated with 3% ISO for 6 h, *HG+SKF+ISO* cells treated with 3% ISO for 6 h after pretreated with with 40 μM SKF96365 for 30 min and cultured with 50 mM glucose for 4 days. **a, b** Cleaved caspase-9 protein expression was detected by western blot. **c, d** Cells in apoptosis are Annexin V-FITC-positive and PI-negative. Summarized data show the apoptotic rates as detected by flow cytometry. Values are the mean ± SEM of n = 3; **P* < 0.05 vs. Con; #*P* < 0.05 vs. HG; &*P* < 0.05 vs. HG + ISO, one-way ANOVA with Tukey correction

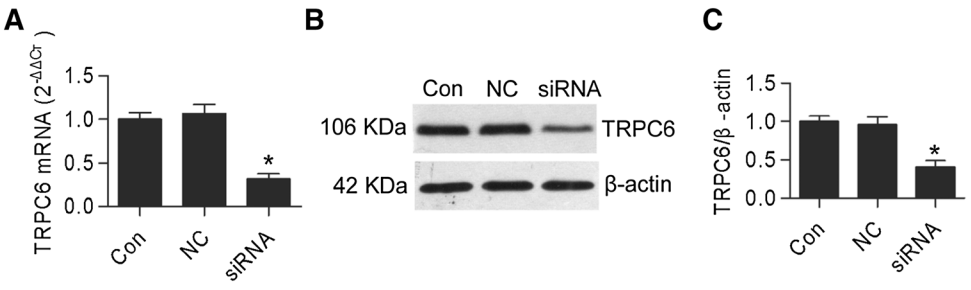

Fig. 7 The mRNA and protein expression of TRPC6 in SH-SY5Y cells with siRNA for knocked-down of TRPC6. *Con* untreated cells, *NC* cells transfected with silencer negative control siRNA, *siRNA* cells transfected with TRPC6 siRNA. **a** The mRNA of TRPC6 was detected by qRT-PCR. **b** The western blot bands show TRPC6 protein expression in SH-SY5Y cells transfected with silencer negative control siRNA or TRPC6 siRNA. **c** Data of TRPC6 protein expression. Values are the mean ± SEM of n = 3, **P* < 0.05 vs. Con, one-way ANOVA with Tukey correction

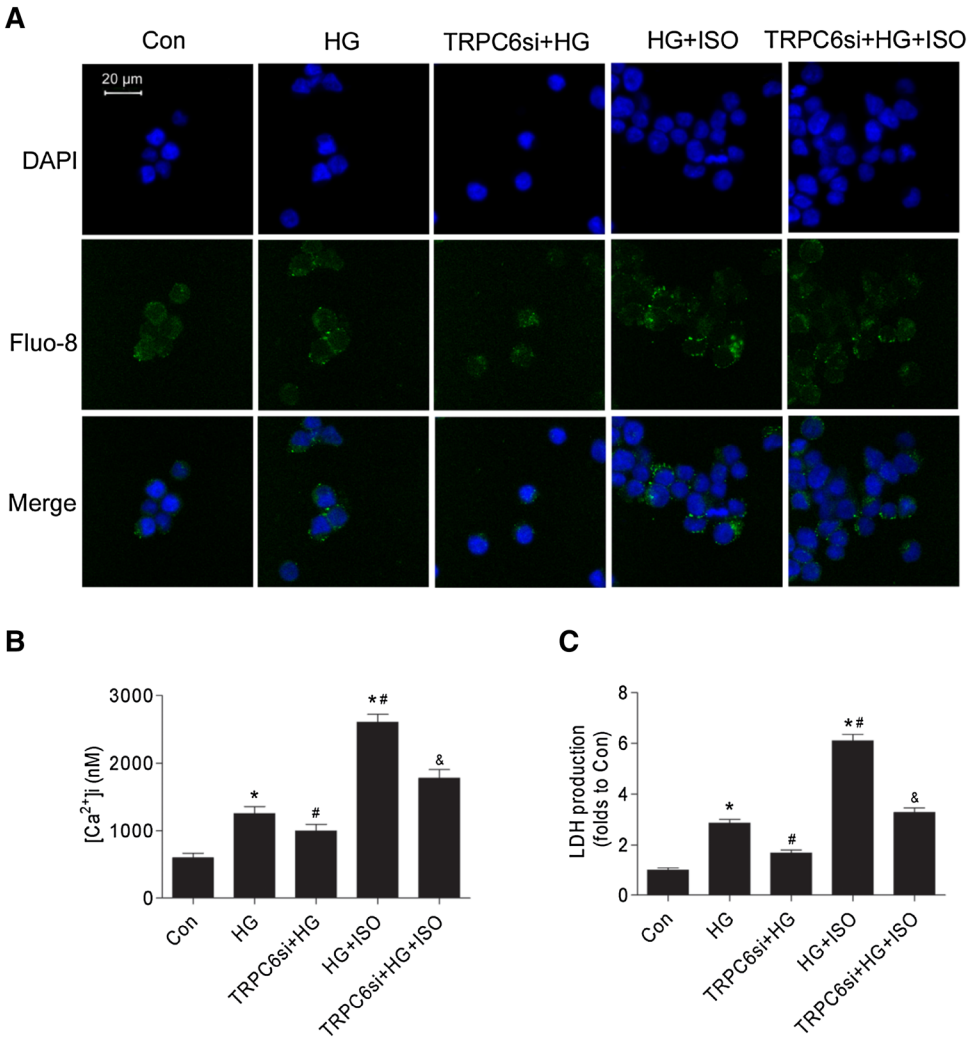

Fig. 8 Knock-down of TRPC6 inhibited the increase of cytosolic Ca^{2+}($[Ca^{2+}]$i) and damage in SH-SY5Y cells treated with high glucose (HG) and isoflurane. *Con* cells transfected with silencer negative control siRNA, *HG* cells transfected with silencer negative control siRNA and cultured with 50 mM glucose for 4 days, *TRPC6si* + *HG* cells transfected with TRPC6 siRNA and cultured with 50 mM glucose for 4 days, *HG* + *ISO* cells transfected with silencer negative control siRNA and cultured with 50 mM glucose for 4 days and then treated with 3% isoflurane (ISO) for 6 h, *TRPC6si* + *HG* + *ISO* cells transfected with TRPC6 siRNA and cultured with 3% ISO for 6 h after treatment with 50 mM glucose for 4 days. **a** Representative confocal scanning laser microscopy image. **b** [Ca2+]i levels measured with Quest Fluo-8 AM ester flow cytometry. **c** LDH production. Values are the mean ± SEM of n = 3; $*P < 0.05$ vs. Con; $\#P < 0.05$ vs. HG; $\&P < 0.05$ vs. HG + ISO, one-way ANOVA with Tukey correction

concentration-dependent manner. Toxic damage was paralleled by the isoflurane-induced increase of $[Ca^{2+}]$i. Our findings showing the ability of isoflurane to induce changes in $[Ca^{2+}]$i are in good agreement with the conclusions of previous study that performed single-channel recordings to assess the effects of isoflurane on IP_3R activity [21]. We observed that isoflurane-induced $[Ca^{2+}]$i overload triggers a cascade of events that eventually leads to cell damage. Next, we cultured cells with 30 or 50 mM glucose for 1, 2, or 4 days to determine its effect on $[Ca^{2+}]$i and neurotoxic damage. The results showed that hyperglycemia also increased $[Ca^{2+}]$i and induced neurotoxic damage in a time- and concentration-dependent manner. Notably, neurotoxic damage was paralleled by the hyperglycemia-induced increase of $[Ca^{2+}]$i. We cultured SH-SY5Y cells with 50 mM glucose for 4 days before exposing them to 3% isoflurane for 6 h. We found that hyperglycemia enhanced the isoflurane-induced increase in $[Ca^{2+}]$i and toxic damage. This result suggested that hyperglycemia enhanced isoflurane-induced neurotoxicity by causing intracellular Ca^{2+} overload. However, the mechanism by which hyperglycemia regulates isoflurane-induced intracellular Ca^{2+} overload remains unknown.

TRPC channel expression levels are altered in diabetic rats [44]. We cultured SH-SY5Y cells with 50 mM glucose for 4 days and quantified mRNA levels of different TRPC subtypes. The results showed that hyperglycemia elevated TRPC1, TRPC5, and especially TRPC6 mRNA levels. TRPC3 and TRPC4 mRNA expressions were not altered by hyperglycemia. Bishara et al. [28] presented evidence that exposing endothelial cells to hyperglycemia results in enhancing TRPC1 expression and agonist-induced Ca^{2+} entry but not Ca^{2+} release. Notably, selective antisense reduction of TRPC1 normalized Ca^{2+} homeostasis. Li et al. [29] reported that early diabetic podocyte injury in a mouse model is caused by up-regulation of TRPC6, which is controlled by the canonical Wnt signal pathway. The effect of isoflurane on TRPC expression remains unknown. We treated cells with 3% isoflurane for 6 h and quantified mRNA levels of different TRPC subtypes. The results

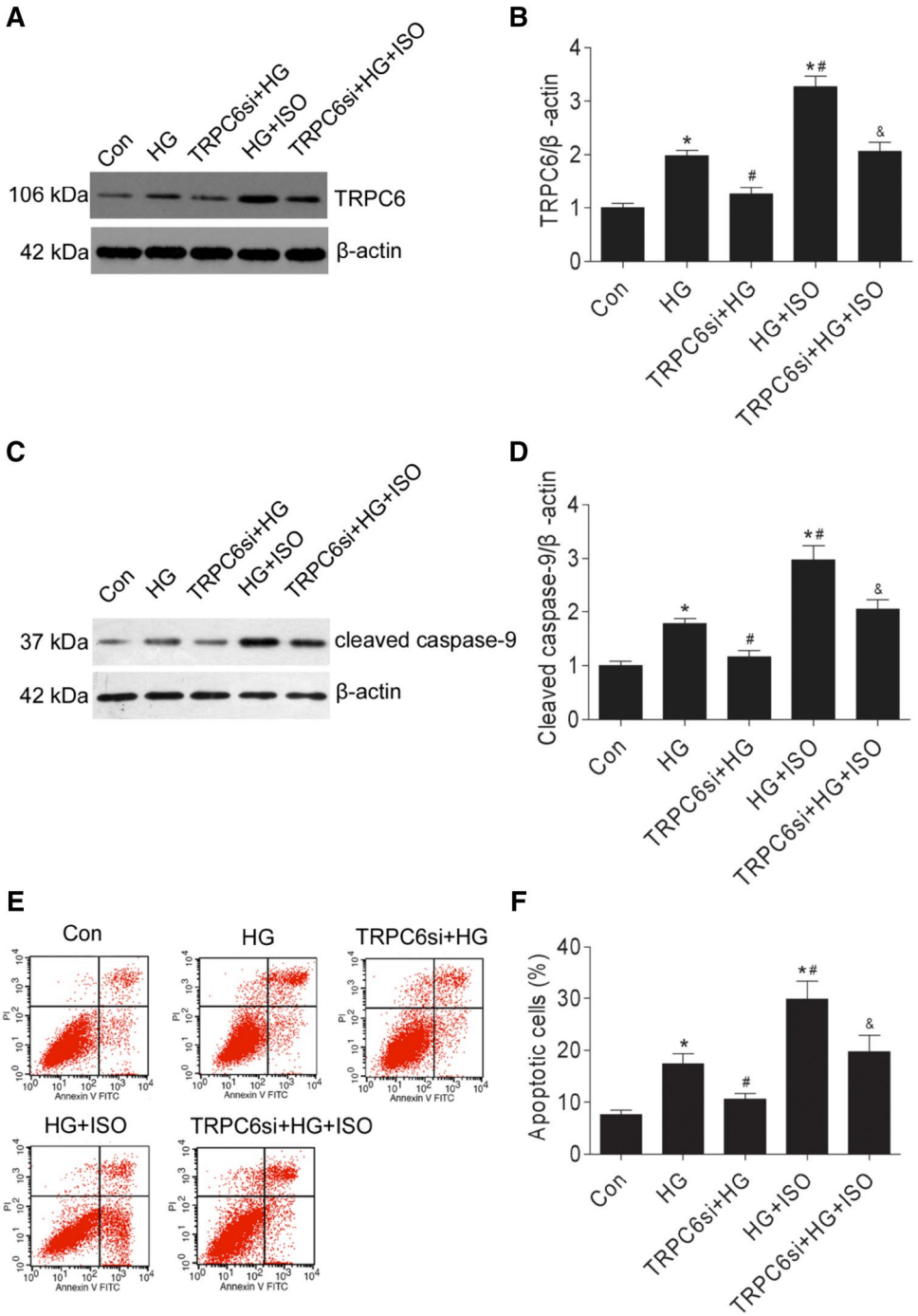

Fig. 9 Knock-down of TRPC6 inhibited isoflurane (ISO-) induced neurotoxicity in SH-SY5Y cells cultured with high glucose. *Con* cells transfected with silencer negative control siRNA, *HG* cells transfected with silencer negative control siRNA and cultured with 50 mM glucose for 4 days, *TRPC6si + HG* cells transfected with TRPC6 siRNA and cultured with 50 mM glucose for 4 days, *HG + ISO* cells transfected with silencer negative control siRNA and cultured with 50 mM glucose for 4 days and then treated with 3% isoflurane (ISO) for 6 h, *TRPC6si + HG + ISO* cells transfected with TRPC6 siRNA and cultured with 3% ISO for 6 h after treatment with 50 mM glucose for 4 days. **a, b** siRNA decreased TRPC6 protein expression induced by isoflurane and high glucose. **c, d** Cleaved caspase-9 protein expression was detected by western blot. **e, f** Cells in apoptosis are Annexin V-FITC-positive and PI-negative. Summarized data show the apoptotic rates as detected by flow cytometry. Values are the mean ± SEM of n = 3; **P* < 0.05 vs. Con; #*P* < 0.05 vs. HG; &*P* < 0.05 vs. HG + ISO, one-way ANOVA with Tukey correction

showed that isoflurane elevated TRPC1, TRPC3, TRPC4, and TRPC5 mRNA levels. Next, we used isoflurane to treat cells cultured in high glucose medium and tested mRNA levels of different TRPC subtypes. The results showed that the increasing mRNA expression of TRPC6 was significantly enhanced. Whether TRPC-dependent Ca^{2+} entry is involved in the hyperglycemia-mediated enhancement of isoflurane-induced neurotoxicity is not clear. The nonselective TRPC inhibitor SKF96365 is often used to research the function of TRPC-dependent Ca^{2+} influx [45]. We used it to determine whether TRPC regulation of Ca^{2+} influx played a crucial role in the hyperglycemia- or isoflurane-induced increase of $[Ca^{2+}]i$. Next, we employed siRNA to knock down TRPC6 and determine whether it played a crucial role in the hyperglycemia-mediated enhancement of isoflurane-induced increase of $[Ca^{2+}]i$. The results demonstrated that blocking TRPC channels and knock-down of TRPC6 could inhibit the hyperglycemia- or isoflurane-induced increase

in $[Ca^{2+}]i$ and decrease isoflurane-induced toxic damage in SH-SY5Y cells cultured in high glucose medium. Collectively, our findings suggest that TRPC involvement in isoflurane-induced increase of $[Ca^{2+}]i$ and TRPC-dependent Ca^{2+} influx is involved in the ability of hyperglycemia to enhance isoflurane-induced neurotoxicity.

Some limitations of this study should be noted. 3% isoflurane for 6 h is beyond a clinical relevant concentration and exposure time. The same effect with a clinically relevant isoflurane dose and exposure time in vivo model needs further study.

In conclusion, hyperglycemia might elevate $[Ca^{2+}]i$ via TRPC-dependent Ca^{2+} influx, thus enhancing isoflurane-induced neurotoxicity.

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