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# Translocator protein 18 kDa ligand alleviates neointimal hyperplasia in the diabetic rat artery injury model via activating PKG

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ABSTRACT

*Aims:* The proliferation of VSMCs is the pathologic basis for intimal hyperplasia after angioplasty in diabetic patients. Translocator protein (TSPO), located in the outer mitochondrial membrane, has been found to regulate redox intermediate components in cell dysfunction. We hypothesized that TSPO may regulate VSMC proliferation and migration, and be involved in the intimal hyperplasia after angioplasty in diabetes.

*Materials and methods:* Cell proliferation was measured by cell counting and MTT assays. Cell migration was measured by Transwell® and scratch-wound assays. TSPO expression in arteries of rats and high glucose-treated A10 cells were detected by immunoblotting and immunofluorescence staining. Neointimal formation of carotid artery was induced by balloon injury in type 2 diabetic rat.

*Key findings:* TSPO expression was increased in the arterial samples from diabetic rats and A10 cells treated with high glucose. Down-regulation of TSPO expression by siRNA decreased the high-glucose-induced VSMC proliferation and migration in A10 cells. This phenomenon could be simulated by using TSPO ligands, PK 11195 and Ro5-4864. cGMP/PKG signals were involved in the TSPO ligand action, since in the presence of cGMP or PKG inhibitor ODQ or KT5823 respectively, the effect of PK 11195 on VSMC proliferation was blocked. Furthermore, PK 11195 significantly inhibited neointimal formation by the inhibition of VSMC proliferation.

*Significance:* This study suggests that TSPO inhibition suppresses the proliferation and migration of VSMCs induced by hyperglycemia, consequently, preventing atherosclerosis and restenosis after angioplasty in diabetic conditions. TSPO may be a potential therapeutic target to reduce arterial remodeling induced by angioplasty in diabetes.

## 1. Introduction

Diabetic patients undergoing surgical revascularization or percutaneous coronary intervention are highly prone to vascular restenosis [1]. Neointimal hyperplasia after vascular injury plays a critical role in the process of vascular restenosis, but its mechanism has not been elucidated [2]. It is commonly accepted that abnormal proliferation and migration of medial vascular smooth muscle cells (VSMCs) are the pathological causes of neointimal formation after tunica intima injury [3]. Hyperglycemia or other conventional vascular risk factors, such as obesity and lipids, promote intimal hyperplasia via increased oxidative stress in lesions [4,5]. Therefore, finding a novel molecule, that could be selectively targeted to inhibit neointimal formation and vascular remodeling, is a promising strategy for the treatment of cardiovascular complications after percutaneous coronary intervention in diabetes.

TSPO is an 18 kDa ubiquitous protein that was previously described as peripheral benzodiazepines receptor [6]. It is mainly localized in the outer mitochondrial membrane and has been shown to be involved in a wide range of important cellular processes, including proliferation [7], apoptosis [8], steroidogenesis [9], immunomodulation [10], gene expression [11] and mitochondrial physiology [12]. The interaction of TSPO with nicotinamide adenine dinucleotide phosphate (NADPH) oxidase links the generation of ROS to the induction of an antioxidant response to maintain redox homeostasis [13]. TSPO is proposed as an

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outer mitochondrial membrane-based pathway to control intracellular $Ca^{2+}$ dynamics and redox transients in neuronal cytotoxicity [14]. Of note, the TSPO component is considered to be a multifaceted molecule that could provide binding sites for synthetic ligands with high affinity [15]. A lot of studies have been demonstrated that TSPO ligands can inhibit the proliferation of various cancer cell lines like melanomas [16], colon [17], esophagus [18], breast [19], astrocytes [20] and mammary gland [21]. However, the role of TSPO drug ligands in vascular diseases, such as coronary artery restenosis and cardiovascular complications of type 2 diabetes mellitus remains elusive. Prototypical TSPO ligand Ro5-4864 has shown beneficial effects in experimental diabetic neuropathy [22]. Recent studies have reported that TSPO function is modulated by selective ligands, leading to changes in glucose homoeostasis and cellular energy production [23]. Based on these, we hypothesized that TSPO may serve as a potent target in the process of neointimal hyperplasia after angioplasty in diabetes. In the present study, we explored the protective effects of TSPO drug ligands PK 11195 and Ro5-4864 on VSMC proliferation and migration, and investigated whether PK 11195 could act as a potential reagent to prevent intimal hyperplasia after angioplasty in diabetes.

## 2. Materials and methods

### 2.1. Materials

1-(2-Chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinolinecarboxamide (PK 11195), 4′-chlorodiazepam (Ro5-4864) and streptozocin (STZ) were purchased from Sigma Co. (St. Louis, MO). Goat polyclonal antibody against TSPO was from Thermo Fisher Scientific (Waltham, MA). Monoclonal α-smooth muscle actin (α-SMA) antibody and proliferating cell nuclear antigen (PCNA) antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). cAMP-dependent protein kinase (PKA) and PKG inhibitors were purchased from Calbiochem (Darmstadt, Germany). cGMP inhibitor ODQ and PKG inhibitor KT5823 were from Thermo Fisher Scientific (Waltham, MA). SDS-polyacrylamide gels were from Pierce (Rockford, IL). Polyvinylidene fluoride (PVDF) and protein gel apparatus were from Bio-Rad (Hercules, CA). All serum, cell media and antibiotics were purchased from Thermo Fisher Scientific (Waltham, MA). All organic solvents were from Solarbio Life Sciences (Shanghai, China).

### 2.2. Cell culture

A10 cells, a smooth muscle cell line from rat thoracic aorta [24], were purchased from ATCC (Hercules, CA). A10 cells were grown in 10% FBS-DMEM containing 1% antibiotics and incubated in a $CO_2$ incubator (5% $CO_2$, 37 °C). Cells were grown to 70 to 80% confluence and serum-deprived in 0.1% FBS for at least 24 h. Quiescent A10 cells were treated with the TSPO drug ligands PK 11195, Ro5-4864 or high glucose at indicated times before RNA or protein extraction, or performance of biochemical assays. PK 11195 and Ro5-4864 were dissolved in DMSO. The cellular data were obtained from at least three independent experiments with three replicates performed in each trial.

### 2.3. Transfection and RNA interference

For the transfection in A10 cells, TSPO siRNAs (siTSPO: 59-GAGA AGGCUGUGGUUCCCC-39) were synthesized by RIBOBIO (Guangzhou, China) based upon previously published sequences [25]. siRNA (50 nM) was transfected when cells reached 80% confluence, by using Lipofectamine 2000 reagent (Thermo Fisher Scientific) according to the manufacturer's protocol. Reduced-Serum Medium (Thermo Fisher Scientific) was used to deliver siRNA. After 48 h, the transfected cells were collected for RNA and protein determination or analyzed for proliferation, migration or other determinations from several sequence detection for the down-regulation of TSPO. The sequence with the best interfering effect was chosen and applied to our experiments.

### 2.4. Proliferation assay

Three different methods were applied to determine A10 cell proliferation including 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide (MTT) (Beyotime Institute of Biotechnology, China), PCNA detection and cell counting assays. A10 cells were seeded into 96-well culture plates for (Corning, Lowell, MA) at a density of $2 \times 10^3$ cells/well and were allowed to grow to subconfluence (70–80%), then were serum starved for another 12 h. Cells were divided into different groups corresponding to the indicated stimulus. After 24 h, 10 μL of MTT (5 mg/mL) were added to each well, and the incubation continued for an additional 4 h at 37 °C. Thereafter, dimethyl sulfoxide (DMSO, 150 μL) was added to each well, and absorbance read at 570 nm on a microplate reader (model 680, Bio-Rad). DNA synthesis in A10 cells was detected by the measurement of PCNA expression via western blot and immunofluorescent staining as described previously [26]. The growth of A10 cells was examined by cell counting. The cells were first made mitogenically quiescence by serum starvation in serum-free medium, then stimulated with the indicated reagents at the indicated times. After incubation, the number of cells were counted in a hemocytometer (trypan blue uptake, which indicates cell death, was observed in < 10% of the cells). Each count is an average of three repeats, and each data point is the average of three experiments.

### 2.5. Cell migration

Cell migration was examined using Transwell® and scratch-wound assays. The Transwell® migration assay was performed using 24-well tissue culture plates (BD Bioscience, Becton, NJ) with an 8-μm-pore polycarbonate membrane. The number of migratory cells was counted in 10 randomly chosen fields of duplicate chambers at a magnification of 200× for each sample.

For the scratch-wound migration assay, A10 cells were seeded in a 6-well plate at a density of $1 \times 10^5$/well, grown to confluence, serum starved for 24 h, then treated with the indicated reagents. The cell monolayer was scratched with a small pipette tip along the ruler and left to recover for the next 24 h in freshly exchanged starvation medium (serum-free DMEM) for 48 h. Cells were visualized on an Olympus IX-70 inverted microscope (Olympus, Tokyo, Japan). The migration area (%) was analyzed in 10 randomly chosen fields under inverted microscope using NIH Image J software, area at 0 h/area at 48 h × 100% was calculated.

### 2.6. Co-localization of TSPO and á-SMA by confocal microscopy

Expression of TSPO in arterial samples from Sprague Dawley (SD) rats were examined by laser scanning confocal microscopy. Concisely, the arterial samples were cleared of blood with ice-cold oxygenated saline and kept in 4% paraformaldehyde for at least 24 h, then embedded in paraffin, sectioned (4 μm), and mounted on slides. The sections were incubated with 5% BSA in PBS for 1 h to block non-specific binding sites. The sections were then incubated at 4 °C overnight with the following primary antibodies: goat polyclonal TSPO antibody (1:100) and rabbit polyclonal anti-α-SMA antibody (1:100). After 3 washes with PBS (3 min/wash), the sections were respectively incubated with cy3-conjugated and FITC-conjugated secondary antibodies corresponding to the primary antibodies at 37 °C for 1 h in the dark. The sections were washed 3 times with PBS and then incubated with DAPI for 2 min in the dark. The sections were analyzed and photographed using a laser scanning confocal microscope. The PCNA expression was performed as described above.

### 2.7. Evaluation of neointimal formation

To explore the morphometric grading of the neointimal formation,

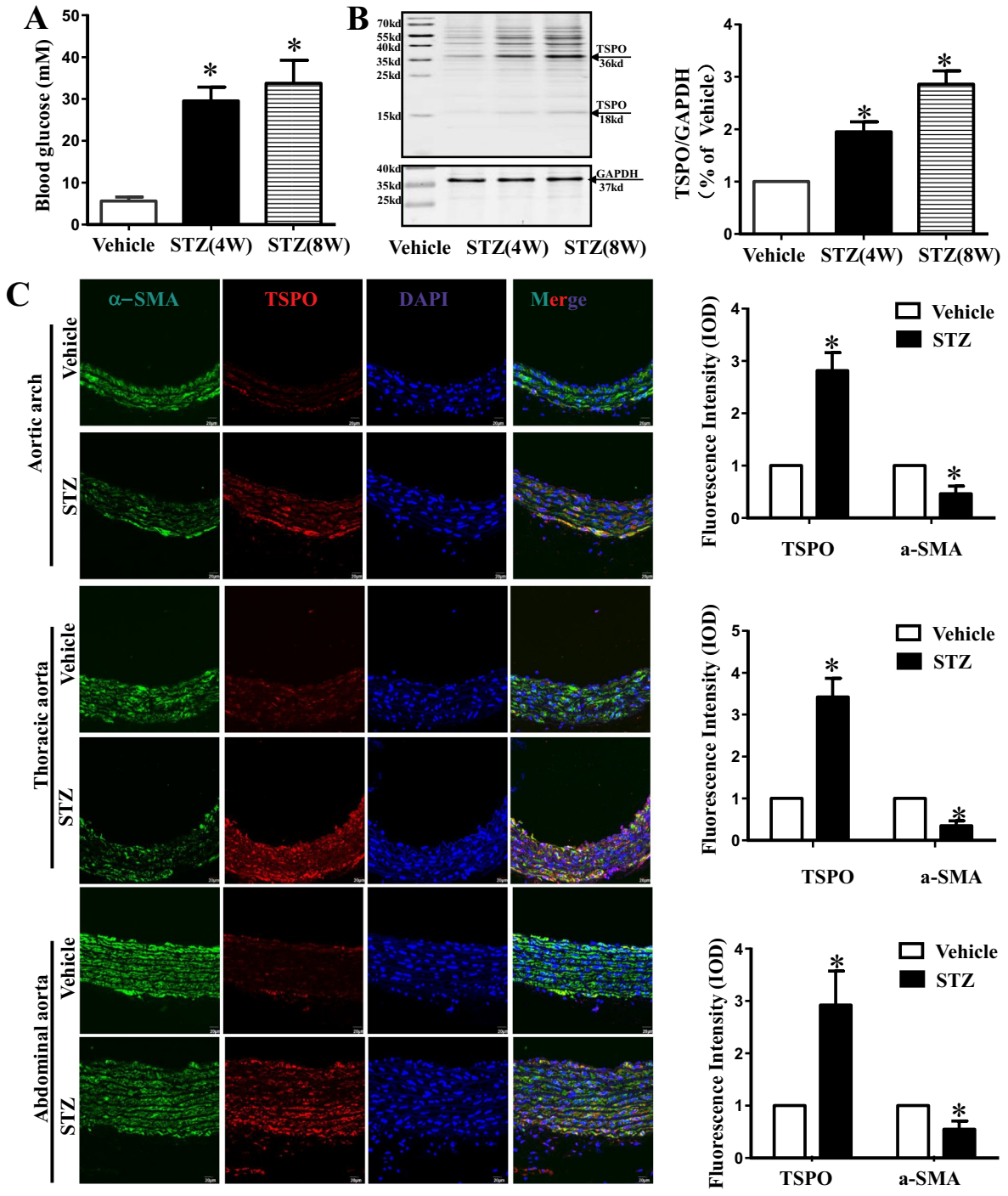


**Fig. 1.** TSPO expression in the arteries of hyperglycemia rats.
Arterial samples were collected from hyperglycemia rats 4 or 8 weeks after STZ injection. (A) Blood glucose level (mM) in hyperglycemia rats. (B) TSPO protein expression in rat aorta, determined by immunoblotting. (C) Immunofluorescence double staining for TSPO (red) and α-SMA (green) in aortic arches, thoracic and abdominal aortas derived from the hyperglycemia rats 4 weeks after STZ treatment. Relative fluorescence intensity of TSPO and α-SMA was shown (n = 5; * $P < 0.05$ vs. vehicle). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the SD rats were euthanized by carbon dioxide inhalation, in accordance with the Animal Welfare Act and institutional guidelines. Arterial tissues from the rats were immediately processed and embedded in paraffin blocks then were sectioned (4 μm thick) from equally spaced intervals in the middle of injured and control common carotid artery segments. These were then stained with hematoxylin and eosin (HE) to identify the contrasting cell types. Fifteen sections from each carotid artery were reviewed and scored under blind conditions. The intimal (I) and medial (M) areas were measured using the NIH Image 1.6 program and I/M ratio was calculated.

### 2.8. Diabetic animal model

Male SD rats weighing 250 ± 50 g were purchased from the Center of Experimental Animals of Third Military Medical University, Chongqing, China. All procedures were approved by the Animal Use Subcommittee of the Third Military Medical University. SD rats were injected intraperitoneally with STZ (60 mg/kg) to induce

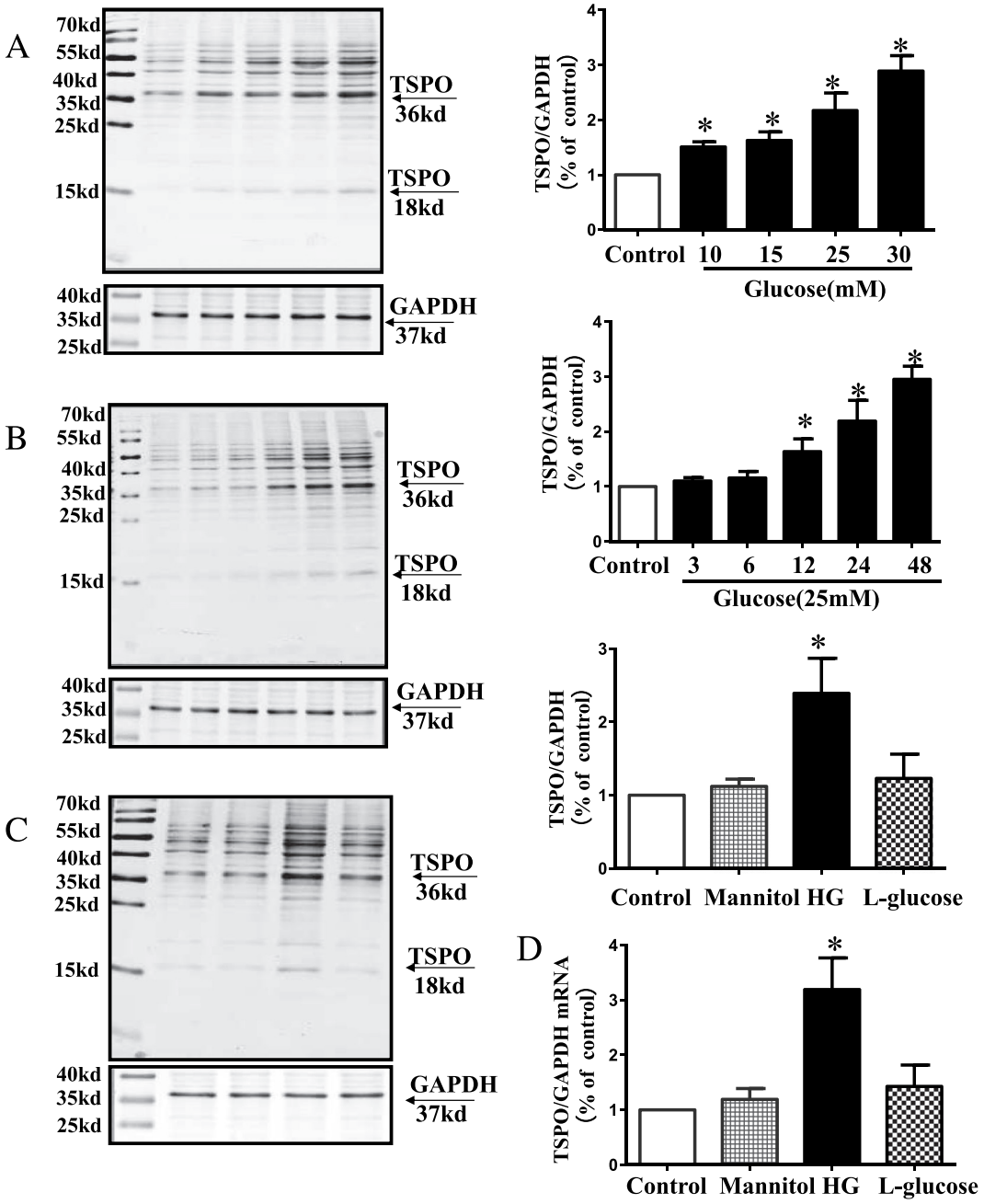


**Fig. 2.** Effect of high glucose on TSPO expression in A10 cells.
(A) Effect of HG on TSPO expression in a concentration-dependent manner in A10 cells. A10 cells were incubated with various concentrations (10, 15, 25, 30 mM) of HG for 24 h. TSPO expression was determined by immunoblotting (n = 8; * P < 0.05 vs. control).
(B) Effect of HG on TSPO expression in a time-dependent manner in A10 cells. A10 cells were incubated with 25 mM HG for the indicated times. TSPO expression was determined by immunoblotting (n = 8; * P < 0.05 vs. control).
(C and D) Effect of osmosis on TSPO expression in A10 cells. A10 cells were treated with HG (25 mM), D-mannitol (25 mM), or L-glucose (25 mM) for 24 h. The expressions of TSPO protein and mRNA were determined by immunoblotting (C) and q-PCR (D) analyses (n = 8; * P < 0.05 vs. control).

hyperglycemia. Type 2 diabetes was induced using low dose injection of STZ (30 mg/kg) and high-fat diet, as described previously [24,27,28]. Rats in control group were injected with the same volume citrate buffer, pH 4.0. The rats with fasting blood glucose concentrations > 16.5 mM were used for the carotid balloon injury experiments. After the balloon-injury, SD rats were injected intraperitoneally twice per week for two consecutive weeks with either vehicle (1% DMSO in safflower oil; n = 6) or 3 mg/kg PK 11195. Details of the balloon-injury model have been described previously [24].

### 2.9. Protein extraction and Western blot analysis

After A10 cells were treated with different ligands at the indicated concentrations and times. They were then washed once in PBS and lysed in redioimmunoprecipitation (RIPA) buffer containing a protease inhibitor mixture. Extraction of proteins, electrophoresis, transfer, immunodetection, and densitometric evaluation were performed as previously described [24,29,30]. The amount of protein transferred onto the membranes was normalized by immunoblotting with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:400).

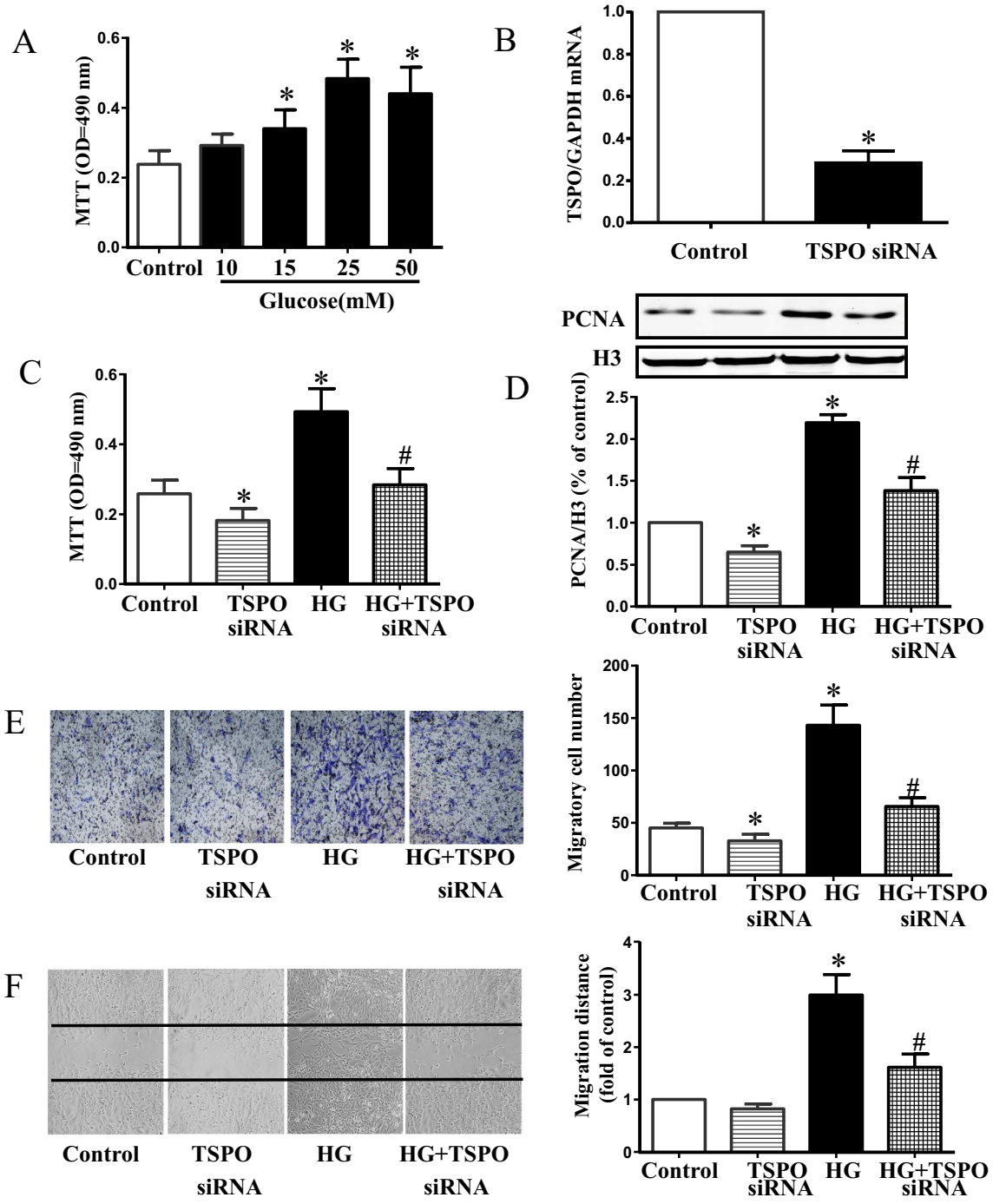


**Fig. 3.** Down-regulation of TSPO on HG-induced A10 cell proliferation and migration.
(A) Effect of HG on the proliferation of A10 cells. A10 cells were incubated with different concentrations (10, 15, 25, 30 mM) of HG for 24 h. Cell proliferation was determined by MTT assay (n = 10, * P < 0.05 vs. control).
(B) Down-regulation of TSPO expression by siRNA. A10 cells were incubated with siRNA against TSPO for 48 h. TSPO expression was determined by q-PCR (n = 8, * P < 0.05 vs. control).
(C and D) Effect of TSPO down-regulation on A10 cell proliferation and migration. A10 cells were transfected with or without TSPO siRNA for 48 h, and then incubated with or without 25 mM HG for another 24 h. Cells were harvested for further assays. Cells proliferation was determined by MTT assay (C). PCNA expression was analyzed by immunoblotting (D). Cells migration was determined by Transwell® (E) and wound healing (F) (n = 10; * P < 0.05 vs. control; # P < 0.05 vs. HG alone).

### 2.10. Reverse transcriptase-PCR

Total RNA from A10 cells was isolated using a Trizol procedure (Invitrogen, Carlsbad, CA). 2 μg of total RNA were used to synthesize cDNA, which served as template for the amplification of TSPO and GAPDH (as housekeeping gene). Primer sequences for TSPO were 5′-CCCGCTTGCTGTACCCTTACC-3′ (forward) and 5′-CACCGCATACATAGTAGTTGAGCACGGTG-3′ (reverse). Primer sequences for GAPDH were 5′-GTGGAGTCTACTGGCGTCTT-3′ (forward) and 5′-GCCTGCTTCACCACCTTCTT-3′ (reverse). The amplification was performed under the following conditions: 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and extension at

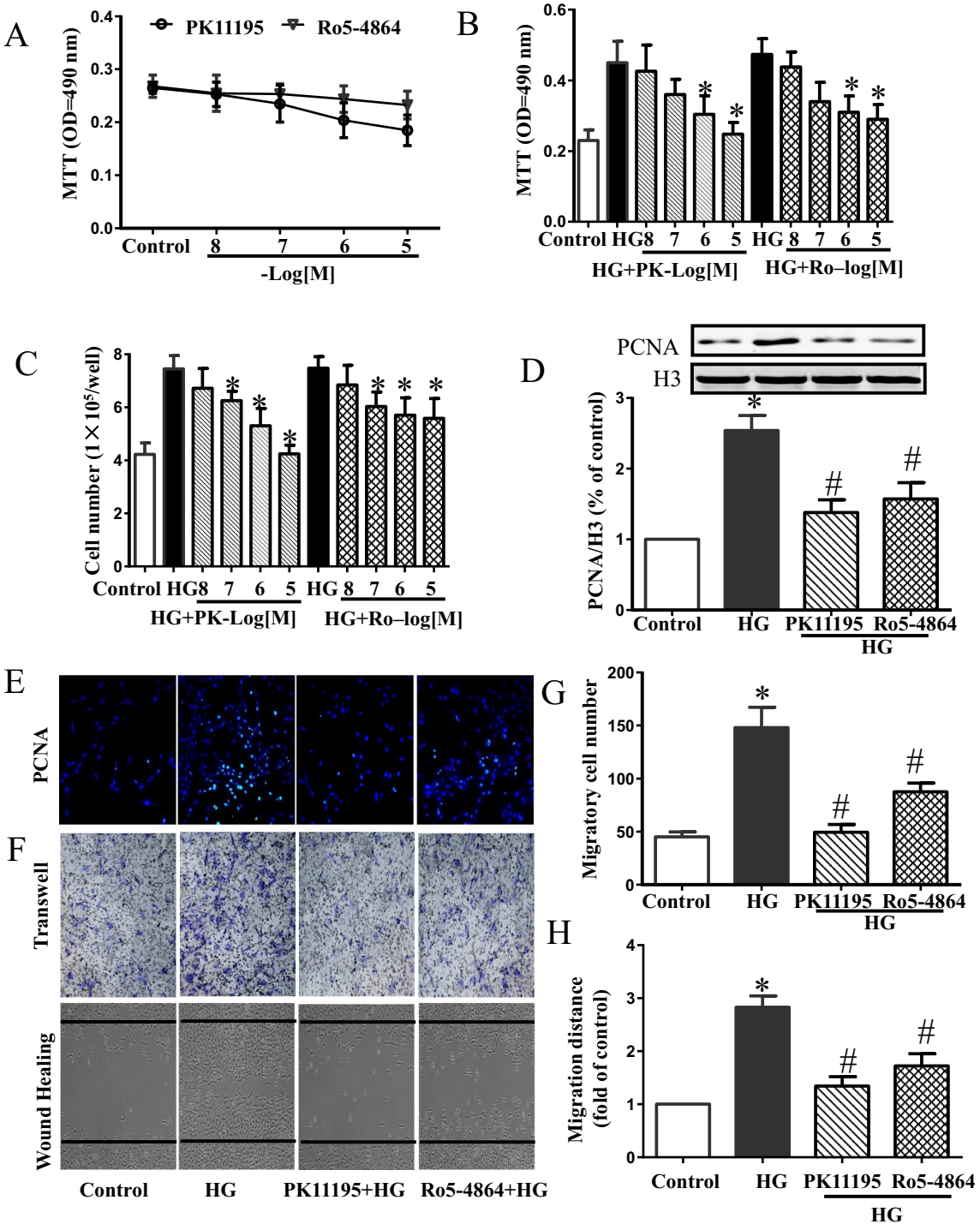


**Fig. 4.** Effects of TSPO ligands, PK 11195 and Ro5-4864 on HG-induced A10 cell proliferation and migration.
(A) Effects of PK 11195 and Ro5-4864 on proliferation of A10 cells. A10 cells were incubated with the indicated concentrations of PK 11195 or Ro5-4864 for 24 h. Cells proliferation was determined by MTT (n = 10, P = NS).
(B and C) Effects of PK 11195 and Ro5-4864 on HG-induced A10 proliferation. A10 cells were co-incubated with 25 mM HG and the indicated concentrations of PK 11195 or Ro5-4864 for 24 h. Cells proliferation was determined by MTT (B) and cell counting (C) (n = 8; * $P < 0.05$ vs. HG alone).
(D) Quantitative analysis of PCNA expression in different groups. Bars showed relative value to control (n = 10; * $P < 0.05$ vs. control; # $P < 0.05$ vs. HG alone).
(E) Effect of HG on HG-induced A10 cells proliferation. A10 cells were co-incubated with 25 mM HG and $10^{-5}$ M PK 11195 or Ro5-4864 for 24 h. Representative images of PCNA staining were shown.
(F) Effect of HG on HG-induced A10 cells migration. A10 cells were co-incubated with 25 mM HG and $10^{-5}$ M PK 11195 or Ro5-4864 for 24 h. Representative images Transwell® and wounding healing tests were shown.
(G and H) Quantitative analysis of migration distance determined by Transwell® and wounding healing. Bars showed relative value to control (n = 10; * $P < 0.05$ vs. control; # $P < 0.05$ vs. HG alone).

72 °C for 45 s. This was followed by a final extension at 72 °C for 10 min. The PCR products were electrophoresed in 2% agarose gels.

### 2.11. Statistical analysis

All data and experiments exhibited in this study were repeated at least 3 times. Results are expressed as mean ± SEM. Comparison within groups was made by repeated measures ANOVA (or paired *t*-test when only 2 groups were compared), and comparison among groups (or *t*-test when only 2 groups were compared) was made by factorial ANOVA using the Holm-Sidak ad hoc test. Values of $P < 0.05$ were considered statistically significant.

## 3. Results

### 3.1. TSPO expression was increased in arterial samples of hyperglycemia rats induced by STZ treatment

Hyperglycemia rat model was established by using STZ (60 mg/kg) injection (Fig. 1A). Immunoblotting revealed that the expression of TSPO protein significantly upregulated in rat aorta, beginning at 4 weeks onward (Fig. 1B). The increased TSPO expression was further confirmed by immunofluorescence staining, which showed the increased TSPO expression in the aortic arches, thoracic, and abdominal aortas derived from hyperglycemia rats after 4 weeks of treatment. Moreover, the increased TSPO accumulation in the VSMCs, is indicated by its co-localization with reduced α-SMA (Fig. 1C).

In addition to the in vivo study, we also investigated the effect of high (25 mM) glucose (HG) on TSPO expression in A10 cells. The TSPO gene encodes 18-kDa isoforms. In our study the 18-kDa band was used for western blot quantitative analysis. Western blotting showed that HG treatment led to significant upregulation of TSPO protein in both concentration- and time-dependent manners (Fig. 2A and B). To eliminate the possible role of osmosis, induced by HG treatment, on the TSPO expression, we treated the A10 cell controls with D-mannitol and L-glucose (25 mM). Western blotting and q-PCR revealed that neither D-mannitol nor L-glucose have effect on TSPO protein and mRNA expressions (Fig. 2C and D).

It is well know that high glucose could potentiate the oxidative metabolism and induce mitogenesis, therefore the number of mitochondria constant may also contribute to increment of TSPO expression. To distinguish possibility whether or not the increased TSPO expression is secondary to the increased mitochondrial number, we performed an experiment showing that, although hyperglycemia induced mitogenesis, indicated by decreased individual mitochondrial area and increased number of mitochondria, in A10 cells, the ratio of TSPO expression and mitochondria number was higher in hyperglycemia group than control (Supplemental Fig. 1A–E), indicating that hyperglycemia did increase TSPO expression in A10 cells.

### 3.2. Down-regulation of TSPO expression inhibited HG-induced A10 cells proliferation and migration

Consistent with our previous study [31], HG promoted A10 cell proliferation in both concentration- and time-dependent manners as determined by MTT assays (Fig. 3A). To test whether TSPO plays a role in this process, we down-regulated TSPO expression in A10 cells by using siRNA interference (Fig. 3B). Knockdown of TSPO decreased A10 cell proliferation induced by HG (25 mM, 24 h), evaluated by MTT analysis (Fig. 3C) and diminished the expression of PCNA, a marker for cell proliferation (Fig. 3D). Moreover, down-regulation of TSPO presented potent inhibitory effect on HG-mediated A10 cell migration which were determined by Transwell® and wound healing tests (Fig. 3E and F).

PK 11195 and Ro5-4864, the two most widely used TSPO antagonists for their high affinity and specificity [32–34], were used in our present study to determine their effects on cell growth in HG-exposed A10 cells. Our findings revealed that treatment with PK 11195 or Ro5-4864 ($10^{-8}$, $10^{-7}$, $10^{-6}$ or $10^{-5}$ mol/L) had no significant effect on the proliferation of A10 cells by itself (Fig. 4A). However, either PK 11195 or Ro5-4864 dose-dependently reduced HG-mediated increased proliferation in A10 cells, as determined by MTT and cell counting analysis (Fig. 4B and C). These effects were further identified by the restoration of HG-induced PCNA levels by PK 11195 or Ro5-4864 ($10^{-5}$ mol/L) (Fig. 4D and E). Moreover, PK 11195 or Ro5-4864 ($10^{-5}$ mol/L) suppressed migration of A10 cells treated with HG, determined by Transwell® and wound healing studies (Fig. 4F, G and H). The above studies indicate that PK 11195 and Ro5-4864 might work as TSPO antagonists.

### 3.3. TSPO ligands inhibited A10 cells proliferation and migration by activating cGMP/PKG signals

Broad evidences have demonstrated that cell proliferative ability is primarily associated with cAMP and cGMP activation [35]. By inference, we hypothesized that TSPO plays an important role in signal transduction by regulating the intracellular activities of cyclic nucleotides. Experiments were carried out to determine which cyclic nucleotide families were involved in the anti-proliferative effect of PK 11195. The inhibitory effect of PK 11195 on A10 cell proliferation was abolished by ODQ, the blocker of cGMP, but not by the cAMP blocker cAMP-Rp (Fig. 5A). Moreover, the role of PKG, which is the downstream mediator of cGMP signals, was investigated by using its inhibitor KT5823; in the presence of KT5823, the inhibitory effect of PK 11195 on HG-mediated proliferation in A10 cells was blocked, as evaluated by MTT assay (Fig. 5B). Consistent with the effect of PK 11195 on cell proliferation, HG decreased cGMP production, which was reversed by PK 11195 treatment; while in presence of ODQ, the stimulatory effect of PK 11195 was blocked (Fig. 5C). All in all, these data identified that cGMP activation was critical for the anti-proliferative effect of PK 11195.

### 3.4. Effect of PK 11195 on neointima formation in vivo

Due to the effect of PK 11195 on VSMC proliferation, we wondered if PK 11195 might have an effect on neointima formation. The role of PK 11195 was explored in the well-established balloon injury model in type 2 diabetic rats. HE staining showed PK 11195 alleviated neointimal formation in the injured carotid arteries (Fig. 6A). The protective effect of PK 11195 on neointimal formation was correlated with its inhibitory effect on VSMC proliferation, as determined by PCNA immunofluorescence staining. It showed that balloon injury increased PCNA positive staining cell number, which was inhibited by PK 11195 (Fig. 6B).

## 4. Discussion

The prevalence of diabetes mellitus has greatly increased in the past three decades, with its resultant heavy global burden of coronary artery disease [36]. Given the complexity of coronary artery disease in patients with diabetes, percutaneous revascularization is challenging [37]. TSPO is highly expressed in the inflammatory regions of atherosclerotic plaques. Several studies have investigated the uptake of [11C] PK 11195, a TSPO radioligand, in atherosclerotic plaques, and has been tested for imaging atherosclerosis [38,39]. VSMC proliferation is the pathological basis for intimal hyperplasia after angioplasty in diabetics. Chronic hyperglycemia promotes VSMC growth and leads to a greater increase in the incidence of vascular restenosis after angioplasty [4]. In this study, we found increased TSPO expression in arterial samples of diabetic rats; of which down-regulation of TSPO alleviated A10 cell proliferation and migration. Additionally, we demonstrated that TSPO ligands PK 11195 and Ro5-4864 could reduce neointimal hyperplasia by inhibition of VSMC proliferation.

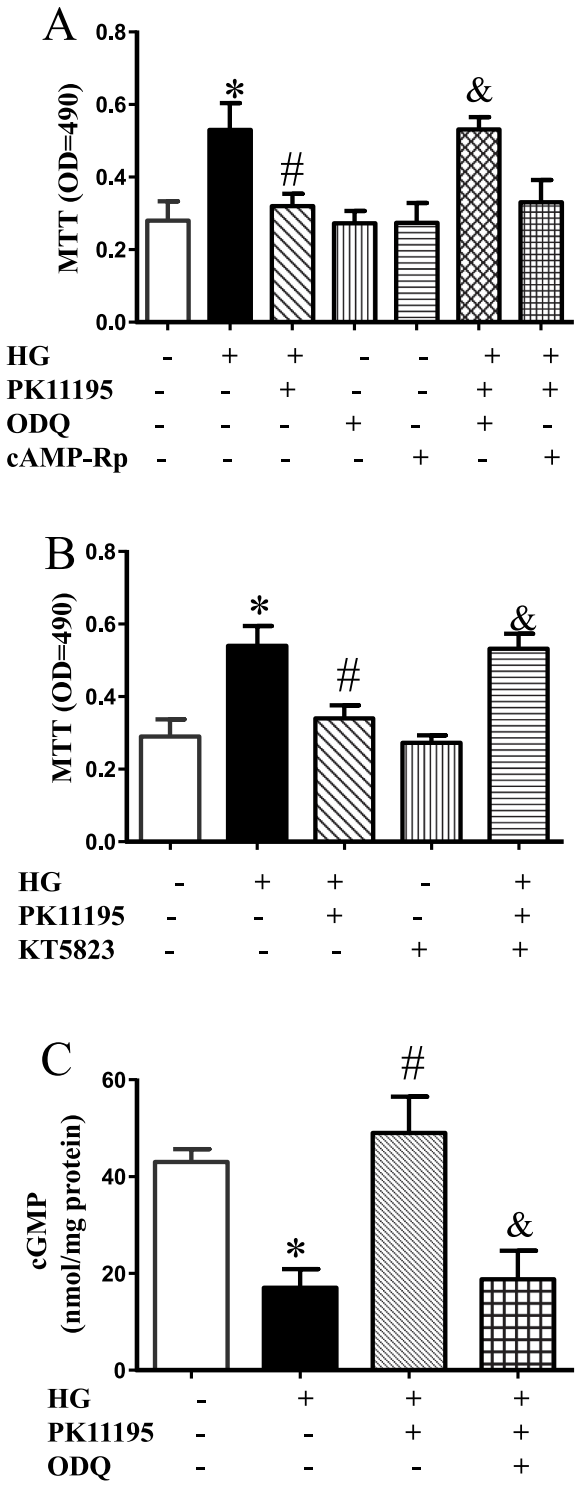


**Fig. 5.** Role of cGMP/PKG in PK 11195-mediated inhibitory effect on A10 cell proliferation.

(A and B) Role of cGMP/PKG on PK 11195-mediated inhibitory effect on A10 cell proliferation A10 cells were treated with 25 mM HG for 24 h in the presence of PK 11195 ($10^{-5}$ mol/L), ODQ (10 mM), cAMP-Rp (10 mM) and/or KT5823 (1 mM). The cell proliferation was determined by MTT assay (n = 8; * $P < 0.05$ vs. control; # $P < 0.05$ vs. HG alone; & $P < 0.05$ vs. HG + PK 11195).

(C) Effect of PK 11195 on cGMP production in the presence of cGMP inhibitor in A10 cells. A10 cells were treated with 25 mM HG and/or $10^{-5}$ mol/L PK 11195 with/without ODQ (10 mM) for 24 h. cGMP activity was determined by ELISA via commercial cGMP activity assay kit (n = 8; * $P < 0.05$ vs. control; # $P < 0.05$ vs. HG alone; & $P < 0.05$ vs. HG + PK 11195).

Several confirmatory evidences suggest that high glucose per se promotes VSMC proliferation and exaggerates intimal hyperplasia [4,40]. HG has been shown to increase ROS, which is a signaling component necessary for cell proliferation and migration. Increased ROS level is probably the culprit in diabetic vascular complications [41]. Our results showed higher TSPO expression in A10 cells after HG stimulation when compared to the control. The mitochondria are the major source of ROS production within the cell. TSPO is mainly localized in the outer mitochondrial membrane, reflecting a key role of TSPO in cellular functions. Issop et al. recently showed that TSPO mediates stabilization of mitochondrial structure and controlling ROS production during TNF-induced inflammatory phenotype [42]. The function of TSPO was modulated by TSPO ligands [6,43]. We showed for the first time that TSPO ligands PK 11195 and Ro5-4864 prevented HG-mediated VSMC proliferation and migration. Moreover, down-regulation of TSPO expression by siRNA exhibited similar effects on VSMC growth.

In line with previous studies [44,45], VSMCs underwent phenotypic changes in response to vascular injury determined by the reduction of α-SMA expression in our diabetic rat model. The synthetic or proliferative phenotype of VSMCs contributes to the progression of neointimal hyperplasia or restenosis. cGMP/PKG pathway has an important role in the process of VSMCs phenotype switching in diabetics [46,47]. Previous studies have shown that the activation of PKG by sildenafil suppresses cardiac hypertrophy and modulates cardiac remodeling [48]. It also has been shown capacity of PKG activation to prevent abnormal VSMC proliferation in diabetics [49]. Consistently, our findings revealed that the inhibitory effects of TSPO ligands PK 11195 or Ro5-4864 on VSMC proliferation and migration were induced by cGMP/PKG signaling pathway stimulation. However, we have not investigated the upstream signaling molecular of cGMP/PKG pathway that could be targeted by TSPO drug ligands, which warrants further experiments in the future.

In our present study, the type 2 diabetic rats were induced by high fat diet and intraperitoneal injection of STZ, characterized by the sharply elevated blood glucose level and insulin resistance. Long term stimulation of hyperglycemia leads to increased macrovascular complications and increases the risk of atherosclerosis in coronary, cerebral, and peripheral arteries [50]. Coronary atherosclerosis is the leading cause of cardiovascular events in diabetic patients [51]. Therefore, minimizing cardiovascular complications is the main goal of diabetes treatment. TSPO ligands exert beneficial effects on a large spectrum of local events, including diabetic neuropathy, hyperglycemia-induced microvascular leakage in the retinas, and adipogenesis [22,52–54]. Our findings demonstrated that PK 11195 evidently alleviates neointimal formation induced by carotid balloon injury in type 2 diabetic rats, through the down-regulation of PCNA expression and attenuation of VSMC dysfunction. However, we have not explored whether or not PK 11195 has a direct impact on glucose and lipid homeostasis. In particular, the existence of endogenous ligands that are regulated by metabolism suggests that TSPO functions to adapt mitochondrial to cellular metabolism [55]. Whether or not these effects are correlated with TSPO's ability in binding and transferring cholesterol into the mitochondria, probably for the needs of membrane biogenesis, requires further investigation.

## 5. Conclusion

Together, this study revealed the crucial role of TSPO in balloon injury-induced neointimal hyperplasia in type 2 diabetic rats. We are the first to show that the TSPO ligand PK 11195 can attenuate neointimal hyperplasia through its anti-proliferative effects on VSMCs via cGMP/PKG pathway. TSPO could be a potential therapeutic target to prevent cardiovascular complications after angioplasty in diabetic patients.

Supplementary data to this article can be found online at https://

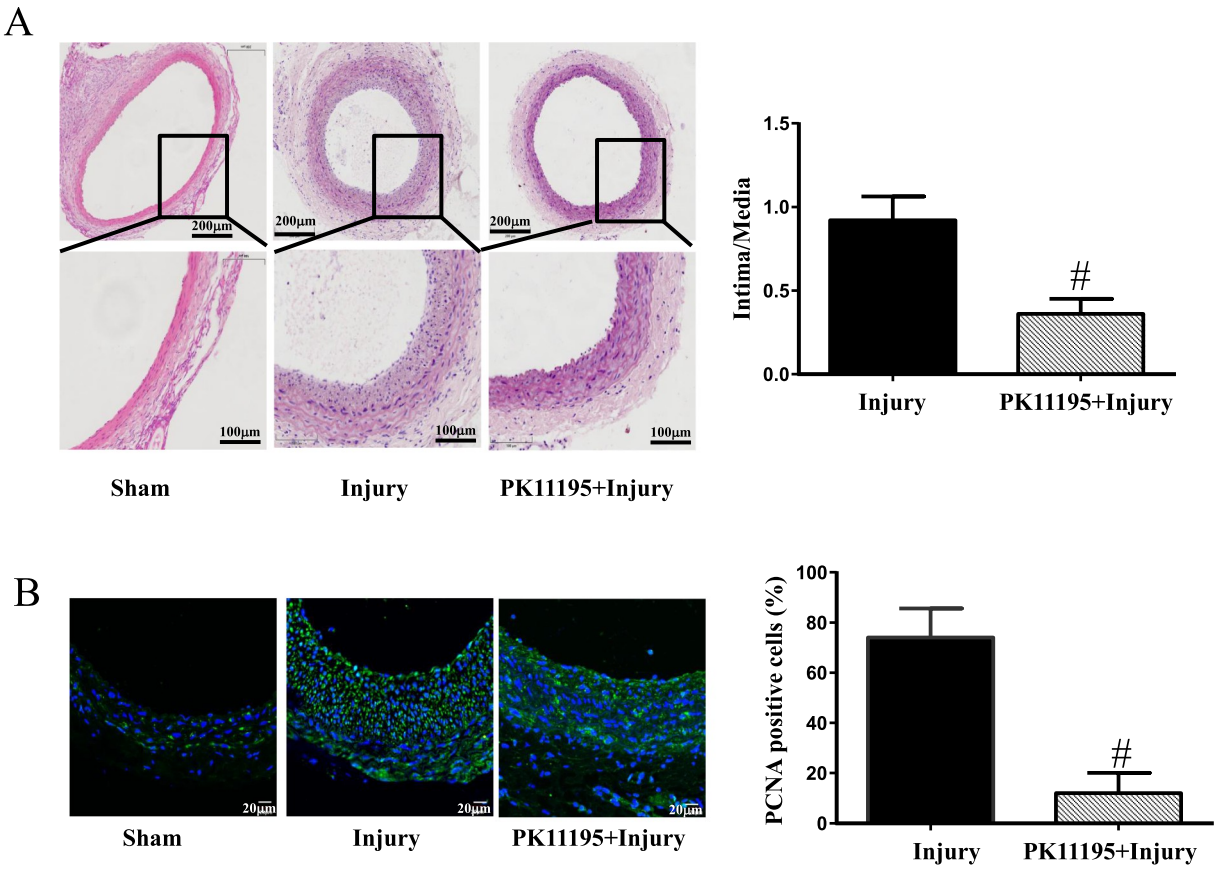


**Fig. 6.** Effect of PK 11195 on neointima formation after balloon-injured arteries in type 2 diabetic rats.
Using balloon injury to induce carotid neointima formation in type 2 diabetic rats, simultaneously injected with vehicle or PK 11195 (3 mg/kg) twice per week for two weeks. After five times injection, carotid artery was harvested for neointima examination. (A) Representative cross-sections of hematoxylin-eosin (HE) in different groups. The bar graph showed the area of the intima to media ratio. (B) Representative immunofluorescence staining of PCNA (green) and DAPI (blue). The bar graph showed the percent of PCNA positive cells (n = 6; * P < 0.05 vs. control; # P < 0.05 vs. injury alone). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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## Abbreviations

| | |
|---|---|
| TSPO | translocator protein |
| PKG | cGMP-dependent protein kinase |
| cGMP | cyclic guanosine monophosphate |
| VSMC | vascular smooth muscle cell |
| ROS | reactive oxygen species |
| SD rat | Sprague Dawley rat |
| STZ | streptozotocin |
| HE | hematoxylin-eosin |
| NADPH | nicotinamide adenine dinucleotide phosphate |
| PKA | cAMP-dependent protein kinase |
| cAMP | cyclic Adenosine monophosphate |
| PCNA | proliferating cell nuclear antigen |
| α-SMA | α-smooth muscle actin |
| FITC | fluorescein isothiocyanate |
| PVDF | polyvinylidene fluoride |
| DMSO | dimethyl sulfoxide |
| FBS | fetal bovine serum |
| BSA | albumin from bovine serum |
| PBS | phosphate buffer saline |
| RIPA | redioimmunoprecipitation |
| GAPDH | glyceraldehyde 3-phosphate dehydrogenase |
| HG | high glucose concentration (25 mM glucose) |

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## Competing interests

The authors declare that they have no competing interests. This manuscript is an original contribution not previously published, and not under consideration for publication elsewhere.

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## Authors' contributions

ZG, YH and LW designed the study, analyzed and interpreted the data, and drafted the paper. TX, HR, DY, DG, HW, CH and DH contributed to data acquisition. LZ and CZ designed the study and drafted the paper. All authors read and approved the final manuscript.

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