Abstract.
ATP-sensitive potassium (KATP) channels are well characterized in cardiac, pancreatic and many other muscle cells. The purpose of this study was to determine if KATP channels play a role in diabetic nephropathy (DN). In the present study, functional expression of the KATP channel was examined in rat mesangial cells with or without high glucose (HG) stimulation. The mesangial cell proliferation and the release of matrix metalloproteinase (MMP)-2 and fibronectin in response to high glucose with a selective opener of KATP (diazoxide, DZX), or with a selective inhibitor of KATP (5-hydroxydecanoate, 5-HD) were also measured. The cell proliferation was observed using Cell Counting Kit-8 assay, and the mRNA expressions of KATP subunit, including Kir6.1, Kir6.2, sulfonylurea receptor 1 (SUR1), SUR2A and SUR2B, were assessed using quantitative real-time PCR. MMP-2 and fibronectin release was measured by ELISA. The present study clarified expression of SUR subunit of KATP in plasma. HG treatment could cause increased cell proliferation and release of MMP-2 and fibronectin in a dose-dependent manner. HG also significantly decreased the expression of Kir6.1, SUR2A and SUR2B. Pretreatment of DZX markedly decreased the expression of SUR1, SUR2A and SUR2B, but had no effect on Kir6.1 expression compared with HG alone, while these changes were inhibited by 5-HD pretreatment. Moreover, DZX also inhibited cell proliferation and release of MMP-2 and fibronectin in HG-induced rat mesangial cells, and that was corrected by 5-HD. These data suggest that HG stimulates mesangial cell proliferation and cellular matrix release via inhibiting KATP channel activity, leading us to propose that KATP channel dysfunction may be involved in the development of DN.

Introduction
Diabetic nephropathy (DN), one of the most serious microvascular complications of diabetes mellitus, is the leading cause of end-stage renal disease in developed countries and major cause of diabetes-related death (1,2). It is characterized by kidney hypertrophy, glomerulus and tubular basement membrane thickening, tubular interstitial fibrosis and arteriosclerosis (3,4), which is largely due to mesangial cell proliferation, extracellular matrix (ECM) deposition. Therefore, searching for effective methods of inhibiting mesangial cell proliferation and ECM accumulation may be of great clinical importance for intervention in DN.

Hyperglycemia plays a central role in the development and progression of DN (5). High glucose (HG) promotes mesangial cell proliferation and fibronectin expression \textit{in vitro}. HG induces renal mesangial cell proliferation and fibronectin expression through JNK/nuclear factor (NF)-κB/NADPH oxidase/ROS pathway (6). HG induced mesangial cell proliferation and arrested cell cycle progress through upregulation of p-p38MAPK (7). Mesangial cells also play a role in the synthesis, as well as the degradation of the ECM, which is mediated by proteinases such as matrix metalloproteinases (MMPs), including type IV collagen, fibronectin and proteoglycans (8). Increased levels of both MMP-2 and MMP-9 have been demonstrated in serum from patients with type 1 diabetes (9), and the level of urine MMP-9 was correlated with the degree of albuminuria (10).

ATP-sensitive potassium (KATP) channels were first identified in cardiac cell (11), inhibited by intracellular ATP and activated by MgADP (12), and coupled cellular energy status to electrical activity playing a critical role in regulating numerous cellular functions, such as cardiac preconditioning, vasodilatation, neuroprotection (13) as well as glucose homeostasis by regulating insulin secretion (14). High glucose conditions have led to the recruitment of KATP channels to the β-cell plasma membrane in a Ca$^{2+}$ and PKA-dependent manner, resulting in an increase in KATP currents (15), whereas a protein kinase C activator facilitated endocytic trafficking of KATP, resulting in decreased KATP currents (16). At molecular level, the KATP channel is composed of subunits from a combination of inwardly rectifying K$^+$ channels (Kir6.1 or Kir6.2) and sulfonylurea receptors (SUR1, SUR2A and SUR2B). KATP channel closure occur by binding to the pore-forming subunit Kir6.2, yet activate channel opening by interacting with the

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regulatory subunit SUR in a Mg$^{2+}$-dependent manner (17). It is believed that different Kir combine with different SUR to form the various native KATP channels (18). Different KATP channels exhibit different pharmacological profiles dictated by the SUR subtypes they are composed of. However, the existence subtypes and role of KATP channels in DN is still not well defined.

In the present study, we investigated the effects of HG on cell proliferation and release of MMP-2 and FN in rat mesangial cells, and elucidated the role of KATP channels in HG-induced DN.

Materials and methods

Isolation and culture of mesangial cells. The experiments were performed in accordance with the Animal Ethics Committee of the Second Military Medical University. Primary rat mesangial cells were harvested from male Sprague-Dawley rat (150-200 g) (the Second Military Medical University Animal Center, Shanghai, China) by filtration with ice-cold 0.9% NaCl solution through a 200-, 120- and 80-µm nylon mesh. Those retained on the sieve were collected, washed by centrifugation (4°C; 800 x g; 6 min), and incubated with 4 mg/ml collagenase type IV for 10 min at 37°C under constant, gentle shaking. Mesangial cells were placed on 25-cm² culture flasks in Dulbecco’s modified Eagle’s medium (DMEM)/F12 (HyClone-Thermo Fisher Scientific, Inc., San Jose, CA, USA) at 37°C in humidified 5% CO$_2$-95% air. The culture media was not changed for the first 7 days. Thereafter, the medium was changed every other day until confluence and passages 11 to 17 were used in this study. Mesangial cells at ~80% confluence were cultured in 1% fetal bovine serum (FBS) DMEM for 24 h for synchronization, and then were exposed to low glucose (5.6 mM), high glucose (30 mM), with or without the pretreatment of diazoxide (DZX) (50 µM) or 5-hydroxydecanoate (5-HD) (200 µM). The cell phenotypes were determined by light microscopy.

Quantitative real-time PCR. Total RNA was extracted and purified from 1x10$^6$ cells using QIAzol reagent (Qiagen, Valencia, CA, USA). Total RNA (1 µg) was reverse-transcribed into cDNA according to the instructions of PrimeScript RT Master Mix (Takara Bio Inc., Shiga, Japan). Real-time PCR was performed to measure the mRNA levels of Kir6.1, Kir6.2, SUR1, SUR2A, SUR2B and GAPDH, an internal control, for each sample in separate wells in duplicate on an ABI-7300 Sequence Detection system using 2 µl of cDNA, 300 nM primers and SYBR-Green PCR Master Mix (all from Applied Biosystems, Foster City, CA, USA). The experiments were performed in accordance with the Animal Ethics Committee of the Second Military Medical University. All investigations were performed in triplicate. The data were assessed by SPSS version 11.5 (IBM SPSS, Armonk, NY, USA). All values were expressed as mean ± SD. Quantitative analyses of data were performed by one-way analysis of variance (ANOVA) and Student’s-t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

KATP subunit expression and subcellular localization. To investigate the role of KATP in mesangial cells, the expression of KATP subunit, including Kir6.1, Kir6.2, SUR1, SUR2A and SUR2B, was measured by quantitative real-time PCR. As shown in Fig. 1A, in all detected KATP subunit, the highest mRNA expression was detected in Kir6.1 and the lowest mRNA expression was detected in SUR1. Moreover, the expression of SUR in plasma membranes was also found using a laser scanning confocal microscopy incubated with BODIPY-glibenclamide and DiIC18(3) (Fig. 1B).

High glucose increases mesangial cell proliferation and release of MMP-2 and fibronectin. After different concentrations of high glucose (HG; 10, 20, 30 or 40 mM) treatment in mesangial cells, the cell proliferation was measured by CCK-8 assay. As shown in Fig. 2A, HG with concentration
ranges from 10 to 30 mM significantly increased mesangial cell proliferation in a dose-dependent manner in 12 and 24 h, while 40 mM HG inhibits mesangial cell proliferation, compared with control mesangial cells. The phenotypes of mesangial cells in response to 30 mM HG for 12 and 24 h are shown in Fig. 2B.
Furthermore, to investigate the effect of HG on matrix components, the production of MMP-2 and fibronectin was measured by ELISA. Our results showed that HG with concentration ranges from 10 to 30 mM significantly increased MMP-2 and fibronectin production in a dose-dependent manner, while 40 mM HG inhibits MMP-2 and fibronectin production, compared with control mesangial cells (Fig. 2C and D).

**DXZ inhibits high glucose-induced KATP subunit expression.** To investigate the effect of HG on KATP subunit, the expression of Kir6.1, Kir6.2, SUR1, SUR2A and SUR2B was detected. We found that 30 mM HG significantly decreased the mRNA expression of Kir6.1, SUR2A and SUR2B, but had no effect on Kir6.2 and SUR1 expression compared with controls (Fig. 3A). These results suggest that HG induced mesangial cell proliferation and MMP-2 and fibronectin production may be through inhibiting KATP channel activity. Therefore, a selective opener of KATP (DZX) and a selective inhibitor of KATP (5-HD) were also introduced in our study. As shown in Fig. 3B, DZX significantly decreased the mRNA expression of SUR1, SUR2A and SUR2B in HG-induced mesangial cells. However, the changes in SUR1, SUR2A and SUR2B mRNA expression were significantly reversed by 5-HD. Whereas, DZX or 5-HD treatment had no effect on the mRNA expression of Kir6.2 KATP subunit (data not shown).

**DXZ inhibits high glucose-induced mesangial cell proliferation and MMP-2 and fibronectin production.** As shown in Fig. 4A and B, DZX significantly decreased mesangial cell proliferation, while 5-HD increased mesangial cell proliferation under HD condition at 12 and 24 h with DZX pretreatment. Additionally, DZX significantly decreased the production of MMP-2 and fibronectin in HG-induced mesangial cells (Fig. 4C). However, the changes in MMP-2 and fibronectin production were significantly reversed by 5-HD.
Discussion

The KATP channel serves as a metabolic sensor, coupling cellular metabolism to electrical activity in a wide range of tissues. Opening of KATP channels under conditions of low metabolism leads to membrane hyperpolarization and switches off cellular functions, and close when metabolism increases, producing a membrane depolarization that leads to cellular responses such as hormonal secretion, neurotransmitter release and contraction, conversely (14). A previous result has indicated that KATP channels seem to play an essential role in murine myometrial motility via activation of SUR2B and Kir6.2 (20). Moreover, the main KATP channel in cardiac myocytes is composed of Kir6.2/SUR2A, and the primary role of KATP channels in heart is cardioprotection under metabolic stress, such ischemia, anoxia or metabolic inhibition (21,22). Despite the large amount of evidence confirming the role of KATP channels in cardioprotection during metabolic stress, the SUR1− and SUR2− knockout mice were found to be more tolerant of global ischemia-reperfusion than control mice (23,24). However, the roles of KATP channels in HG-induced DN are not elucidated. In the present study, we found that activating KATP channels significantly suppressed HG-induced cell proliferation and release of MMP-2 and fibronectin in cultured mesangial cells.

Several clinical studies have demonstrated that elevated blood glucose level was a risk for the development of microvascular complications of diabetes, including DN. Increased mesangial cell proliferation and excessive accumulation of ECM proteins synthesized by mesangial cells are major pathologic features in the early stage of DN (25,26). Our results showed that HG enhanced mesangial cell proliferation and release of MMP-2 and fibronectin in a dose-dependent manner range from 10 to 30 mM. As one of the most important ECM proteins, glucose-induced fibronectin expression in MCs results in the accelerated progression of glomerulosclerosis (27).

Previous studies have implied that MMPs play an important role in regulating physiological homeostasis and pathological disorders of the kidney through modulating the decomposition of ECM components, including fibronectin (28).

Most studies have focused on direct regulation, such as changes in the open probability or the ATP sensitivity of the channels (29), but little is known about how channel numbers at the surface membrane are regulated. Our results demonstrated that all the subunits of KATP were expressed in rat mesangial cell, and the SUR subunit was observed in plasma granules. Cell Metab 6: 217-228, 2007.

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In conclusion, the present study provides novel evidence that the impairment of KATP channels is associated with HG-induced multiple renal injuries, including increased mesangial cell proliferation and excessive accumulation of ECM proteins. Taken together, we could conclude that one of the molecular mechanisms involved in renal protective effects was activation of KATP channels. Our results also provided a potential target in treatment of DN.

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