

## Original Article

# Chronic exposure to high glucose enhances expression of mitochondrial carbonic anhydrase VA and induces apoptosis in renal tubular epithelial cells

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**Abstract:** Chronic hyperglycemia has been causally implicated in initiation and progression of diabetic kidney complications. There is growing evidence suggesting that chronic exposure to high glucose induces cell death but the underlying molecular mechanisms remain largely unknown. Here we examined the correlation between high glucose exposure, apoptosis and oxidative stress in renal proximal tubule epithelial cell line (TH1). Our result show that high dose of glucose induces caspase-3 cleavage, cytochrome c release and apoptosis in TH-1. Interestingly, chronic exposure to a high dose of glucose significantly enhances expression of mitochondrial Carbonic Anhydrase VA (CA5A) gene, which is associated with overproduction of free radicals and oxidant stress. Knockdown of CA5A potentially abolishes high glucose-induced production of reactive oxygen species (ROS), supporting its role in mediating oxidative stress caused by chronic hyperglycemia in kidney cells. As expected, reduced expression of CA5A significantly inhibits cytochrome c release and reduces cell death, supporting a link between oxidative stress and cell death in type 2 diabetes (T2D). This is in line with deregulation of mitochondrial redox balance being important in the pathogenesis of T2D. Together, these results support the role of CA5A in high glucose-induced oxidative stress and apoptosis, and suggest that CA5A is a putative therapeutic target for diabetic kidney complications.

**Keywords:** CA5A, high glucose, oxidative stress, apoptosis

## Introduction

Diabetic nephropathy (DN) is one of the common Diabetes-related complications as well as the leading cause of the major direct cause of death in patients with diabetes mellitus [1-3]. Over one-third of all patients with diabetes have DN, which is associated with long term damaging effects of kidney loss and enhanced risk of end-stage renal disease [4]. Previous studies suggest that several cellular dysfunctions and molecular defects are associated with T2D, such as  $\beta$ -cell malfunction, loss of insulin secretion and function, chronic hyperglycemia, as well as several other deregulations in systematic metabolism [5-7]. There is growing evidence suggesting that kidney dysfunction is tightly associated with cell injury and apoptosis induced by diabetes associated hyperglycemia [8]. It is well known that chronic hyperglycemia is toxic to various cell types in

patients with type 2 diabetes. However, the mechanism underlying cellular dysfunction and the resulting apoptosis via glucose toxicity are not fully characterized.

One possible mechanism is that excess glucose in diabetes leads to an increasing energy expenditure and oversupply of electrons in the mitochondrial transfer chain, resulting in mitochondrial membrane hyperpolarization and the overproduction of ROS [9, 10]. These free radicals cause cellular oxidative stress and induce the release of mitochondrial proapoptotic factors that activate the cysteine protease family of caspases and propagate the apoptotic signaling cascade [8, 10-13]. Accordingly, it has been suggested that oxidative stress induced by hyperglycemia is a key factor in the pathogenesis of diabetic complications. In line with this hypothesis, it has been shown that reducing oxidative stress in the diabetic animal model

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could largely prevent the deleterious effects of cell death caused by chronic hyperglycemia [14-17].

Tubular epithelial cells account for 90% of the total kidney volume. It has been shown that injury of renal tubular epithelial cells is closely related to the renal dysfunction in diabetic patients. In the current study, we investigated the mechanisms of high glucose-induced cell death in kidney cells [18]. We hypothesize that activation of pathways that rid the cells of the constitutively increased flux of glucose might enhance ROS formation and kidney injury in diabetic patients [11-13, 19, 20]. Given the tight link between high glucose-induced cell death and oxidative stress, we focused our research on CA5A gene, which is involved in the catalytic reaction of conversion of pyruvate to oxaloacetate [15, 21, 22]. Given its central role in regulating the respiration rate, CA5A and is considered to be a broad and powerful regulator of mitochondrial function and ROS production [15, 21]. In the present study, we determined a novel mechanism by which high dose of glucose induces the expression of a mitochondrial carbonic anhydrase CA5A, leading to overproduction of ROS, enhanced oxidative stress as well as induced cell death.

### Materials and methods

#### *Cell cultures*

Human renal proximal tubule epithelial cell line TH1 was cultured in DMEM and 10% FBS (Life Technologies) in a 5% CO<sub>2</sub>, 37°C incubator. For treatment with different doses of glucose, cells were cultured in six well plate (~10<sup>6</sup> cells per well) with basal medium (5 mM glucose) with or with high glucose (50 mM glucose) for up to five days.

#### *Flow cytometry analysis for apoptosis*

After treatment with normal glucose or high glucose for the indicated time, cells were harvested by 0.25% trypsin and cell precipitates were washed once with PBS. Apoptosis was determined using a FITC-Annexin V and propidium iodide (PI) double staining assay kit (Thermo Fisher) according to the manufacturer's instructions. Briefly, cells were resuspended in 200 µl binding buffer at density of 1×10<sup>6</sup>/mL, and then

incubated with Annexin V and PI reagents for 20 min at room temperature. Different fluorescent signals were counted with an Accuri C6 flow cytometry (BD Biosciences). One million cells were counted per sample and three parallel samples were measured.

#### *Knockdown of CA5A gene*

ShRNA targeting CA5A were cloned into pENTR/U6 using:

Top Strand: 5'-CACCGGGTACACGCCAGTCCAC-AGTCGAAACTGTGGACTGG CGTGTACCC-3' and Bottom Strand: 5'-AAAAGGGTACACGCCAGTCC-ACAGTTTCGACTGTGGACTGGCGTGTACCC-3'.

ShRNA targeting GFP was used as a control. Cells were transfected with control or CA5A shRNA vector and selected for two weeks with 1 µg/mL puromycin in the growth medium to establish stable cell lines.

#### *Immunofluorescence assay*

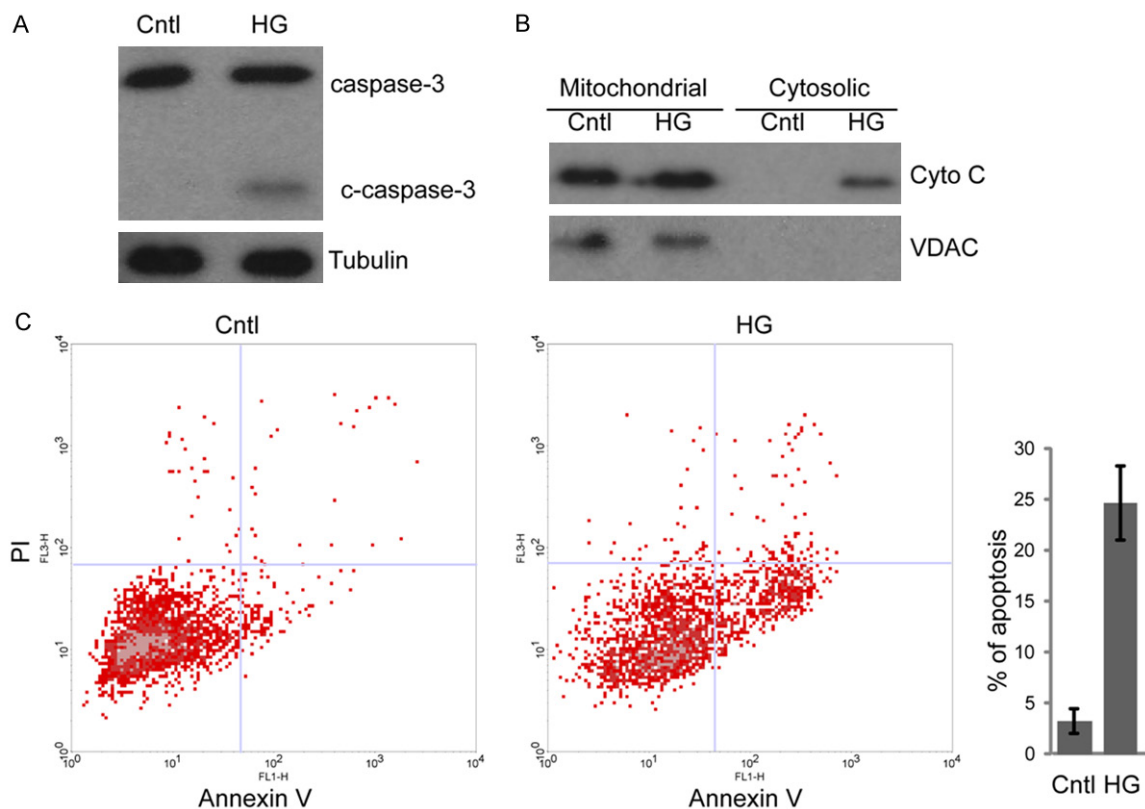
Cells were cultured on glass bottom mattek dish (MatTek) and were treated as indicated in the figures. After removing medium, cells were washed once with PBS, fixed in 4.0% formaldehyde, and then blocked with 5% BSA in PBS. Immunofluorescence staining was performed by incubating the fixed cells with anti-CA5A antibody (1:500) at four degree overnight, followed by incubation with FITC (fluorescein isothiocyanate)-conjugated secondary antibody (1:1000, Protein Tech). Cells were viewed with an Olympus FV1000 Laser Scanning Confocal Microscope (Olympus).

#### *Western blot analysis*

After indicated treatments, cells were washed with ice cold PBS, scraped and solubilized in RIPA buffer supplemented with protease and phosphatase inhibitors.

The whole cell lysates or nucleus/cytosol fractions were harvested and protein content was measured by the BCA assay. For western blotting, proteins were boiled in Laemmli buffer, separated by SDS-PAGE and transferred onto PVDF membrane. Membranes were incubated with primary antibodies at four degree overnight. CA5A antibody (Thermo Fisher, 1:1000),

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**Figure 1.** Chronic exposure to HG induces apoptosis in TH-1 cells. A. Western-blot of caspase 3 and cleaved caspase 3 in cells treated with normal and high dose of glucose for five days. B. Western-blot of cytochrome (Cyto) C release from mitochondria to cytosol in cells treated with HG. VDAC was used as mitochondria marker. C. Flow cytometric analysis of cells treated with normal or high glucose. Histogram of cell apoptotic percentage are presented as the mean  $\pm$  standard deviation (n=3).

caspase-3 antibody (Abcam, 1:2000), Cyto-c antibody (Abcam, 1:2000), VDAC (Abcam, 1:2000) and Tubulin (Sigma, 1:3000) were used.

### Measurement of ROS production

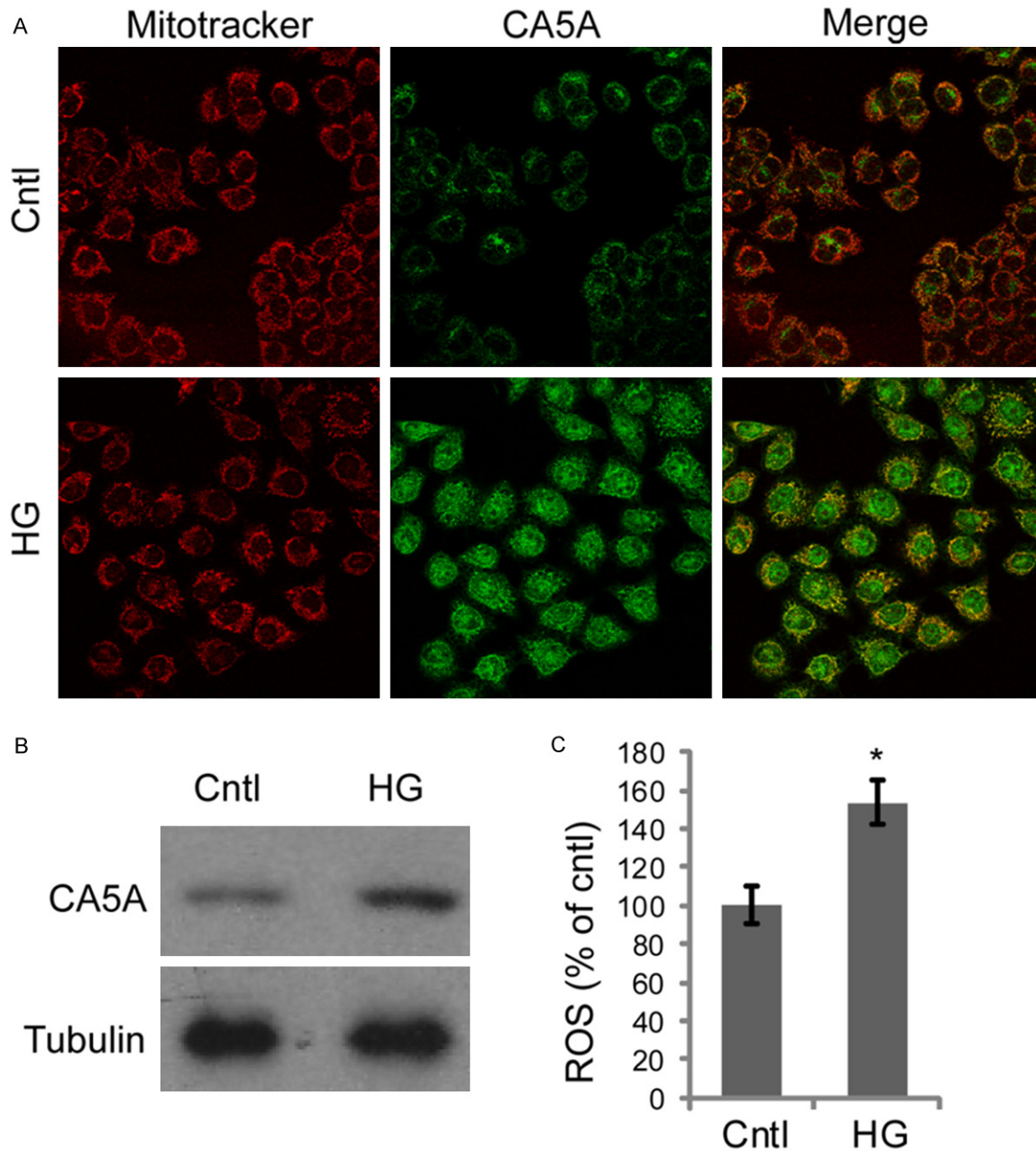
Intracellular ROS production was measured using a ROS activity assay kit (Cell Meter™ Fluorimetric Intracellular Total ROS activity assay kit, Thermo Fisher) according to the manufacturer's instructions. Briefly, cells were seeded in Costar black wall/clear bottom 96-well plate at a density of  $1 \times 10^5$  cells per well in 100  $\mu$ l of growth media with different dose of glucose. After treatment, 100  $\mu$ l of assay loading solution was added to each well and the incubations were continued in a 5% CO<sub>2</sub>, 37°C incubator for 1 hour. Fluorescence at excitation and emission wavelengths of 490 and 520 nm, respectively, were measured using a fluorescence plate reader (BioTek). Each sample was run in triplicate and the mean value and SD were calculated.

### Results

#### *Chronic exposure to high glucose induces cell death in TH-1 cells*

Previous studies suggest that renal tubular epithelial cell injury is causally linked to the pathogenesis of DN. To examine whether treatment of high glucose (HG) directly induces cell death in epithelial cells, TH-1 cells were treated with normal (5 mM) or high dose (50 mM) of glucose for five days. As shown in **Figure 1A**, exposure to HG induced marked activation and cleavage of Caspase-3, indicating induced apoptosis in these cells. In line with this result, a significant mitochondrial release of cytochrome C (Cyto C) into the cytosol was also observed. Furthermore, apoptosis rates of TH-1 cells treated with different dose of glucose were determined by Annexin V/PI staining followed by flow cytometry (**Figure 3C**). The results showed that chronic exposure to HG significantly increased the rate of apoptotic cells as compared with control

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**Figure 2.** Chronic exposure to HG enhances CA5A expression and ROS production. A. Immunofluorescence analysis of CA5A expression in cells treated with normal or high glucose. Mitochondria are marked with Mitotracker. B. Western-blot analysis of CA5A expression in cells treated with normal or high glucose. C. Histogram of ROS production presented as percentage of control cells. Data are presented as the mean  $\pm$  standard deviation (n=3).

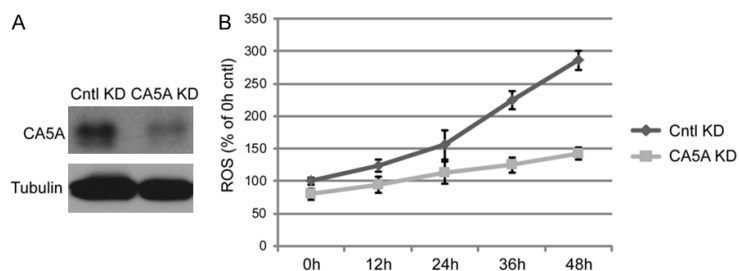
cells ( $24.63 \pm 3.63\%$  vs.  $3.25 \pm 1.25$ ,  $P < 0.001$ , Student's t-test).

Together, these results provide evidence that chronic exposure to HG could directly induce cell death in TH-1 Cells, which might be causally linked to injury of renal tubular epithelial cells as well as dysfunction of renal tube in diabetic patients with DN.

*Chronic exposure to high glucose enhances the expression of CA5A and oxidative stress*

It is well known that enhanced glucose flux might alter cellular metabolism and induce oxidative stress in the cell, leading to apoptosis. In response to global metabolic disturbances, the cells may alter the expression of a number of key regulators in metabolic pathways. We then

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**Figure 3.** CA5A knockdown abolishes HG-induced ROS production. A. Western-blot analysis of CA5A expression in control knockdown and CA5A knockdown cells. B. ROS production in CA5A or control knockdown cells overtime. Data are presented as the mean  $\pm$  standard deviation ( $n=3$ ).

asked whether chronic exposure to high glucose affects the expression of a key regulator of mitochondrial respiration, namely, CA5A. Under normal condition, CA5A mainly colocalized with mitochondrial marker, Mito-Tracker CMXRos. Interestingly, after chronic exposure to HG for five days, we observed a significant increase in CA5A signal in TH-1 Cells (**Figure 2A**). Interestingly, some CA5A proteins were released from mitochondrial to cytosol and nucleus, probably as a result of permeabilization of the mitochondrial membrane during apoptosis, which is similar to Cyto C release. Consistently, enhanced expression of CA5A was confirmed by western-blot (**Figure 2B**).

Abnormal glucose metabolism induced by high glucose and overexpression of CA5A might result in an abnormal increase of ROS production and oxidative stress. To test this hypothesis, we measured intracellular ROS in normal control cells and those treated with high HG. As expected, we observed a significant increase in ROS production in HG treated cells as compared with control ( $100 \pm 9.85\%$  vs.  $153.6 \pm 11.45\%$ ,  $P < 0.001$ , Student's t-test).

### *CA5A is a critical regulator of ROS production in cells treated with HG*

We further examined whether the enhanced respiration rate and ROS overproduction were regulated by upregulation of mitochondrial CA5A. To this end, using shRNA-based gene silencing, we established stable TH-1 cell line with efficient knockdown of CA5A (**Figure 3A**). CA5A knockdown cells and control knockdown cells were treated with HG for up to four days and ROS production were monitored. As expected, CA5A silencing resulted in significantly reduced ROS production overtime upon treatment with HG (**Figure 3B**). This result provided

direct evidence that CA5A is playing a critical role in regulating intracellular glucose metabolism and ROS production. As oxidative stress resulted from increased ROS production may immediately lead to mitochondrial dysfunction and renal damage, we thus suggested that CA5A might play a critical role in the pathogenesis of DN in patients with diabetes.

### *CA5A knockdown attenuates HG-induced apoptosis*

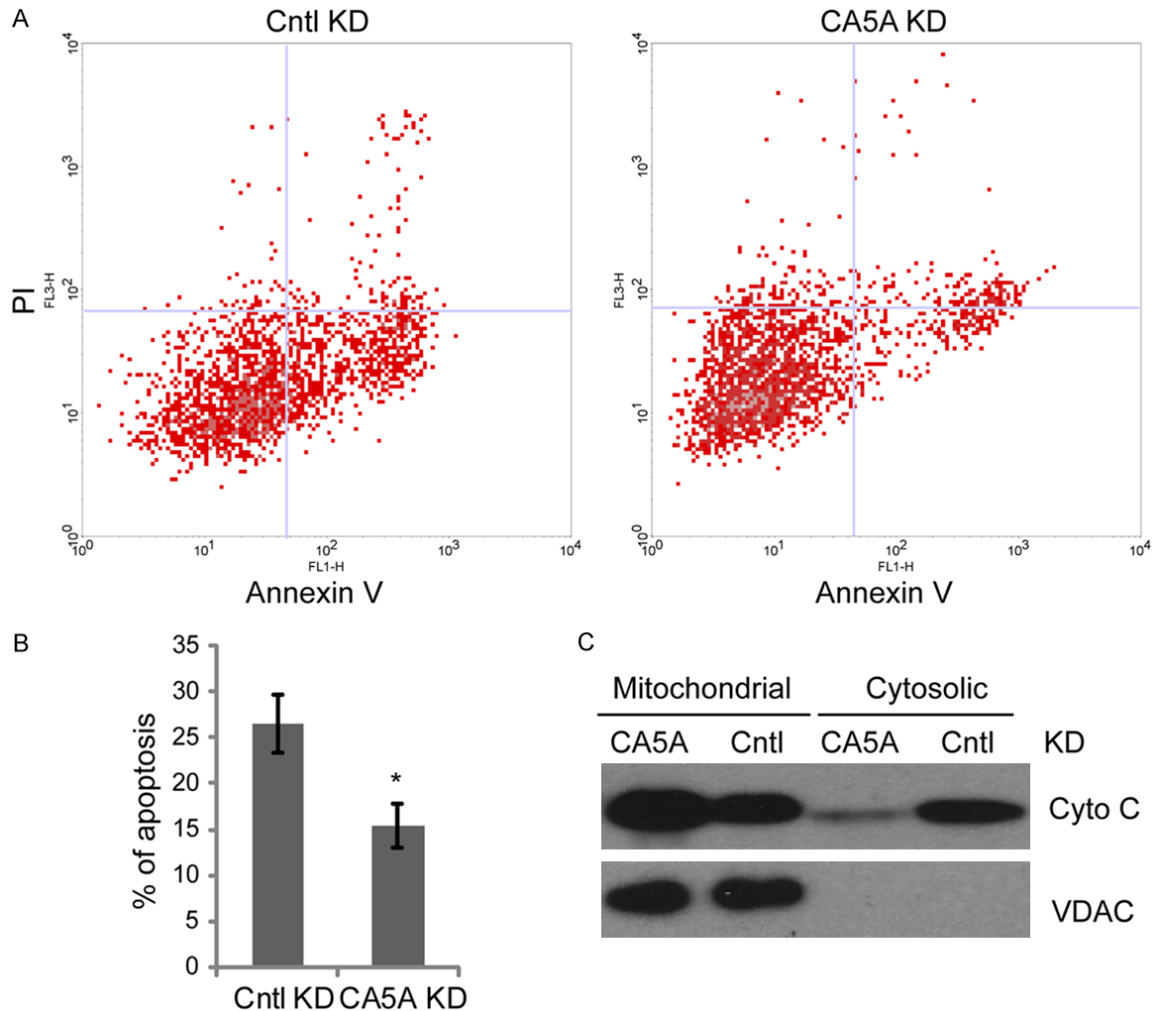
As overproduction of ROS is immediately relative to cell death, CA5A knockdown may reduce ROS production and attenuate HG-induced apoptosis, leading to longevity and acute stress resistance of renal tubular epithelial cells. This prediction we proved is true. As shown in **Figure 4A** and **4B**, FACS results suggested that CA5A knockdown significantly reduced the proportion of apoptotic cells upon HG treatment as compared with control knockdown ( $26.53 \pm 3.22\%$  vs.  $15.16 \pm 2.39\%$ ,  $P < 0.001$ , Student's t-test). Consistently, knockdown of CA5A potentially reduced Cyto C release from mitochondria to cytosol following HG treatment (**Figure 4C**).

These results suggest that overexpression of CA5A induced by HG increases the susceptibility of cells to apoptosis by increasing ROS. Accordingly, CA5A overexpression and oxidative stress are detrimental for renal tubular epithelial cells and may contribute to the vascular complications of diabetes. As a result, pharmacological inhibition or silencing of CA5A may have a protective effect against high glucose-induced tubular damage by slowing the rate of respiration, blocking excess ROS production and inhibit epithelial cell death.

## Discussion

The pathogenesis of DN, which eventuates in renal failure in diabetic patients, is very complex and has not yet been fully elucidated. Abnormally increased protein trafficking through the glomerulus marks the onset of DN. Other pathological features of the disease include renal hypertrophy, basement membrane thickening, matrix protein accumulation as well as tubulointerstitial fibrosis. Previous studies

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**Figure 4.** CA5A knockdown rescued HG-induced cell death. A. Flow cytometric analysis of CA5A knockdown or control knockdown cells treated with high dose of glucose. B. Histogram of cell apoptotic percentage in (A) presented as the mean  $\pm$  standard deviation (n=3). \*P<0.01, student's t-test. C. Western-blot of cytochrome (Cyto) C release from mitochondria to cytosol in CA5A knockdown or control knockdown cells treated with HG. VDAC was used as mitochondria marker.

suggested that damage to the renal proximal tubular epithelial cell plays an important role in the pathogenesis of the disease. Chronic exposure to hyperglycemia and high concentration of glucose may activate a number of metabolic signaling pathways and induce oxidative stress, leading to epithelial cell death [18, 23]. Examining the effect of high dose of glucose on expression of proteins involved in respiration over time, we observed an acute increase in CA5A expression in TH-1 cells. Knockdown of CA5A cells significantly reduced ROS production and inhibited HG-induced cell death, indicating that CA5A plays an important role in mediating tubular epithelial cell damage in diabetic patients.

Our result is supported by several previous studies in vivo and in vitro. It has been shown that pharmacological inhibition of CA5A rescues hyperglycemia-induced cerebral pericytes apoptosis in mouse model. CA5A silencing also reduces HG-induced oxidative stress and apoptosis in cultured brain pericytes in vitro. Consistently, overexpression of CA5A significantly increased oxidative pressure and pericyte cell death in cell culture. The role of elevated ROS in the pathogenesis of microvascular complications of diabetes has been well documented. In our study, we showed that knockdown of CA5A using shRNA resulted in significant Caspase-3 activation as well as release of Cyto C from mitochondria, providing strong evidence that

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high expression of CA5A is sufficient to induce kidney injury. Collectively, these data and our research support a pivotal role of CA5A in regulating ROS production and renal epithelial cell death in diabetes-induced renal dysfunction.

It is noteworthy that the acute and chronic effects of high glucose may have different effect on cell proliferation and apoptosis. It has been shown that in vitro, short exposure to high glucose can promote DNA synthesis and cell cycle progression from G1 to S phase, leading to enhanced cell proliferation [24]. In contrast, our study demonstrated that chronic exposure to high glucose could result in accumulation of ROS overtime and lead to cell death in the long run, consistent with that long-term prognosis of DN is associated with the severity of tubulointerstitial damage.

Accordingly, the early invention that inhibit CA5A abnormal activation in diabetic patients may have a protective role in impairing diabetic renal tubular epithelial cell death and help to control the progression of DN. For example, CA5A inhibitor Topiramate may potentially be used to inhibit oxidative stress and to protect the microvasculature of the kidney from hyperglycemia-induced damage [15]. This provides a prove-of-the-concept that key regulators of gluconeogenesis and oxidative stress could be promising therapeutic targets for diabetic complications such as DN.

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### Disclosure of conflict of interest

None.

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