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The mitochondrial Na^+/Ca^{2+} exchanger may reduce high glucose-induced oxidative stress and nucleotide-binding oligomerization domain receptor 3 inflammasome activation in endothelial cells

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Abstract

Background The mitochondrial Na^+/Ca^{2+} exchanger, NCLX, plays an important role in the balance between Ca^{2+} influx and efflux across the mitochondrial inner membrane in endothelial cells. Mitochondrial metabolism is likely to be affected by the activity of NCLX because Ca^{2+} activates several enzymes of the Krebs cycle. It is currently believed that mitochondria are not only centers of energy production but are also important sites of reactive oxygen species (ROS) generation and nucleotide-binding oligomerization domain receptor 3 (NLRP3) inflammasome activation. **Methods & Results** This study focused on NCLX function, in rat aortic endothelial cells (RAECs), induced by glucose. First, we detected an increase in NCLX expression in the endothelia of rats with diabetes mellitus, which was induced by an injection of streptozotocin. Next, colocalization of NCLX expression and mitochondria was detected using confocal analysis. Suppression of NCLX expression, using an siRNA construct (siNCLX), enhanced mitochondrial Ca^{2+} influx and blocked efflux induced by glucose. Unexpectedly, silencing of NCLX expression induced increased ROS generation and NLRP3 inflammasome activation. **Conclusions** These findings suggest that NCLX affects glucose-dependent mitochondrial Ca^{2+} signaling, thereby regulating ROS generation and NLRP3 inflammasome activation in high glucose conditions. In the early stages of high glucose stimulation, NCLX expression increases to compensate in order to self-protect mitochondrial maintenance, stability, and function in endothelial cells.

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1 Introduction

Diabetes mellitus is a leading cause of death globally and a chronic disability, resulting in enormous social and economic burden. Angiopathy is a major contributor to the progression of diabetes and its associated complications.[1] While the pathophysiology of diabetic endothelial dysfunction is incompletely characterized, it seems to be multifactorial. In the past few decades, hyperglycemia-induced oxidative stress has been increasingly identified as a trait of diabetic vasculature.[2,3] Mitochondria are well known as storehouses for intracellular calcium, a source of reactive oxygen species (ROS), and a sensor of oxidative stress.[4, 5] Furthermore, mitochondrial Ca^{2+}($[Ca^{2+}]_{mito}$) can be dynamically changed upon stimulation by various ligands, for example, during high glucose (HG) conditions, $[Ca^{2+}]_{mito}$ plays an important role in regulating mitochondrial as well as cellular functions, such as oxidative stress,[6] inflammasome activation[7, 8] and apoptosis.[9] Recent findings suggest that many stimuli engage Ca^{2+} signaling as an intermediate step to trigger mitochondrial instability, and result in generating mitochondrion-associated ligands to activate the NLRP3 inflammasome.[10] Thus, it is essential to fully understand how glucose-dependent $[Ca^{2+}]_{mito}$ is regulated and how it affects mitochondrial and cellular functions in endothelial cells.

Ca^{2+} enters the mitochondria mainly via a Ca^{2+} selective channel and the Ca^{2+} uniporter, and this is driven by a large negative membrane potential.[11–13] $[Ca^{2+}]_{mito}$ is extruded by the Na^+-Ca^{2+}exchanger and/or H^+-Ca^{2+} exchanger.[14,15] Although the activity of the mitochondrial Na^+/Ca^{2+} exchanger was discovered more than 40 years ago, its molecular identity and mechanisms had remained unclear, until 2009 researchers found that NCLX protein (previously known as

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NCKX6) is a long-searched-for mitochondrial Na^+/Ca^{2+} exchanger, which appears to be a gene product of mammalian *SLC8B1*.[16, 17]

The amplitude and duration of $[Ca^{2+}]_{mito}$ elevations reflect the balance between uptake and release mechanisms.[18] Prolonged accumulation of $[Ca^{2+}]_{mito}$ in the matrix space can lead to mitochondrial $[Ca^{2+}]_{mito}$ overload, resulting in the outbreak of oxidative stress and the activation of cell death signals.[5,19] To avoid this, mitochondria possess two membrane systems to extrude Ca^{2+}: the Na^+-Ca^{2+}exchanger and the H^+-Ca^{2+} exchanger. However, De Marchi, *et al*.[20] found that NCLX protein, but not the H^+-Ca^{2+} exchanger, mainly mediated $[Ca^{2+}]_{mito}$ extrusion. These new findings present novel opportunities to study the contributions of $[Ca^{2+}]_{mito}$ to cellular and systemic activity in health and disease.

In this study, we found an increase in the amount of the mitochondrial Na^+/Ca^{2+} exchanger, NCLX, in rats with endothelial diabetes mellitus, which is induced by injection of streptozotocin. Next, we examined whether NCLX expression modulates oxidative stress in HG conditions. Suppression of NCLX expression, using a siRNA construct (siNCLX), enhanced $[Ca^{2+}]_{mito}$ influx and blocked efflux induced by glucose. Unexpectedly, silencing of NCLX expression induced ROS generation and increased activation of the NLRP3 inflammasome.

2 Methods

2.1 Reagents

Cell culture media, supplements, MitoTracker® and Lipofectamine™ RNAiMAX were obtained from Invitrogen. Primary antibodies were obtained from Sigma-Aldrich [anti-actin mouse monoclonal antibody (mAb)], Proteintech [anti-NCLX rabbit polyclonal antibody (pAb)], Abcam (anti-Caspase-1 rabbit pAb, anti-NLRP3 rabbit pAb) and Santa Cruz [anti-PECAM-1 (CD31) mouse mAb]. Secondary antibodies were obtained from Jackson ImmunoResearch Laboratories. Fluorescein isothiocyanate (FITC) and cyanine 3 (CY3) immunofluorescence secondary antibodies were obtained from Beyotime Institute of Biotechnology. SiGENOME SMARTpool Rat Slc24a6 (Lot#130809) was obtained from Thermo Scientific Dharmacon.

2.2 Animals

Male Wistar rats were intraperitoneally (i.p.) injected with streptozotocin (STZ,65 mg/kg) (Sigma-Aldrich, USA), which was freshly dissolved in 0.9% saline, to induce diabetes mellitus (DM). Rats were considered diabetic if their blood glucose was > 16.7 mmol/L. The rats were anesthetized with an injection of sodium pentobarbital (40 mg/kg, i.p.), and all efforts were made to minimize suffering, after maintaining the DM rat for one month. The descending thoracic aorta was isolated and cleaned from the surrounding adipose and connective tissues. The 0.3 to 0.5 cm long aorta was trapped using Tissue-Tek O.C.T Compound (Sakura, CA, USA) in liquid nitrogen. The tissue sample was then cryo-sectioned.

2.3 Isolation, culture, and transfection of rat aortic endothelial cells

Male Wistar rats were anesthetized with an injection of sodium pentobarbital (40 mg/kg, i.p.), and all efforts were made to minimize the suffering. Briefly, the aorta was isolated from the abdominal cavity of the rat. The aorta was cleaned, opened longitudinally, cut into 0.2 to 0.3 cm sections, and placed with the intimal side down onto Matrigel-coated plates, in growth medium RPMI-1640 containing 15% fetal bovine serum (FBS), 0.5 ng/mL of vascular endothelial growth factor (VEGF) (PeproTech Lot#1107436) and 100 U/mL of penicillin-streptomycin. Rat aortic endothelial cells (RAECs) were incubated in a humidified atmosphere containing 5% CO_2, at 37°C. The growth medium RPMI-1640 was added every second day to prevent drying out. After 50–60 h, the pieces were removed and the cells were harvested. The cells were passaged when they reached 90% confluence. The identity of RAECs was confirmed by a positive immunofluorescence staining of platelet endothelial cell adhesion molecule (PECAM-1). RAECs were grown in RPMI-1640 medium supplemented with 10% FBS after a large number of RAECs were harvested. RAECs were transfected for 48 h with siNCLX or siControl using Lipofectamine™ RNAiMAX, according to the manufacturer's reverse transfection protocol. Transfection efficiency was determined using western blot analyses.

2.4 Western blotting analysis

The RAECs were lysed in radioimmunoprecipitation assay (RIPA) buffer [50 mmol/L Tris–HCl (pH 7.6), 5 mmol/L EDTA, 150 mmol/L NaCl, 0.5% NP-40, 0.5% Triton-X-100] containing 1 g/mL leupeptin, 1 g/mL aprotinin, and 1 g/mL antipain, and 0.5 mmol/L phenylmethanesulfonylfluoride (PMSF). Sample protein concentrations were measured using the BCA Protein assay. Next, 50 g of total protein was separated using 8% or 10% SDS–PAGE and then transferred onto a membrane. The membrane was blocked with 5% skimmed milk, probed with a primary antibody overnight at 4°C, and incubated with a horseradish peroxidase-conjugated secondary antibody. Proteins were detected by chemiluminescence using the enhanced chemilumines-

cence (ECL) reagent (Pierce, Rockford, IL, USA). Densitometry was performed using Quantity One (BIO-RED) analysis software. β-actin was used as the normalization control.

2.5 Measurement of interleukin-1β (IL-1β) concentration

The IL-1β concentration of the medium was measured using a colorimetric sandwich ELISA kit (IL-1β Quantikines ELISA kit) from R&D systems according to the manufacturer's instructions. The absorbance was measured at 490 nm using a microplate reader.

2.6 Measurement of $[Ca^{2+}]_{mito}$ using laser confocal microscopy

Changes in $[Ca^{2+}]_{mito}$ were detected through incubation for 30 min, at 37°C in the dark, with the Ca^{2+} probe Rhod-2 AM (Invitrogen) at a final concentration of 4 μmol/L. After incubation, the cells were washed twice with Hanks' Balanced Salt Solution (with Ca^{2+}&Mg^{2+}) (Beyotime Institute of Biotechnology) to remove the probes, and were analyzed using laser confocal microscopy. Fluorescence images were captured using a laser confocal system mounted on an inverted microscope equipped with an argon–krypton laser. Rhod-2 AM fluorescence (excitation at 559 nm) was detected at a wavelength of 577 nm.[21] In this glucose dependent experiment, the cells were pre-washed for 30 min with low glucose (5.56 mmol/L) containing HBSS, followed by HG (35 mmol/L) containing HBSS. The specific $[Ca^{2+}]_{mito}$ line-scanning method was performed over a single horizontal position consisting of 160 lines at 5 s intervals. The Ca^{2+} level was expressed as a pseudo-ratio value (F/F_0) of the actual fluorescence intensity (F) divided by the basal intensity of the $[Ca^{2+}]_{mito}$ at rest (F_0). F_0 was calculated as the average value obtained during the 50–60 s prior to stimulus application.[22]

2.7 Measurement of total intracellular ROS levels

Changes in intracellular ROS levels were detected using the oxidant-sensing fluorescent probe: dichlorofluorescin diacetate (DCFDA), in the DCFDA Cellular ROS Detection Assay Kit (ab113851). This probe was loaded into previously subcultured RAECs, at a final concentration of 25 μmol/L, and the cells were then cultured for 45 min at 37°C, in the dark. After incubation, the culture medium was washed twice with a 1× buffer solution to remove the dyes, and analyzed using laser confocal microscopy. An excitation wavelength of 488 nm (argon laser) and an emission wavelength of 519 nm were used. The ROS line-scanning method was performed over a single horizontal position consisting of 50 lines at 10 s intervals. Changes in ROS levels were expressed as a pseudo-ratio value (F/F_0) of the actual fluorescence intensity (F) divided by the basal intensity of the ROS at rest (F_0). F_0 was calculated as the average value obtained during the 50–60 s prior to stimulus application.[23,24]

2.8 Statistical analysis

Traces from all the fluorescent imaging experiments were plotted using Olympus Fluoview (ver.2.1b FV1000). Influx of Ca^{2+} into mitochondria consistently began after stimulation of RAECs with HG. Similarly, Ca^{2+} efflux out of the mitochondria followed the influx phase, as previously described. Hence, the fluorescent ratio signals were normalized to the average signal obtained at the beginning of the measurements. The influx and efflux rates were calculated as the slope of the linear fit of the fluorescence change during the 30 s following the administered HG.[16]

Data were expressed as mean ± SD of at least three independent experiments, using 15–30 cells in each experiment. The statistical significance for all experiments was determined using one-way ANOVA test followed by Bonferroni post-hoc analysis. $P < 0.05$ was considered statistically significant.

3 Results

3.1 Increased NCLX expression in RAECs by high glucose stimulation

Recently, NCLX was identified as the mitochondrial Na^+/Ca^{2+} exchanger in several cell types.[16, 25]

We first investigated whether NCLX was expressed in rat aortic endothelia, using double immunofluorescence labeling. Confocal microscopy analysis was performed on aorta cryosections. PECAM-1(CD31) was used to mark the endothelial level (red) (Figure 1A). Next, we found that NCLX expression was significantly increased in DM RAECs. This was consistent with the *in vitro* results for the RAECs, which were induced by HG (HG, 35 mmol/L) stimulation. Western blotting analysis ($P < 0.05$, Figure 1B) detected a major band of 100 kDa and a weaker band at 50 kDa, which correlated to the SDS-stable NCLX. Next, immunofluorescence double staining was performed using MitoTracker®, which indicated that NCLX was expressed in the mitochondria of RAECs[26] (Figure 1C, Pearson's colocalization coefficient = 0.68 ± 0.06, $n = 15$), a result consistent with previous studies.[27]

3.2 The role of NCLX in high glucose-induced $[Ca^{2+}]_{mito}$ transport and ROS formation

NCLX knockdown using siRNA caused a 47% ± 13.61%

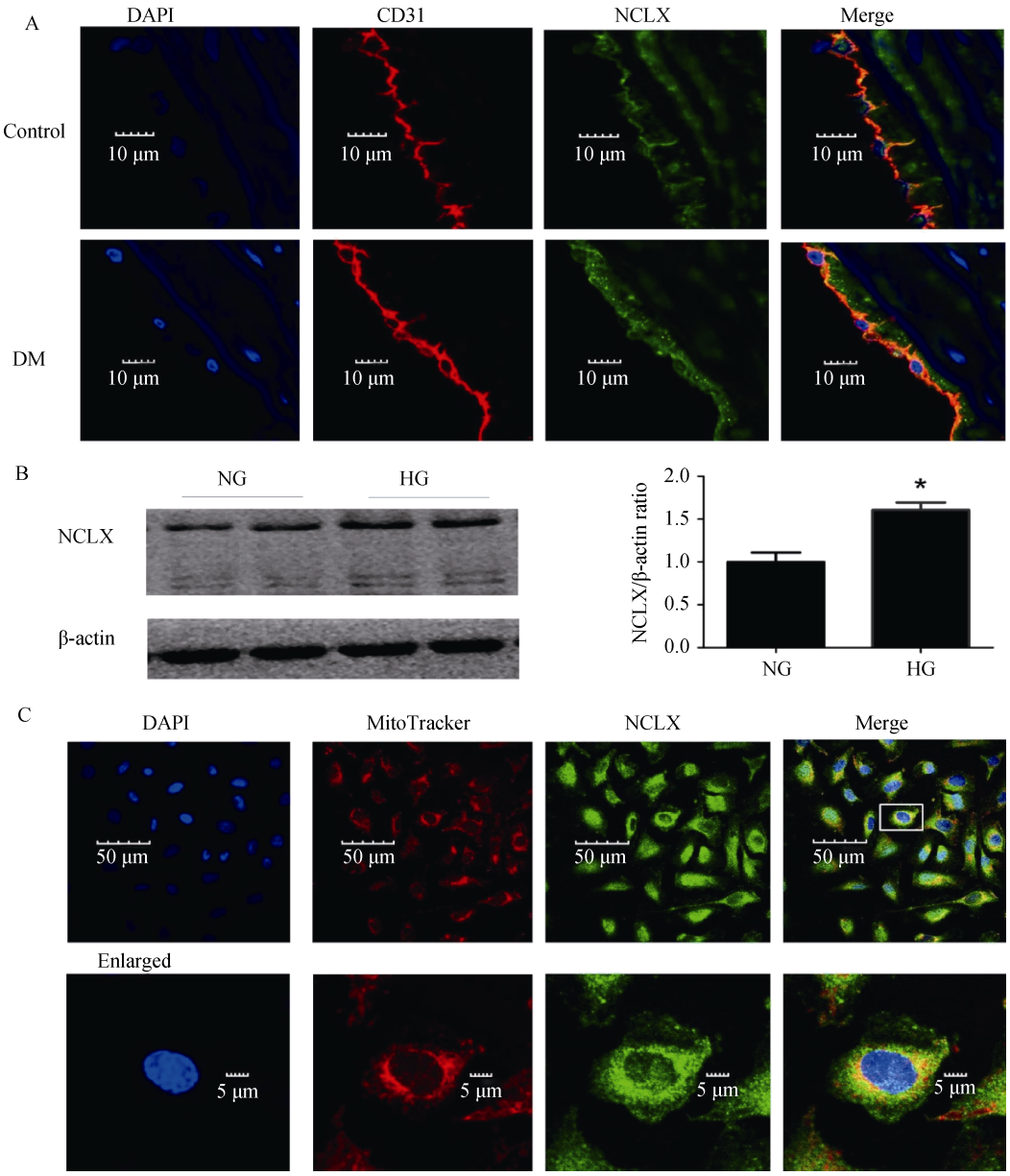

Figure 1. The expression and location of NCLX. (A): NCLX is expressed in rat aortic endothelial and is further increased in DM rats; (B): Western blot analysis of NCLX expression in confluent RAECs which were cultured in medium containing normal glucose (5.5 mmol/L) or high glucose (35 mmol/L) levels; and (C): NCLX (green) and Mito Tracker (red) were co-localized. The nucleus was stained by DAPI (blue). DM: diabetes mellitus; HG: high glucose; NG: normal glucose; RAECs: rat aortic endothelial cells. *$P < 0.05$.

reduction in NCLX protein expression (Figure 2A). $[Ca^{2+}]_{mito}$ was monitored in RAECs, which were initially superfused with low glucose (5.5 mmol/L), followed by application of HG (35 mmol/L) with HBSS (with Ca^{2+} and Mg^{2+}). Application of HG triggered robust mitochondrial Ca^{2+} transients (Figure 2B). Knockdown of NCLX expression was followed by a time-dependent increase in Ca^{2+} influx (Figure 2C) and a time-dependent decrease in Ca^{2+} efflux (Figure 2D). Silencing of NCLX expression blocked glucose-dependent $[Ca^{2+}]_{mito}$ efflux. Comparison of the peak amplitude of $[Ca^{2+}]_{mito}$ signals of siNCLX *vs*. siControl, using HG stimulation, demonstrated an increase in cells transfected with siNCLX (Figure 2E).

Mitochondria are important sites of ROS generation, and numerous studies have confirmed that HG levels generate ROS.[28,29] Next, we determined the effect of NCLX expression on ROS production. ROS production was markedly increased by HG stimulation, in the siNCLX group (Figure 2F).

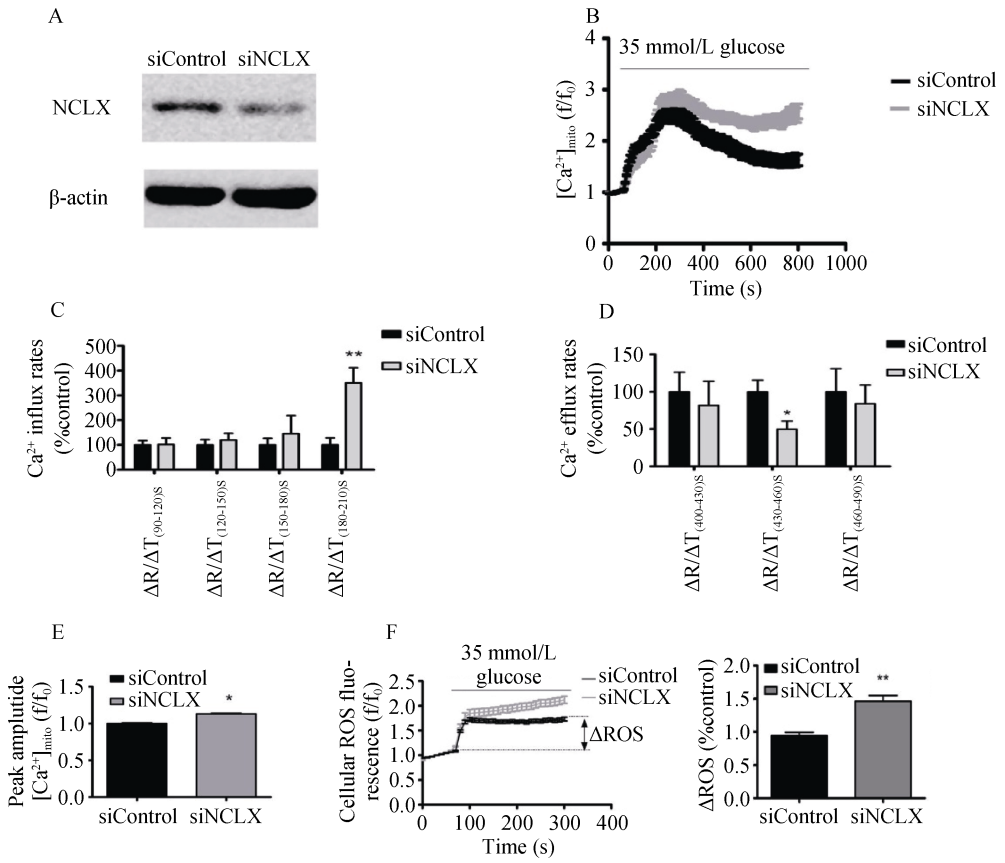

Figure 2. The role of NCLX in high glucose-induced $[Ca^{2+}]_{mito}$ transport and ROS formation. (A): Western blotting analysis of NCLX expression in siNCLX *vs*. siControl (50 μg) transfected RAECs; (B): silencing of NCLX expression blocks the glucose dependent mitochondrial Ca^{2+} efflux; (C): time–dependent average rates of mitochondrial Ca^{2+} influx after high glucose conditions, $n = 15$ ($P_{\Delta T180\text{-}210s} < 0.01$); (D): time–dependent average rates of mitochondrial Ca^{2+}efflux, $n = 15$ ($P_{\Delta T430\text{-}460s} < 0.05$); (E): average mitochondrial Ca^{2+} response peak amplitude, $n = 15$ ($P < 0.05$); (F): silencing of NCLX expression makes total intracellular ROS response more severe *vs*. siControl, by stimulation with high glucose levels ($P < 0.01$). Data are represented as the mean ± SD, of four independent experiments for each group. RAECs: rat aortic endothelial cells; ROS: reactive oxygen species; siNCLX: siRNA construct. *$P < 0.05$; **$P < 0.01$.

3.3 Knockdown of NCLX expression results in activation of the NLRP3 inflammasome

RAECs, which were cultured in medium containing normal glucose (NG), were used as the control group. We first compared the changes in total intracellular ROS production and NLRP3 expression in RAECs treated with siNCLX or siControl, which were cultured in HG (35 mmol/L) for 24 h. Total intracellular ROS production and NLRP3 expression were significantly increased in HG+siNCLX *vs*. HG+siControl cells (Figure 3 A&B). However, the change between the NG and the HG+siControl group was not notable. We next determined caspase-1 expression and IL-1β release, and there is the same result with NLRP3 expression among the three groups (Figure 3 C&D).

4 Discussion

The major findings of the present study include evidence that demonstrates that the mitochondrial Na^+/Ca^{2+} exchanger, NCLX, is richly expressed in rat aortic endothelial cell mitochondria. NCLX expression in RAECs was increased in HG conditions. Unexpectedly, knockdown of NCLX induced ROS generation and increased activation of the NLRP3 inflammasome. The results of our study demonstrated that NCLX was linked to elevated glucose, ROS generation, and activation of the NLRP3 inflammasome by mediating $[Ca^{2+}]_{mito}$ efflux. Our findings indicated that NCLX may protect against oxidative stress and inflammasome activation in HG conditions at an early stage.

NCLX is a member of the NCX family and is phylogenetically and functionally distinct from other known NCX or NCKX family members.[30,31] The abbreviated term NCLX stands for Na/Li/Ca exchanger. Palty, *et al*.[16] found that NCLX can transport either lithium (Li^+) or sodium (Na^+), in exchange for Ca^{2+}, while the plasma membrane exchangers NCX and NCKX do not transport Li^+. Bongju

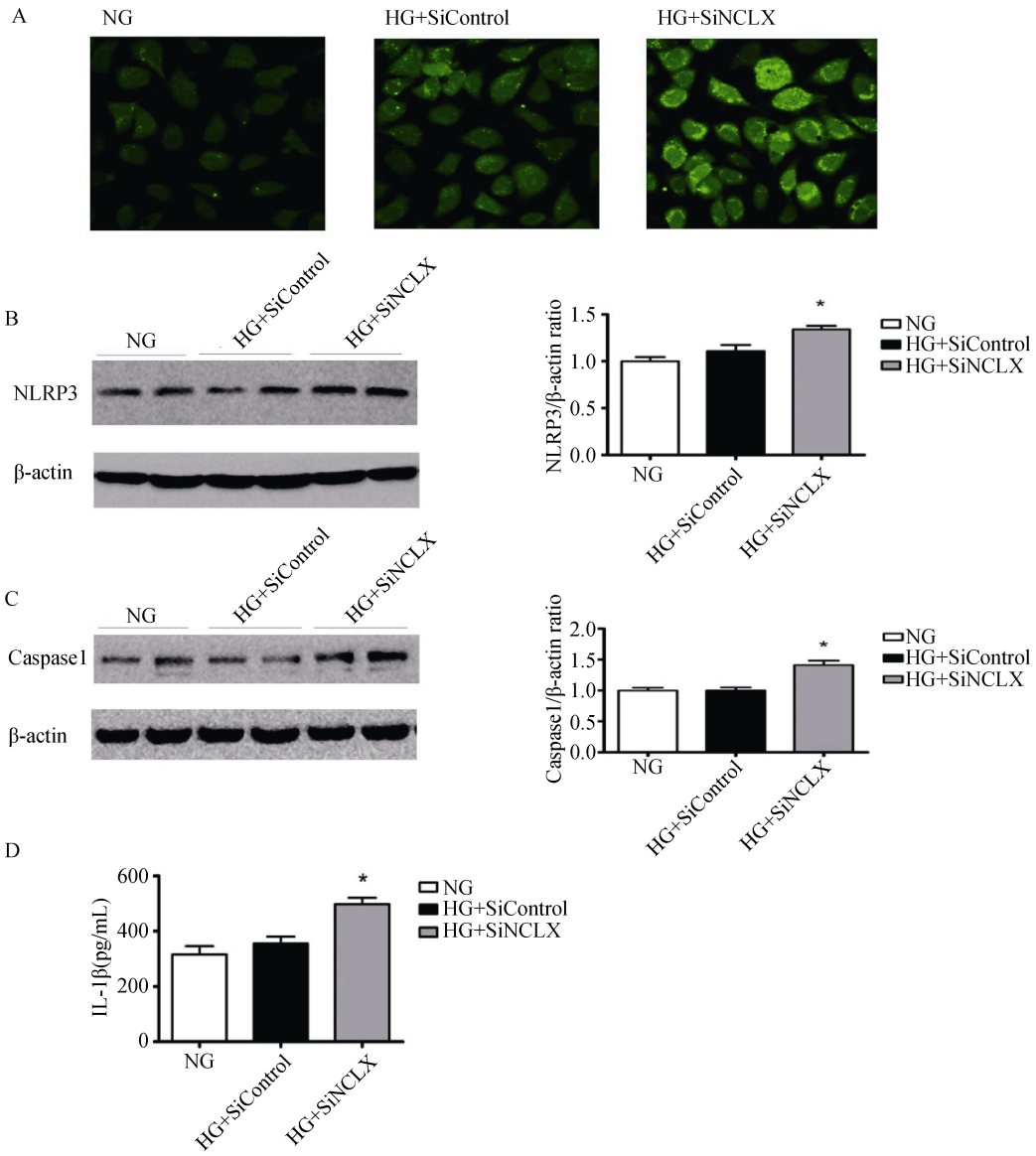

Figure 3. NLRP3 inflammasome activation. (A): Representative images confocal microscopy for total intracellular ROS production; (B & C): Western blotting analysis of NLRP3 and caspase-1 expression ($P < 0.05$); (D): ELISA of IL-1β secretion ($P < 0.05$). HG: high glucose; NG: normal glucose; NLRP3: nucleotide-binding oligomerization domain receptor 3; siNCLX: siRNA construct; ROS: reactive oxygen species. *$P < 0.05$.

Kim's experiments using EGFP-labeled NCLX in NIH/3T3 fibroblasts suggested that NCLX was exclusively expressed in mitochondria. Our experimental data were not only consistent with these previous studies, but also demonstrated for the first time that NCLX was expressed in endothelial cell mitochondria and was increased by HG stimulation. The balance between Ca^{2+} influx and efflux across the mitochondrial inner membrane is crucial for establishing Ca^{2+} homeostasis within the cell, and therefore, specific Ca^{2+} transport mechanisms must exist, one of which is the newly identified NCLX. NCLX expression accelerates $[Ca^{2+}]_{mito}$ efflux activity, whereas silencing of NCLX expression attenuates this process. Ca^{2+} efflux was fully rescued in NCLX-silenced cells by concomitant overexpression of NCLX.[16] The expression of NCLX is essential and sufficient to functionally complement mitochondrial Na^+/Ca^{2+} exchange.[32] Despite the profound effect of NCLX expression on $[Ca^{2+}]_{mito}$ efflux, it did not affect the steady-state resting $[Ca^{2+}]_{mito}$ level.[16] Our results indicated that NCLX plays an important role in $[Ca^{2+}]_{mito}$ responses triggered by HG conditions in endothelial cells. Importantly, knockdown of NCLX expression increased the Ca^{2+} influx rate, particularly in the late stages of absorption, increased mitochondrial peak Ca^{2+}, and blocked the Ca^{2+} efflux rate. Therefore,

the NCLX Ca^{2+} extrusion system can prevent long lasting $[Ca^{2+}]_{mito}$ elevations. Our results were consistent with Nita's study using Min6 cells. He also found that knockdown of NCLX expression was followed by a minor increase in resting $[Ca^{2+}]_{mito}$ levels, in the Min6 cells.[32] However, this finding was not detected in our test with RAECs. One possibility is that this may be dependent on cell type, as the tissue distribution of NCLX has a unique pattern (high in the pancreas, skeletal muscle and stomach, and low in the kidney and lung). Further, mitochondria from different tissues exhibit large differences in their permeability to Ca^{2+}.[33]

Importantly, we found that knockdown of NCLX expression increased ROS generation induced by HG stimulation, in compared to the siControl. Mitochondrial metabolism is likely to be affected by the activity of NCLX because Ca^{2+} activates several enzymes of the Krebs cycle.[34] It is currently thought that mitochondria are not only centers of energy production but also important sites of ROS generation.[35,36] Stimulation of the tricarboxylic acid cycle and oxidative phosphorylation by Ca^{2+} enhances ROS generation.[37] On the one hand, calcium overload in mitochondria was the major cause of ROS production.[36,38] On the other hand, some studies have found ROS production prior to oxidative stress, and that ROS regulated Ca^{2+} release.[39] In conclusion, there is a complex and close link between $[Ca^{2+}]_{mito}$ and ROS generation. Our results showed that blocking mitochondria Ca^{2+} efflux resulted in an increase in HG-induced ROS generation. HG-induced apoptosis is highly likely to be due to a time-dependent increase in Ca^{2+} overload and ROS production in mitochondria.[29] NCLX, by accelerating $[Ca^{2+}]_{mito}$ shuttling, increases the duration of $[Ca^{2+}]_{mito}$ transients and is thus likely to prevent calcium overload in HG conditions. Over-generation of ROS, via the mitochondrial electron transport chain, contributes to diabetic vascular injury.[40–42] Thus, in order to compensate for this and for self-protection, the mitochondria maintains its stability and function via an increase in NCLX expression in HG-induced early stages of action.

Ca^{2+} controls many aspects of mitochondrial biology. Ca^{2+} mobilization may modulate inflammasome activation by affecting other aspects of mitochondrial function. Several studies have identified that NLRP3 inflammasomes were activated in a manner dependent on Ca^{2+} signaling.[43] The NLRP3 inflammasome is a sensor of specific pathogen, host, and environmental danger molecules. Upon activation, NLRP3 recruits caspase-1, which cleaves and subsequently activates precursor interleukin-1β (IL-1β) and IL-18 to initiate immune responses. Zhou, *et al*.[7] first linked mitochondrial damage to activation of the NLRP3 inflammasome. They found that the accumulation of damaged mitochondria, by blocking mitophagy, increased activation of the NLRP3 inflammasome.[7] Mitochondria have been considered as central regulators of NLRP3 function by several recent studies.[7,10,44] The mechanisms of NLRP3 activation also included generation of ROS.[45]

Many stimuli engage Ca^{2+} signaling as an intermediate step to trigger mitochondrial destabilization and this destabilization plays a fundamental role in apoptosis of endothelial cells. Translocation of soluble adenylyl cyclase (sAC) to mitochondria can influence the development of apoptosis by cytochrome C release and caspase-9 cleavage in endothelial cells.[46] Overexpression of Bcl-2 and Bcl-XL prevents the decrease in mitochondrial membrane potential and protects endothelial cells against apoptosis.[47] The cellular mechanisms of the apoptotic and anti-apoptotic actions are complex. Our study demonstrated that silencing of NCLX expression blocked $[Ca^{2+}]_{mito}$ efflux, resulting in an accumulation of damaged mitochondria, subsequent increased ROS generation and activation of the NLRP3 inflammasome, followed by caspase-1 cleavage in endothelial cells. Elevated ROS production was proposed to be a relevant signal of mitochondrial damage. Disruption of the electron transport chain, which resulted in an induction of ROS, was also sufficient to activate the NLRP3 inflammasome and caspase-1 cleavage. Therefore, NCLX localized to mitochondria seem to be an important target in anti-apoptosis in endothelial cells, by regulating $[Ca^{2+}]_{mito}$ in the matrix influence over the NLRP3 inflammasome is conferred.

Overall, NCLX was linked to elevated glucose levels, ROS generation, and activation of the NLRP3 inflammasome. Thus, further studies characterizing the molecular basis and regulatory mechanisms of NCLX will enable a better understanding of the physiological role of this protein on the pathogenesis of diabetic vascular disease. In the future, development of drugs that modulate the activity of NCLX could represent a new strategy for the treatment of diabetes mellitus or its chronic complications.

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