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Contribution of redox-dependent activation of endothelial Nlrp3 inflammasomes to hyperglycemia-induced endothelial dysfunction

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Abstract

Recent studies indicate that inflammasomes serve as intracellular machinery to initiate classical cytokine-mediated inflammatory responses and play a crucial role in the pathogenesis of cardiovascular diseases. However, whether or not the activation of endothelial inflammasomes directly causes cell dysfunction or tissue injury without recruitment of inflammatory cells is unknown. We explored the role of endothelial cell inflammasome activation in mediating tight junction disruption, a hallmark event of endothelial barrier dysfunction leading to endothelial hyperpermeability in diabetes. We used confocal microscopy to study the formation and activation of NOD-like receptor family pyrin domain containing-3 (Nlrp3) inflammasomes and expression of tight junction proteins in coronary arteries of streptozotocin-treated diabetic wild type and *Nlrp3* gene-deleted mice. We found that *Nlrp3* ablation prevented inflammasome activation and tight junction disassembly in the coronary arterial endothelium of diabetic mice. Similarly, *Nlrp3* gene silencing prevented high glucose-induced down-regulation of tight junction proteins in cultured mouse vascular endothelial cells (MVECs). The high glucose-induced tight junction disruption and consequent endothelial permeability were attributed to increased release of the high mobility group box protein-1 (HMGB1), which is dependent on enhanced Nlrp3 inflammasome activity. Mechanistically, reducing reactive oxygen species (ROS) production abolished high glucose-induced inflammasome activation, tight junction disruption, and endothelial hyperpermeability in MVECs. Collectively, the ROS-dependent activation of endothelial Nlrp3 inflammasomes by hyperglycemia may be an important initiating mechanism to cause endothelial dysfunction. These effects could contribute to the early onset of endothelial injury in diabetes.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interests.

Keywords

Nlrp3 inflammasome; High glucose; Endothelium; Tight junction proteins; Diabetes

Introduction

Recently studies have demonstrated a central role of inflammasome, in particular, the Nlrp3 inflammasome, in the pathogenesis of cardiovascular diseases including atherosclerosis, hypertension, vascular inflammation, cardiac remodeling, and associated end-stage organ diseases [1–6]. The Nlrp3 inflammasome monomer is a multi-subunit protein, which consists of pattern recognition receptor Nlrp3, the adaptor protein ASC (apoptotic speck-like protein), and inactive pro-caspase-1 [7–9]. Oligomerization of Nlrp3 inflammasome monomers forms a high-molecular-weight inflammasome complex, in which pro-caspase-1 is converted to its active form, caspase-1, and subsequently cleaves its substrates such as pro-interleukin-1 β (IL-1 β) to bioactive IL-1 β . So far, it is well documented that inflammasome exerts its effects through the production of IL-1 β that instigates inflammatory cells (monocytes and T lymphocytes) leading to local tissue sterile inflammation. These canonical cytokine and inflammatory cell-mediated inflammatory responses can cause or exacerbate the vascular inflammation and injury contributing to the development of cardiovascular diseases such as atherosclerosis [9–12]. On the other hand, accumulating evidence indicate that inflammasome activation can also cause cell dysfunction or injury via non-canonical actions through pyroptosis, a specific form of cell death that combines characteristics of apoptotic and necrotic death pathways [11], interference with cytoskeleton arrangement [13], lipid handling [14], or direct regulation of synthesis, metabolism, or secretion of functional proteins [15, 16]. In this respect, we recently reported that inflammasome activation causes endothelial junction disruption during obesity [1], which adds to the repertoire of non-canonical actions of inflammasomes in mammalian cells. However, it is imperative to know whether such non-canonical role of inflammasome is involved in endothelial dysfunction and injury in other diseases.

Vascular complications are major causes of disability and death in patients with diabetes mellitus [17]. Endothelial dysfunction is a pathological change in response to cardiovascular risk factors at early stage of cardiovascular diseases and also plays a crucial role in the pathogenesis of vascular abnormalities in diabetes mellitus [18, 19]. However, it remains unclear how endothelial dysfunction is initiated to develop the vascular injury at early stage of diabetes mellitus. Hyperglycemia is the major risk factor for the development of various diabetes-associated pathologies including vasculopathy [20, 21]. High glucose induces production of reactive oxygen species (ROS) and disruption of the cellular calcium homeostasis in vascular cells [22, 23]. Because these high glucose-induced effects are implicated in inflammasome signaling in non-vascular cells [2, 3, 9], it is of interest to examine whether high glucose activates inflammasome in vascular endothelial cells thereby contributing to endothelial dysfunction in diabetes mellitus.

To test this hypothesis, we first determined whether the Nlrp3 inflammasome is formed and activated in coronary arterial endothelium in streptozotocin diabetic mice in vivo. Our data

demonstrated that genetic deficiency of Nlrp3 in mice results in protective effects on tight junction disruption in coronary arterial endothelium. Then, in cultured mouse vascular endothelial cells (MVECs), we showed that high glucose-induced tight junction disruption is dependent on Nlrp3 inflammasome-derived HMGB1 release. Further, we revealed a role of ROS in high glucose-induced Nlrp3 inflammasome activation, tight junction disruption, and enhanced endothelial permeability. Together, our study implicates the clinical potential of targeting inflammasome signaling axis for prevention of the early onset of diabetic vasculopathy.

Materials and methods

Animal procedures

Wild type C57BL/6J (*Nlrp3*^{+/+}) and *Nlrp3*^{-/-} mice (4 weeks of age, male) were bred from breeding pairs from The Jackson Laboratory, Bar Harbor, ME, USA. All protocols were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University. Streptozotocin was injected intraperitoneally at 200 mg/kg for the study groups. The control group was injected intraperitoneally with an equal volume of citrate buffer (pH 4.5). The study groups (STZ group) were injected streptozotocin and fed high-fat diet to produce diabetes as described previously [24]. After 2 weeks, mice were sacrificed and heart tissues were harvested for immunofluorescence examinations. The fasting blood glucose was determined in a 65 µl blood sample using ABL 90 FLEX blood gas analyzer (Radiometer, Westlake, OH).

Confocal microscopy of inflammasome proteins in coronary arterial endothelium of mice

Confocal immunofluorescence analysis was performed to detect co-localization of different proteins in the coronary arterial endothelium as described [1]. Briefly, the mouse hearts were frozen in Tissue-Tek OCT and cut by cryostat into 10 µm sections and mounted on Superfrost/Plus slides. After fixation with acetone, the frozen section slides were incubated with following primary antibodies: rabbit or mouse anti-Nlrp3 (1:100; Abcam, Cambridge, MA), anti-caspase-1 (1:200; Abcam, Cambridge, MA), anti-ZO-1 (1:100, Invitrogen, Grand Island, NY), anti-ZO-2 (1:100, Invitrogen, Grand Island, NY). Endothelium was also visualized by co-staining slides with sheep anti-vWF (Von Willebrand Factor) (1:200; Abcam, Cambridge, MA). After incubation with primary antibodies, the slides were washed and labeled with corresponding Alexa Fluor-488 and Alexa Fluor-555 conjugated secondary antibodies (Invitrogen). Then, the slides were washed, mounted, and visualized through sequentially scanning on an Olympus laser scanning confocal microscope (Fluoview FV1000, Olympus, Japan). Co-localization was analyzed by Image Pro Plus software, and the co-localization coefficient was represented by Pearson's correlation coefficient.

In situ analysis of caspase-1 activity in coronary arterial endothelium of mice

FLICATM (Fluorescent Labeled Inhibitor of Caspases) probes were used to detect active caspase-1 enzyme in coronary arterial endothelium [1]. FLICA probe FAM-YVAD-FMK consists of three moieties (1) YVAD (tyrosine-valine-alanine-aspartic acid), a caspase-1 recognition sequence, which binds to active caspase-1; (2) FMK (fluoromethyl ketone) moiety irreversibly binds with caspase-1; (3) FAM (carboxyfluorescein) reporter, which acts

as a green fluorescent tag. FLICA™ probes enter the cells and become covalently bound to the active cas-pase-1, while any unbound FLICA™ probes diffuse out of the cells and are washed away. The green fluorescent signal from FLICA™ probes is used to determine the relative enzyme activity of caspase-1 in cell or tissue samples. To detect caspase-1 activity in coronary arterial endothelium, frozen section slides were first fixed in acetone then incubated overnight at 4 °C with sheep anti-vWF (1:200; Abcam). Slides were then co-stained with fluorescence-conjugated anti-sheep secondary antibody and FLICA reagent (1:10) from a FLICA™ caspase 1 assay kit (ImmunoChemistry Technologies, LLC, Bloomington, MN) for 1.5 h at room temperature. The slides were washed, mounted, visualized, and analyzed by confocal microscopy as described above. Co-localization was analyzed by Image Pro Plus software, and the co-localization coefficient was represented by Pearson's correlation coefficient.

Cell culture and treatment

The mouse vascular endothelial cell (MVEC) line EOMA was purchased from ATCC. The cell line was originally isolated from mouse hemangioendothelioma. MVECs were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA), containing 10 % of fetal bovine serum (Gibco, USA) and 1 % penicillin–streptomycin (Gibco, USA). The cells were cultured in a humidified incubator at mixture at 37 °C with 5 % CO₂ and 95 % air. Cells were passaged by trypsinization (Trypsin/EDTA; Sigma, USA), followed by dilution in DMEM medium containing 10 % fetal bovine serum. To stimulate the cells by hyperglycemia, the cells were treated with or without high glucose (10–30 mM) and then incubated for 24 h. In case of inhibitors used, the cells were pretreated with glycyrrhizin (GLY, 130 μM) or N-acetyl-L-cysteine (NAC, 10 μM) for 30 min.

Immunofluorescence microscopic analysis of MVECs

Cells grown on glass coverslips were treated as indicated and then fixed in 4 % paraformaldehyde for 15 min. Cells were washed in phosphate-buffer saline (PBS) and then incubated at 4 °C for 2 h with rabbit and/or mouse anti-Nlrp3 (1:100; Abcam, Cambridge, MA), anti-ASC (1:100; Abcam, Cambridge, MA), or anti-caspase-1 (1:200; Abcam, Cambridge, MA), anti-ZO-1 (1:500, Invitrogen, Grand Island, NY), anti-ZO-2 (1:500, Invitrogen, Grand Island, NY). After incubation with primary antibodies, the slides were washed and labeled with corresponding Alexa Fluor-488 and Alexa Fluor-555 conjugated secondary antibodies (Invitrogen). The slices were visualized through sequentially scanning on an Olympus laser scanning confocal microscope (Fluoview FV1000, Olympus, Japan). Co-localization was analyzed by Image Pro Plus software, and the co-localization coefficient was represented by Pearson's correlation coefficient as described [25].

Immunoblotting

MVECs were washed twice with ice-cold PBS and homogenized in ice-cold HEPES buffer containing 25 mM Na-HEPES, 255 mM sucrose, 1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride (pH 7.4). After centrifugation at 1000×g for 10 min at 4 °C, the supernatants containing the membrane protein and cytosolic components, termed homogenates, were frozen in liquid N₂, and stored at –80 °C until use. Cell homogenates were denatured with reducing Laemmli SDS-sample buffer and boiled for 5 min. Samples

were run on SDS-PAGE gel, transferred into PVDF membrane and blocked. The membranes were probed with primary antibody of anti-caspase-1 (1:500) overnight at 4 °C followed by incubation with secondary antibody, and then conjugated to horseradish peroxidase-labeled immunoglobulin G. The immunoreactive bands were enhanced by chemiluminescence methods and imaged on Kodak Omat film. β -actin served as a loading control. The intensity of the bands was quantified by densitometry.

Nucleofection

Transfection of shRNA plasmids was performed using a 4D Nucleofector X-Unit (Lonza, CA, USA) according to the manufacturer's instructions [26]. The Nlrp3 shRNA plasmid was obtained from Origene. Briefly, MVECs were trypsinized and centrifuged at $80\times g$ for 10 min. The cell pellet was resuspended in 100 μ L P1 Nucleofection solution (Lonza) for Nucleofection (with the program code CM137). The program was chosen based on the fact that Nucleofection efficiency was over 80 % as analyzed by flow cytometry using control GFP plasmids. For each Nucleofection sample, 2 μ g plasmid DNA was added in 100 μ L P1 Nucleofection solution. After Nucleofection, cells were cultured in DMEM medium for 24 h.

Electronic spin resonance spectrometric detection of O_2^-

Electronic spin resonance (ESR) detection of O_2^- was performed as we described previously [27]. In brief, 20 μ L cell homogenates were incubated in modified Krebs-HEPES buffer containing deferoxamine (100 μ M; Sigma) and diethyldithiocarbamate (5 mmol/L; Sigma). The samples were then mixed with 1 mM spin trap 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH) (Noxygen, Elzach, Germany) with in the presence or absence of 100 units/mL polyethylene glycol (PEG)-conjugated superoxide dismutase (SOD). The reaction mixture loaded in glass capillaries was immediately analyzed for O_2^- production for 10 min using a Miniscope MS400 ESR spectrometer (Magnetech, Germany). The ESR settings were as follows: biofield, 3360; field sweep, 60G; microwave frequency, 9.78 GHz; microwave power, 20 mW; modulation amplitude, 3G; 4096 points of resolution; receiver gain, 50; and kinetic time, 10 min. The SOD-inhibitable signals were normalized by protein concentration and expressed as the fold change of treatment group versus control.

Endothelial permeability assay

MVECs were cultured in 24-well transwell plates (Corning) and treated without or with high glucose and inhibitors as indicated for 24 h. The transwell inserts were moved into non-used wells with 200 μ L fresh media. Then 100 μ L solution (1 mg/mL) of fluorescein isothiocyanate (FITC)-dextran (40 kDa) (Sigma) was added into each insert. The plate with inserts was incubated at 37 °C for 2 h to allow FITC-dextran molecules flow through the endothelial cell monolayer. The inserts were then removed and fluorescent intensity in each well was determined at excitation/emission of 485/530 nm using a fluorescent microplate reader (FL $\times$ 800, BIO-TEK Instruments). The arbitrary fluorescence intensity was used to calculate the relative permeability.

Statistics

Data are presented as means $\pm$ SE. Significant differences between and within multiple groups were examined using ANOVA for repeated measures, followed by Duncan's multiple-range test. A Student *t* test was used to detect significant difference between two groups. $P < 0.05$ was considered statistically significant.

Results

Formation and activation of Nlrp3 inflammasomes in coronary arterial endothelium of streptozotocin-induced diabetic mice

Nlrp3 inflammasome has been demonstrated as a principal machinery to trigger the inflammatory responses in a variety of mammalian cells including endothelial cells [28]. We first examined whether hyperglycemia-induced endothelial activation and dysfunction are associated with the formation and activation of inflammasomes in coronary arterial endothelium in vivo. A mouse model of diabetes mellitus was established by intraperitoneal injection of streptozotocin (STZ) in either *Nlrp3*^{+/+} or *Nlrp3*^{-/-} mice. Two weeks after STZ injection, the blood glucose concentrations was 14.7 ± 2.2 mM in STZ-treated *Nlrp3*^{+/+} mice, which was significantly higher than that in vehicle-treated *Nlrp3*^{+/+} mice (4.6 ± 0.7 mM). Similar increase in blood glucose concentration was observed in *Nlrp3*^{-/-} mice with 14.5 ± 2.4 in STZ group and 4.7 ± 0.8 in vehicle group (data in mean $\pm$ SD, $N = 5-6$). These data suggest that Nlrp3 gene deletion does not affect STZ-induced hyperglycemia in mice in our experimental setting. To characterize the formation of Nlrp3 inflammasome in the coronary arterial endothelium of *Nlrp3*^{+/+} mice, the co-localization of Nlrp3 with caspase-1 was analyzed because these inflammasome components are aggregated to form inflammasome complexes upon activation. As shown in Fig. 1a, the co-localization of Nlrp3 with caspase-1 was barely observed in vehicle-treated control mice, whereas such co-localization was significantly increased as indicated by intense yellow spots or patches in the intima of the arterial wall of STZ-treated mice. The Pearson correlation coefficient of Nlrp3 with caspase-1 showed the quantitative co-localization of Nlrp3 with caspase-1 (Fig. 1b) in coronary arteries of mice. The increased co-localization of Nlrp3 with caspase-1 suggests the enhanced formation of Nlrp3 inflammasomes in the coronary arterial endothelium of diabetes mice. However, it is also possible that increased co-localization of Nlrp3 with caspase-1 is due to increased protein expression of pro-caspase-1 rather than increased formation of Nlrp3 inflammasome complex.

To further examine whether pro-caspase-1 is converted to active caspase-1 upon inflammasome formation in STZ mice, we detected caspase-1 activation in the coronary arterial endothelium by using FLICATM probes. FLICATM probe is a green fluorescent and specifically binds to the active form of caspase-1 protein but not pro-caspase-1. The endothelium was visualized by staining the coronary arteries with vWF, an endothelial cell marker. As shown in Fig. 1c, d, immunofluorescence studies demonstrated that the treatment of mice with streptozotocin markedly increased caspase-1 activity in coronary arteries of *Nlrp3*^{+/+} mice as shown by enhanced green fluorescence of FLICA. It was found that streptozotocin increased co-localization of FLICA with vWF suggesting that streptozotocin increased caspase-1 activity primarily in the arterial endothelium. In contrast, such

streptozotocin-induced activation of endothelial caspase-1 was significantly reduced in the coronary arteries of *Nlrp3*^{-/-} mice. Together, these results suggest that Nlrp3 inflammasomes are formed and activated in the coronary arterial endothelium in mice with hyperglycemia.

Nlrp3 deficiency abolishes tight junction disruption in coronary arterial endothelium of mice with streptozotocin-induced diabetes mellitus

Decreased expression of tight junction proteins leads to tight junction disruption, which is a marker event for increased endothelial permeability during vascular dysfunction [29]. Here, we examined whether hyperglycemia-induced endothelial Nlrp3 inflammasome activation contributes to endothelial tight junction disruption in coronary arteries. As shown in Fig. 2a–d, immunofluorescence studies demonstrated that treatment of mice with streptozotocin markedly decreased the expression of tight junction protein ZO-1 and ZO-2 in the endothelium layer of coronary arteries from *Nlrp3*^{+/+} mice, whereas the streptozotocin-induced down-regulation of ZO-1 and ZO-2 was significantly attenuated in coronary arteries of *Nlrp3*^{-/-} mice.

High glucose induces the Nlrp3 inflammasome formation and activation in MVECs

Next, we determined whether high glucose induces Nlrp3 inflammasome formation and activation in cultured endothelial cells in vitro. We first examined the formation of inflammasome complex by analyzing the co-localization of Nlrp3 inflammasome components. As shown in Fig. 3a–b, high glucose increased the co-localization between Nlrp3 (green) and ASC (red) as shown by increased yellow staining and co-localization coefficient (maximal co-localization was observed after 30 mM glucose stimulation). Similar increase in co-localization was found between Nlrp3 and caspase-1.

We further demonstrated that high glucose increased Nlrp3 inflammasome activity in MVECs as shown by enhanced cleavage of pro-caspase-1 proteins to their bioactive forms (p20 subunit) (Fig. 4c–d). Such enhanced conversion of inactive pro-caspase-1 to active form of caspase-1 in MVECs was further confirmed by detecting increased release of IL-1 β , a primary caspase-1 substrate, in cell supernatants as measured by a commercially available ELISA kit (R&D System, Minneapolis, MN) (Fig. 4e).

Effect of Nlrp3 shRNA on high glucose-induced tight junction disruption in MVECs

We further examined whether Nlrp3 inflammasome activation by high glucose causes disassembly of junction proteins in cultured endothelial cells in vitro. As shown in Fig. 4, high glucose (30 mM) treatment markedly decreased the expression of tight junction protein ZO-1 and ZO-2 at cell junctions in endothelial cell monolayers. The changes in ZO-1 and ZO-2 expression in cell junctions were further represented by the relative fluorescence intensity across cell-cell contacts (as shown by white lines that cross two cell-cell contacts). In contrast, such high glucose-induced down-regulation of ZO-1 and ZO-2 was prevented in MVECs transfected with Nlrp3 shRNA plasmids.

HMGB1 mediates high glucose-induced tight junction disruption in MVECs

As shown in Fig. 5, high glucose treatment induces HMGB1 release in the culture medium of MVECs as quantified by ELISA (MyBioSource), which is inhibited by *Nlrp3* gene silencing. To confirm the role of HMGB1 in high glucose-induced tight junction disruption, confluent endothelial cell monolayers were pretreated with HMGB1 activity inhibitor glycyrrhizin. It was found that inhibition of HMGB1 by glycyrrhizin (GLY group) could preserve tight junction structure in the presence of high glucose as shown by intact honeycomb expression pattern of ZO-1 and ZO-2 (Fig. 6a–b) and similar fluorescence intensity across cell-cell contacts compared to untreated control cells (Fig. 6c–d). These results suggest that *Nlrp3* inflammasome-dependent HMGB1 release plays an important role in high glucose-induced tight junction disruption in endothelial monolayers.

ROS production contributes to high glucose-induced inflammasome activation and tight junction disruption in MVECs

As shown in Fig. 7a–c, inhibition of ROS production by N-acetyl-L-cysteine markedly attenuated high glucose-induced increases in cleavage of pro-caspase-1 proteins to active caspase-1 (p20 subunit) and production of IL-1 β . ESR spectrometry data further demonstrated that high glucose-induced production of superoxide free radicals was abolished in MVECs by N-acetyl-L-cysteine (Fig. 7d). More importantly, high glucose-induced down-regulation of ZO-1 and ZO-2 were mostly prevented in MVECs treated with N-acetyl-L-cysteine (Fig. 8).

Inhibition of ROS production or HMGB1 or *Nlrp3* gene silencing blocks high glucose-induced endothelial permeability

Tight junction proteins are required to coordinate the passage of macromolecules through endothelium [30]. It is well established that decreases in expression of tight junction proteins ZO-1/ZO-2 result in tight junction disruption leading to increased permeability of endothelial monolayer. By analyzing the relative permeability of endothelial monolayer to FITC-dextran, we demonstrated that high glucose-induced endothelial permeability was blocked in the presence of ROS inhibitor N-acetyl-L-cysteine or HMGB1 inhibitor glycyrrhizin (Fig. 9a). Moreover, we demonstrated that *Nlrp3* gene silencing also significantly attenuated high glucose-induced endothelial permeability (Fig. 9b).

Discussion

The present study demonstrated that loss of function of *Nlrp3* inflammasomes prevents tight junction disruption either in endothelial cells under high glucose condition or in the coronary arterial endothelium of streptozotocin diabetic mice. Our results prove a hypothesis that high glucose induces HMGB1 release through the aggregation and activation of *Nlrp3* inflammasomes, which are mediated by ROS signaling pathway. Blockade of *Nlrp3* inflammasome activation by ROS inhibitor or inhibition of inflammasome-derived HMGB1 activity may improve endothelial function by preventing endothelium hyperpermeability in diabetes.

Endothelial dysfunction is developed at the very early stage of cardiovascular diseases with various coronary risk factors such as diabetes mellitus [31, 32]. Hyperglycemia-induced endothelial dysfunction is multifactorial, which includes altered cell signaling [33], increased oxidative stress [34], pro-inflammatory activation [35], and mitochondrial dysfunction [36]. Recent studies indicate that the inflammasomes play important roles in regulating inflammatory cell functions during the development of metabolic diseases including diabetes [21] and obesity [37]. In the present study, our data from streptozotocin diabetic mice demonstrated that hyperglycemia induces the formation and activation of the Nlrp3 inflammasome complex in the endothelium. Further, the Nlrp3 inflammasome-dependent caspase-1 activity in coronary arterial endothelium was determined by evaluating FLICA™ staining in vWF-positive intima area. Our data showed that the increase in caspase-1 activity in coronary arterial endothelium was almost abolished in mice when Nlrp3 gene is absent (Fig. 1c, d). We also detected a basal-level FLICA™ staining in Nlrp3-deficient mice with or without streptozotocin treatment, which suggests that the Nlrp3 gene deletion did not completely reduce the caspase-1 activity (Fig. 1c, d). Such basal-level caspase-1 activity may be associated with activities from other inflammasome isoforms such as Nlrp1 or Nlrp4 [38]. Nonetheless, our data support the view that Nlrp3 inflammasome serves as an intracellular sensor in endothelial cells for the endogenous danger signals in the plasma such as hyperglycemia in diabetes.

Vascular endothelium has important functions to regulate vascular smooth muscle tone, angiogenesis, and tissue fluid hemostasis [39]. The endothelium forms an interface and serves as a semi-permeable barrier controlling the passage of macromolecules and fluid between the blood and interstitial space. Enhanced endothelium permeability represents one of the initial pathologic processes leading to endothelial dysfunction [40]. It has been well characterized that decreased expression of tight junction proteins ZO-1/ZO-2 reduces their adhesive properties resulting in disruption of their interaction in the inter-endothelial junctions [29, 41]. In this regard, down-regulation of ZO-1/ZO-2 is often considered as a marker event for enhanced endothelial permeability in vascular dysfunction. Recent studies have revealed a role of Nlrp3 inflammasomes in mediating the impairment of tight junction integrity in renal tubules induced by albumin [42] and in vascular endothelium during obesity [1]. In the present study, our fluorescence microscopic data demonstrate that the protein expressions of ZO-1/ZO-2 in coronary arterial endothelium were significantly reduced in diabetic mice, whereas such decreases in ZO-1/ZO-2 were significantly attenuated in Nlrp3-deficient mice. Thus, our data strongly suggest that loss of inter-endothelial junction integrity and, possibly, the consequent endothelial barrier dysfunction in diabetes are associated with endothelial inflammasome activation.

The present study further determined whether Nlrp3 inflammasome activation induced by high glucose causes tight junction disassembly and enhances endothelial permeability in endothelial cells in vitro. Our confocal microscopic data first demonstrated that high glucose stimulation induced the formation of the Nlrp3 inflammasome complex in cultured MVECs, as shown by co-localization of Nlrp3 with ASC or Nlrp3 with caspase-1. Molecular and biochemical analysis further demonstrated that high glucose increased the caspase-1 activity and IL-1 β production in MVECs. Therefore, these results indicate that the Nlrp3 inflammasomes are functioning in MVECs and that high glucose stimulation can lead to

their activation. Our results are consistent with previous findings that Nlrp3 inflammasomes are present in various non-immune cells such as endothelial cells and other residential cells in the brain, heart, kidney, and vessels [6, 37, 43, 44]. Nlrp3 inflammasomes can be activated in endothelial cells under different pathological conditions such as ATP, shear stress and adipokine visfatin [37, 45, 46]. Consistent with these findings, the present study provides the first evidence that high glucose condition serves as an independent danger factor to activate Nlrp3 inflammasomes in MVECs. In contrast, a recent study reported that high glucose alone did not activate Nlrp3 inflammasome in rat aortic endothelial cells [47]. This discrepancy may be due to the different sources of endothelial cells isolated. Rat aortic endothelial cells are isolated from large conductance arteries [47], whereas MVEC line (EOMA) is derived from the microvessels (i.e., aortic vs. microvascular). Consistent with the findings in diabetic mice, our in vitro data demonstrated that Nlrp3 gene silencing prevents high glucose-induced down-regulation of tight junction protein ZO-1/ZO-2 in cultured endothelial monolayers. To our knowledge, these data for the first time link Nlrp3 inflammasome activation to high glucose-induced endothelial tight junction disruption. Together, our data indicate that high glucose-induced activation of Nlrp3 inflammasomes may be an important pathogenic mechanism responsible for endothelial inflammation and dysfunction, at least in microvasculature, in diabetes.

Our data further demonstrated that tight junction disruption induced by high glucose is mediated by Nlrp3-dependent HMGB1 release. HMGB1 is a ubiquitous nuclear and cytosolic protein and released by damaged or necrotic cells during sterile inflammation and infection [48]. It has been shown that caspase-dependent HMGB1 release is regulated by Nlrp3 inflammasomes [49]. Consistently, our data also show that high glucose can stimulate HMGB1 release in endothelial cells via activation of Nlrp3 inflammasomes (Fig. 5). Recent studies have shown that HMGB1 can serve as novel permeability factor in vitro and in vivo [1, 50, 51]. HMGB1 increases the permeability of enterocytic monolayers and impairs intestinal barrier function in mice [50]. In human and mouse endothelial cells, HMGB1 enhances permeability of endothelial monolayers possibly via its autocrine or paracrine action on receptor for advanced glycation end products (RAGE)-mediated pathway [1, 6]. Consistent with these previous studies, our data demonstrated that HMGB1 inhibitor glycyrrhizin could block the high glucose-induced tight junction protein disruption and enhanced permeability in endothelial monolayers of MVECs. Given that hyperglycemia increases HMGB1 and RAGE expression in the primary human aortic endothelial cells [52], it is plausible that such HMGB1-RAGE axis also contributes to high glucose-induced tight junction disruption in MVECs.

It has been well documented that ROS generation is a general mechanism for activation of Nlrp3 inflammasomes in mammalian cells including immune cells and vascular cells [37, 53]. Consistently, our data demonstrated that ROS inhibitor N-acetyl-L-cysteine (NAC) prevents high glucose-induced caspase-1 activation and IL-1 β production in these cells suggesting that high glucose induces Nlrp3 inflammasomes in MVECs via ROS production. It has been demonstrated that high glucose induces ROS production in endothelial cells via either eliciting Ca²⁺ and ERK1/2 pathways leading to mitochondrial fission or activating NADPH oxidases [22, 54]. Further, ROS induced by high glucose trigger the dissociation of thioredoxin-interacting protein (TXNIP) from thioredoxin, which allows TXNIP bind to the

Nlrp3 protein leading to formation and activation Nlrp3 inflammasome complexes [53, 55, 56]. Together, these findings support the view that endothelial Nlrp3 inflammasome is activated through ROS-dependent mechanisms, possibly via ROS/TXNIP/Nlrp3 signaling axis. Excessive ROS production in hyperglycemia stimulates non-vascular cells including glial cells to secrete cytokines such as TNF- α and IL-1 β or growth factors such as vascular endothelial growth factor, which in turn disturb tight junctions, increase permeability, and allow vascular leakage of liquid and molecules into retinal parenchyma [57]. ROS can also alter brain endothelial tight junction dynamics and blood-brain barrier integrity possibly via protein kinase B signaling pathway [58]. These studies indicate that ROS production modulates the dynamics of endothelial tight junctions; however, the relative contribution of ROS-dependent inflammasome to this tight junction modulation remains unclear. In an in vitro permeability model, the present study demonstrated that inhibition of ROS production, HMGB1 activity, or Nlrp3 gene silencing attenuates high glucose-induced tight junction disruption and enhanced permeability of endothelial monolayers in MVECs. Therefore, our data support the view that ROS/Nlrp3 inflammasome/HMGB1 axis is critically involved in high glucose-induced endothelial barrier dysfunction. It has been well documented that endothelial dysfunction is an early event in the development of cardiovascular diseases. However, future studies should investigate the pathological roles of Nlrp3 inflammasome-dependent barrier dysfunction in in vivo animal models such as left ventricle analysis for diabetic cardiomyopathy as described previously [59].

In summary, the present study revealed that high glucose induces endothelial Nlrp3 inflammasome activation in vitro and in vivo. The injurious effect of high glucose stimulation on the endothelial barrier function is associated with Nlrp3 inflammasome-derived HMGB1 activity. Our study provide novel insights that endothelial Nlrp3 inflammasome activation may serve as a novel early event that leads to endothelial dysfunction, ultimately initiates or contributes to the development of diabetes-associated vasculopathy.

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Key message

- Endothelial tight junction disruption in diabetes requires Nlrp3 inflammasomes.
- High glucose activates Nlrp3 inflammasome in endothelial cells via ROS production.
- Activation of endothelial inflammasome by high glucose triggers release of HMGB1.
- Blockade of Nlrp3/HMGB1 axis inhibits high glucose-induced endothelial permeability.

Fig. 1.

Formation and activation of Nlrp3 inflammasomes in mouse coronary arterial endothelium of streptozotocin-treated mice. Wild type (*Nlrp3*^{+/+}) or Nlrp3-deficient (*Nlrp3*^{-/-}) mice were treated with vehicle (*Ctrl*) or streptozotocin (STZ) as described. **a** Frozen sections of mouse hearts were stained with Alexa488-anti-Nlrp3 and Alexa555-anti-caspase-1. Representative merged images displayed *yellow dots* or patches indicating the co-localization of Nlrp3 (*green*) with caspase-1 (*red*). **b** The summarized data show the co-localization coefficient of Nlrp3 with caspase-1 ($n = 6-12$). * $P < 0.05$ vs. Ctrl. **c** Frozen sections of mouse hearts were stained with FLICA, a green fluorescent probe specific for active caspase-1, and Alexa555-anti-vWF against an endothelium marker vWF. Representative merged images displayed *yellow dots* or patches indicating the co-localization of FLICA (*green*) with vWF (*red*). **d** The summarized data show the co-localization coefficient of FLICA with vWF ($n = 5-10$). * $P < 0.05$ vs. *Nlrp3*^{+/+} Ctrl; # $P < 0.05$ vs. *Nlrp3*^{+/+} with STZ. Enlarged images of area of interest (AOI) in merged images are shown. Scale bar = 50 μ m.

Fig. 2.

Nlrp3 gene deletion inhibits disassembly of tight junction proteins in mouse coronary arterial endothelium of streptozotocin-treated mice. Wild type (*Nlrp3*^{+/+}) or *Nlrp3*-deficient (*Nlrp3*^{-/-}) mice were treated with vehicle (*Ctrl*) or streptozotocin (STZ) as described. Frozen sections of mouse hearts were stained with Alexa488-anti-vWF and Alexa555-anti-ZO-1 or Alexa555-anti-ZO-2. **a** Representative fluorescent images show the expression of ZO-1 (*red*) in the endothelium visualized by vWF staining (*green*). **b** The summarized data show the fluorescence ratio of ZO-1 over vWF ($n = 5-7$). **c** Representative fluorescent images show the expression of ZO-2 (*red*) in the endothelium visualized by vWF staining (*green*). **d** The summarized data show the fluorescence ratio of ZO-2 over vWF ($n = 5-8$). * $P < 0.05$ vs. *Nlrp3*^{+/+} Ctrl; # $P < 0.05$ vs. *Nlrp3*^{+/+} with STZ. Enlarged images of area of interest (AOI) in merged images are shown. Scale bar = 50 μ m.

Fig. 3.

High glucose increases Nlrp3 inflammasome, caspase-1 activation, and IL-1 β production in MVECs. **a** Representative confocal fluorescence images depict the effect of high glucose (10–30 mM; 24 h) on the co-localization of Nlrp3 with ASC or caspase-1. **b** Summarized data show the co-localization efficiency of Nlrp3 with ASC (*black bars*) or Nlrp3 with caspase-1 (*gray bars*) ($n = 4-8$). **c** Western blot analysis shows the effect of high glucose on pro-caspase-1 (Pro-casp1) and cleaved caspase-1 (Cle-casp1) expression. **d** Summary of Western blot results ($n = 4$). **e** Summary of data for IL-1 β production compared with untreated control ($n = 4$). * $P < 0.05$ versus control group.

Fig. 4.

Effect of Nlrp3 gene silencing on high glucose-induced disruption of tight junction proteins in MVECs. MVECs were transfected with scramble or Nlrp3 shRNA plasmids and then stimulated with or without high glucose (*HG*) (30 mM) for 24 h. Representative fluorescence images **a** and **c** show the cell membrane fluorescence of ZO-1 and ZO-2 from at least three independent experiments. Tight junctions are represented by histograms of ZO-1 **b** or ZO-2 **d** fluorescence intensity (*RFI*) as indicated by *dotted lines* across the two cell-cell contacts.

Fig. 5. Nlrp3 gene silencing inhibits high glucose-induced high mobility group box protein 1 (HMGB1) release in MVECs. MVECs were transfected with scramble or Nlrp3 shRNA plasmids and then stimulated with or without high glucose (HG) (30 mM) for 24 h. **a** Western blot documents and summarized data showing the effects of gene silencing on the expression of Nlrp3 proteins ($n = 4$). **b** Summarized data show the concentration of HMGB1 in culture medium of MVECs as measured by ELISA ($n = 3$). * $P < 0.05$ vs. scramble ctrl; # $P < 0.05$ vs. scramble with HG.

Fig. 6.

Inhibition of high mobility group box protein 1 (HMGB1) activity abolishes high glucose-induced disruption of tight junction proteins in MVECs. MVECs were treated with or without high glucose (HG, 30 mM) for 24 h in the presence of PBS (*Vehl*: vehicle) or HMGB1 inhibitor glycyrrhizin (GLY, 130 μM). Representative fluorescence images **a** and **c** show the cell membrane fluorescence of ZO-1 and ZO-2 from at least three independent experiments. Tight junctions are represented by histograms of ZO-1 **b** or ZO-2 **d** fluorescence intensity (*RFI*) as indicated by *dotted lines* across the two cell-cell contacts.

Fig. 7.

High glucose-induced Nlrp3 inflammasome activation depends on ROS production. MVEC cells were treated with or without high glucose (HG, 30 mM) for 24 h in the presence of PBS (Vehl: vehicle) or ROS inhibitor N-acetyl-L-cysteine (NAC, 10 μM). **a** Western blot analysis shows the effect of NAC on high glucose-induced conversion of pro-caspase-1 (Pro-casp1) to cleaved caspase-1 (Cle-casp1). **d** Summary of Western blot results ($n = 6$). **c** Summary of data for IL-1β production ($n = 4$). **d** Summarized data show the superoxide production by MVEC cells as measured by electron spin resonance (ESR) spectrometry ($n = 3$). * $P < 0.05$ versus Vehl ctrl; # $P < 0.05$ vs. Vehl with HG.

Fig. 8.

Inhibition of ROS prevents high glucose-induced disruption of tight junction proteins in MVECs. MVECs were treated with or without high glucose (HG, 30 mM) for 24 h in the presence of PBS (*Vehl: vehicle*) or ROS inhibitor N-acetyl-L-cysteine (*NAC*, 10 μM). Representative fluorescence images **a** and **c** show the cell membrane fluorescence of ZO-1 and ZO-2 from at least three independent experiments. Tight junctions are represented by histograms of ZO-1 **b** or ZO-2 **d** fluorescence intensity (RFI) as indicated by *dotted lines* across the two cell-cell contacts.

Fig. 9.

Effects of HMGB1 or ROS inhibition on high glucose-induced endothelial cell monolayer permeability. **a** MVEC on inserts of transwells were treated with or without high glucose (HG, 30 mM) for 24 h in the presence of PBS (*Veh1*: vehicle), glycyrrhizin (*GLY*, 130 μ M) or N-acetyl-L-cysteine (*NAC*, 10 μ M). FITC-dextran was added to the inserts for the last 2 h. Summarized data show the relative permeability of endothelial monolayers in the inserts for FITC-dextran ($n = 4$). * $P < 0.05$ versus Veh1 ctrl; # $P < 0.05$ vs. Veh1 with HG. **b** MVEC were transfected with scramble or Nlrp3 shRNA plasmids, seeded on transwell inserts, and then stimulated with or without high glucose (HG) (30 mM) for 24 h. Summarized data show the relative permeability of endothelial monolayers in the inserts for FITC-dextran ($n = 4$). * $P < 0.05$ vs. scramble ctrl; # $P < 0.05$ vs. scramble with HG.