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Brief communication (Original)

High glucose enhances CD39 expression in vascular endothelial cells

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Background: Diabetes mellitus (DM) patients lose their ability to control normal blood glucose levels, resulting in high blood glucose levels (hyperglycemia). Hyperglycemia causes DM complications. This involves responses of vascular endothelial cells (VECs) to hyperglycemia, affecting inflammatory process and platelet activity. Ecto-enzyme CD39 is expressed on VECs, catalyzing the hydrolysis of ATP and ADP to AMP and, consequently, regulating inflammatory process and platelet activation.
Objective: We studied whether high glucose concentration has an effect on CD39 expression on VECs.
Methods: Cultured human umbilical vein endothelial cells (HUVEC) were used as a model of study. HUVEC were cultured in different glucose conditions (4, 9, 24, and 34 mM) for 24 hours. Cell viability was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-based assay and expression of CD39 was examined by using SDS-PAGE and western blot techniques.
Results: HUVEC were cultured in normal (4 and 9 mM) or high (24 and 34 mM) glucose concentrations for short term (24 hours). The results showed that high glucose (24 and 34 mM) reduced cell viability to 89.5 ± 11.3 and 86.3 ± 13.5 (mean ± SD), compared with control (4 mM), respectively. High glucose also induced increases in CD39 expression in HUVEC.
Conclusion: High glucose decreases cell viability and increases CD39 expression in HUVEC, suggesting involvement of CD39 in cell responses to high glucose.

Keywords: CD39, cell viability, diabetes mellitus, HUVEC, hyperglycemia, short term, vascular endothelial cell

Diabetes mellitus (DM) is a group of metabolic disorders of carbohydrate metabolism, causing health problems worldwide [1]. Human blood glucose levels are normally dynamic, increasing after meal and returning to normal after the action of insulin. However, blood glucose levels in DM patients are considerably higher than normal. The cause of DM is a deficiency of insulin hormone; therefore, DM patients are unable to maintain normal blood glucose levels, resulting in high blood glucose concentration (hyperglycemia). Chronic hyperglycemia can lead to microvascular and macrovascular complications, including diabetic retinopathy, nephropathy, neuropathy, and cardiovascular diseases [2, 3]. A major player in underlying mechanisms involves vascular endothelial cells (VECs) [4, 5]. Hyperglycemia induces increases in expression of adhesion molecules on VECs, facilitating leukocyte infiltration and progress in pathogenesis of atherosclerosis. Moreover, activated VECs reduce their production of prostacyclin and NO, which are platelet-inhibitory substances. Hyperglycemia induces VECs responses, including oxidative stress, upregulation of adhesion molecules, and decreased production of prostacyclin and NO. Consequently, these effects lead to proinflammatory and prothrombotic states [6]. Because high glucose concentration induces oxidative stress and increased expression of adhesion molecules on VEC, we examined if high glucose concentrations have effects on VEC. First, we tested effects of high glucose concentrations on cell viability, using HUVEC as our model of VECs.

CD39 (EC 3.6.1.5, apyrase, ATPDase, Bgp95, G28-8, NTPDase-1 (nucleoside triphosphate diphosphohydrolase 1), vascular ATP diphospho-hydrolase) is a member of the ecto-nucleoside triphosphate diphospohydolase (E-NTPDase) family, catalyzing hydrolysis of ATP, a proinflammatory mediator, and ADP, a platelet agonist, to AMP [7-9]. CD39 is expressed on several cells, including VECs,

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activated B cells, natural-killer cells, macrophages, dendritic cells, neurons, glial cells and astrocytes. CD39 has roles on inflammatory process and regulation of platelet activation [10-12]. Consequently, CD39 has been proposed as a potential target for treatment of patients with complications caused by platelet activation [11-13]. CD39 is an ecto-enzyme expressed on VECs and is an important player in the regulation of inflammation and platelet activation, associated with DM complications [14, 15]. However, it has not been reported if hyperglycemia has effects of CD39 in VECs. In the present study, we examined if high glucose can modulate CD39 expression in VECs.

Materials and methods

Cell culture

Human umbilical vein endothelial cells (HUVEC) were used as a model of VECs. They were purchased from Invitrogen (Carlsbad, CA, USA) and cultured in M200 media supplemented with of 2% low serum growth supplement (LSGS) at 37°C in 5% CO_2. The media was changed every 48–72 hours. At confluence, the media was changed to M200 media supplemented with 1% LSGS. Random blood glucose levels are normally lower than 11.1 mM. Therefore, we chose 2 normal (4 and 9 mM) and 2 high (24 and 34 mM) concentrations of glucose and tested their effects. After treatment for 24 hours, cells were analyzed for cell viability. Glucose (5, 20 and 30 mM) was then added to the media where the basal glucose concentration was 4 mM, giving final concentrations of 4, 9, 24, and 34 mM. To test if hyperosmolarity in high glucose condition causes effects on VEC, osmolarity control was conducted by adding mannitol (30 mM), representing the same osmolarity as 34 mM glucose. Cells were then incubated at 37°C for 24 hours, and analyzed for cell viability and expression of CD39.

Cell viability

After treatment, cell viability was examined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-based assay [16, 17]. MTT solution (5 mg/mL, Sigma-Aldrich, St. Louis, MO, USA) was added to cultured cells. After 4 hours incubation, MTT formazan was solubilized with DMSO and absorbance was determined at 570 nm and 630 nm as a reference wavelength. Percent cell viability was calculated by comparison with control by setting the mean absorbance of controls as 100%.

Expression of CD39

Treated cells were harvested, lysed, and analyzed using SDS-PAGE and western blotting. Briefly, cell lysate was loaded onto a 10% SDS-polyacrylamide gel and separated proteins were then transferred to a nitrocellulose membrane. After that, the membrane was blocked with 5% albumin-containing Tris-buffered saline (TBS), washed with TBS, incubated with primary antibody against CD39 (1:500, Santa Cruz, Santa Cruz, CA, USA) or β-actin (1:500, Santa Cruz), washed with TBS and finally incubated with anti-rabbit or anti-mouse IgG horse radish peroxidase (HRP) conjugates (1:10,000), respectively. The protein signals were then developed with peroxide and luminol enhancer solution (ECL SuperSignal Substrate kit, Pierce, Rockford, IL, USA) and visualized by exposure to X-ray films.

Results

We found that cell viability after treatment of glucose at 9, 24, and 34 mM was 110.1 ± 22.0%, 89.5 ± 11.3%, and 86.3 ± 13.5% (mean ± SD), respectively, compared with 4 mM glucose (**Figure 1**). Moreover, cell viability of HUVEC treated with mannitol, as osmolarity control, was 94.7 ± 15.7%, which was not significantly different from normal glucose effects. A *t* test showed the effects of 4 and 9 mM glucose on cell viability were not significantly different, while effects of 24 mM and 34 mM glucose were significantly different from 9 mM glucose ($p < 0.05$), but not different from 4 mM glucose ($p = 0.09$ and 0.05, respectively).

We observed that short-term exposure of high glucose (34 mM) caused increases in expression of CD 39 in HUVEC, while mannitol, an osmolarity control, did not induced changes (**Figure 2**).

Discussion

We found that cell viability reduced significantly by high glucose concentration (24 and 34 mM). This finding is similar to that of previous studies, showing that high glucose concentrations decrease VEC viability and increase apoptosis [18, 19]. However, the earlier studies used long-term exposure to high glucose concentrations. In the present study, we used short-term exposure (24 hours) and observed similar results. This reveals that short-term exposure to hyperglycemia can also cause adverse effects to VECs. Previous studies also reported effects of short-term exposure to hyperglycemia [20-22]. High glucose induces oxidative stress in endothelial cells after 3-

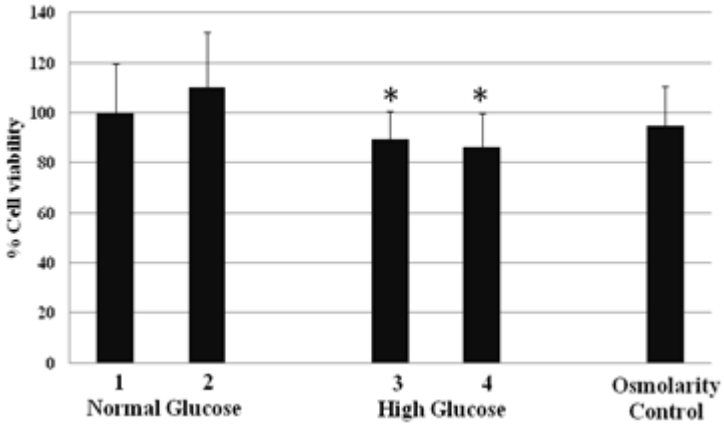

Figure 1. Effects of high glucose on cell viability. HUVECs were cultured in different glucose concentrations for 24 hours; 4 (1) and 9 (2) mM as normal glucose levels and 24 (3) and 34 (4) mM as high glucose levels. Osmolarity control was achieved using mannitol at the same concentration as 34 mM glucose. Cell viability was determined by the MTT method. The data are shown as percent, compared with basal medium glucose at 4 mM (100%). *Cell viability significantly different from normal glucose ($p < 0.05$). The data are from three triplicate experiments.

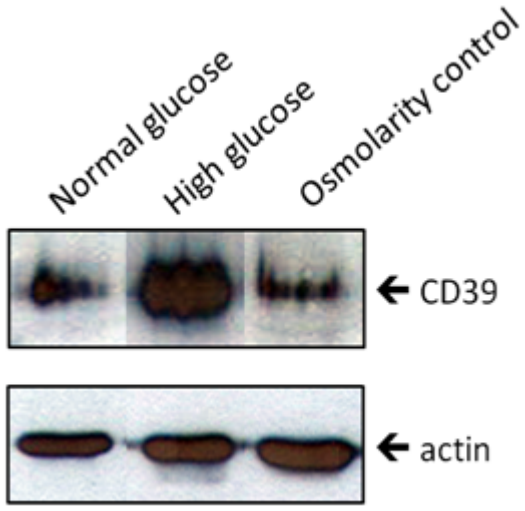

Figure 2. Effects of high glucose on CD39 expression. HUVECs were cultured in normal (4 mM), high (34 mM) glucose concentrations or mannitol (osmolarity control) for 24 hours. CD39 expression was assessed by the SDS-PAGE and western blotting. The upper panel shows CD39 bands and the lower shows bands from β-actin loading controls. The data represent 6 different experiments.

hour exposure and enhances expression of adhesion molecules (E-LAM-1, VCAM-1 and ICAM-1) after 24-hour exposure [20, 22]. In addition, an in vivo study in C57B1/6J mice showed that acute hyperglycemia results in increases in oxidative stress and worsened myocardial infarction [21]. Moreover, another in vivo study in DM patients revealed that induction of oxidative stress is associated with acute glucose fluctuation, indicated by using the mean amplitude of glycemic excursion (MAGE, arithmetic mean of the difference between peak and nadir glucose levels). Acute glucose fluctuation reflects exposure to high glucose, at least, for a short period of time [23]. They also showed that the oxidative stress is not associated with long-term exposure of high glucose (HbA_{1C}). This suggests that short-term exposure to high glucose concentrations, including controlled DM patients, can have impact on the patients. These reports support our finding that short-term exposure to high glucose can reduce HUVEC viability.

We found that a high glucose concentration induced an increase in CD39 expression after short-term exposure (24 hours) to high glucose (34 mM). Moreover, hyperosmolarity (osmolarity control) did not cause a reduction of cell viability or change of CD39 expression; therefore, these effects appear to be associated with high glucose, not increased osmolarity.

In the present study, high glucose caused several fold increases in CD39 expression, compared with that of controls, while high glucose induces about 10%–20% decreases in cell viability (cell death). The later result is consistent with previous reports. Because the changes of responses in cell viability and CD39 expression are in different magnitude, we consider that cell viability or cell death is not a causes of change in CD39 expression. However, further study is required to understand how high glucose induced increases in CD39. It is possible that augmentation of CD39 is a mechanism to protect vascular endothelial cells from thrombosis as a result of inflammation and platelet activation, because CD39 helps hydrolyze the inflammatory mediator ATP and platelet agonist ADP. CD39 helps prevent diabetic nephropathy by suppressing upregulation of adhesion molecules on endothelial cells, resulting in reduction of leukocyte infiltration, suppression of scavenger receptors, limitation of foam cell formation, a step in atherogenesis, and prevention of platelet activation [10]. This concept is supported by studies showing that CD39 is upregulated by several insults to endothelial cells, including lipopolysaccharide and proinflammatory cytokines [24, 25]. In addition, it has been shown that increases in CD39 on glial cells of retina in diabetic mice [26], and on platelets and peripheral blood mononuclear cells (PBMC), isolated from diabetic patients [27, 28].

Conclusion

CD39 with a role in endothelial cell responses to insults, is modulated by high glucose conditions, suggesting its involvement in cell responses to high glucose or hyperglycemia. Short-term exposure to high glucose mediates effects on HUVEC and suggest that CD39 is involved in HUVEC responses to the exposure to hyperglycemia.

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