**High glucose and interleukin 1 β -induced apoptosis in human umbilical vein
endothelial cells involves in down-regulation of monocarboxylate transporter 4**

Authors: Dong Wang^{1§}, Qingjie Wang^{1§}, Gaoliang Yan¹, Yong Qiao¹, Ling Sun³,
Boqian Zhu¹, Chengchun Tang¹, Yuchun Gu²

1. Department of Cardiology, Zhongda Hospital of Southeast University Medical
School, Dingjiaqiao Road, 210009, Nanjing, Jiangsu, China

2. Institute of Molecular Medicine, Peking University, Yiheyuan Road, 100871,
Beijing, China

3. Department of Cardiology, Changzhou Hospital of Nanjing Medical University,
Gehu Road, 213004, Changzhou, Jiangsu, China

§ These authors contributed equally to this work

Corresponding author:

Chengchun Tang (E-mail: tangchengchun@medmail.com.cn)

Yunchun Gu (E-mail: ycgu@pku.edu.cn)

Abstract

Hyperglycaemia and inflammatory can induce apoptosis in vascular endothelial cells, which contributes to the development of vascular complications in diabetes. Endothelial cells depend on glycolysis for their energy metabolism, and monocarboxylate transporters (MCTs) regulate intracellular pH by mediating the influx and efflux of lactate. Here, we evaluate the role of MCT4 in high glucose (HG) and interleukin 1 β (IL-1 β)-induced apoptosis in human umbilical vein endothelial cells (HUVECs). We demonstrate that aortic endothelium damage is severe in *db/db* mice by using scanning ion conductance microscopy (SICM). HG and IL-1 β decrease MCT4 and its location on plasma membrane, as well as increase lactic acid accumulation and apoptosis in HUVECs. Knockdown of MCT4 blocks lactate efflux to result in lactic acid accumulation and pH dropping, which is involved in triggering apoptosis in HUVECs.

Key words: scanning ion conductance microscopy, endothelium damage, endothelial cells, monocarboxylate transporter 4, lactate

Introduction

Vascular complications are known as the leading cause of mortality in diabetes patients [1]. Type 2 diabetes mellitus (T2DM) is an inflammatory disease and numerous inflammatory factors such as interleukin-1 (IL-1), IL-6 and C-reactive protein, elevate in the blood of T2DM patients [2]. However, most inflammatory factors are regulated by IL-1, and blocking IL-1 activity by IL-1 receptor antagonist reduce their concentrations [3].

Vascular endothelium plays a pivotal role in maintaining vascular health and homeostasis under physiological conditions [4]. Endothelial cells (ECs) dysfunction and damage are early physiopathological clinical events for the development of vascular complications during diabetes [5]. The experimental process of traditional techniques to observe vascular endothelium damage is complex, such as immunofluorescence. Scanning ion conductance microscopy (SICM) has been used to study living cells for several years [6]. SICM is a powerful tool for high-resolution topographic imaging on a nanometre scale. One of the great advantages of SICM lies on its ability to perform contact-free scanning [7], which makes it possible to detect endothelium damage without fixation. However, only a limited number of reports have examined endothelium damage using SICM [8].

Similar to tumour cells, ECs depend on glycolysis for their energy metabolism in normoxia [9]. Lactate is the final product of glycolysis, and its influx and efflux through the plasma membrane is mediated by monocarboxylate transporters (MCTs) isoforms 1-4 [10]. Thus, MCTs play an essential metabolic role in the lactate acid regulation. MCT4 expression is elevated under hypoxia in tumour cells, which is consistent with the preferential role of MCT4 in mediating lactic acid efflux in glycolytic cells [11]. However, if the lactate acid production becomes excessive but the efflux reduces, intracellular acidification and then lactic acidosis may occur to cause pH dropping, which is dangerous to the tissues and cells [12]. Several research showed that the pathological overexpression or mutation of MCTs result in some diseases, including pancreatic β -cell malfunction [13] and chronic fatigue syndromes [14]. However, it is unknown whether the efflux of lactic acid from glycolysis is blocked to result in ECs dysfunction and damage in diabetes.

In this study, we initially examined endothelium damage in T2DM model (*db/db* mice) by using SICM and immunofluorescence. Next, we investigated the apoptosis, lactate level and MCT4 expression under high glucose (HG) and IL-1 β condition in cultured human umbilical vein ECs (HUVECs). Finally, we evaluated the effects of MCT4 on intracellular lactate level, pH and apoptosis in HUVECs.

Methods

Animals

Animal experiments conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (DHWE publication No. 96-01, revised in 2002) and was approved by the Ethics Review Board for Animal Studies of Institute of Southeast University, Nanjing, China. Male *db/db* mice (*C57BL/6-lepr^{db}/lepr^{db}*) and *C57BL/6* mice were obtained from Southeast University Animal Center. In all experiments 10-12 weeks old mice were employed.

Reagents

Recombinant human IL-1 β and endothelial cell growth medium were purchased from Promocell (Heidelberg, Germany). 2',7'-bis(2-carboxethyl)-5(6)-carboxy-fluo-rescein-acetoxymethyl (BCECF-AM) was purchased from life technologies (OR, USA). Goat polyclonal MCT4, goat polyclonal β -actin, mouse monoclonal Bcl-2 (B-cell lymphoma 2) and rabbit polyclonal BAX (Bcl2-associated X protein) antibodies were purchased from Santa Cruz Biotechnology (CA, USA). Rabbit polyclonal CD31 antibody was purchased from Abcam (Cambridge, UK).

HUVECs culture

Primary HUVECs were purchased from Promocell and grown in endothelial cell growth medium containing supplement mix at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. All of the experiments were performed using 2-5 passages of HUVECs.

Aortic endothelium imaging by scanning electron microscope (SEM) and SICM

Mice were anesthetized using chloral hydrate (350 mg/kg, i.p.) and aortic arch was exposed. Phosphate buffer saline (PBS) was injected through the left ventricle. After that, 5 % glutaraldehyde, 5 % glucose and 0.2 % silver nitrate were injected one after another. Then, the aortic arch was isolated carefully and fixed with 5 % glutaraldehyde overnight. The aorta was freezing dried and coated with platinum before the SEM analysis. The morphology images of endothelium were taken by SEM (JSM-IT300, Japan).

Electrodes were fabricated following manufacture's instruction (Sutter Instruments). Quartz capillaries (Sutter Instruments, Novato, CA., O.D:1.0 mm, I.D: 0.58 mm) were pulled using a CO₂ laser pipet puller (P-2000, Sutter Instruments, Novato, CA) to form nanopipets in two cycles: (1) HEAT = 325, FIL= 3, VEL= 30, DEL= 200, PUL= 0, and (2) HEAT = 340, FIL= 3, VEL= 30, DEL= 180, PUL= 250. It produces nanopipets with inner diameters of ~30 nm and an outer diameter of ~100 nm. The pipet electrode (PE) consists of an Ag/AgCl wire back-inserted into the pipet-electrode, which was filled with endothelial cell growth medium. Mice were anesthetized using chloral hydrate (350 mg/kg, i.p.), aortic arch was exposed and isolated carefully. Aortic arch was sliced open, washed with growth medium. Cell culture dishes (Corning Inc., NY, USA) were coated by silicone elastomer (Dow

corning, MI, USA), and then aorta was fixed by micro needles with endothelium faced up in growth medium. Vessel surface was scanned with ScanIC SICM (Ionscope, London, UK) using the hopping mode to random scan more than five images via a standard procedure [15]. Acquired topography data were bilinear-interpolated using Scanning Probe Image Processor (SPIP, Ionscope, Version 6.3.5) to produce final images.

Immunofluorescence and the terminal deoxynucleotide transferase-mediated dUTP nick end labeling (TUNEL) staining

For immunofluorescence, paraffin tissue sections from mice aortic were deparaffinized and rehydrated. After pre-treatment with pepsin for 30 min (1% pepsin in 0.1% HCl) for antigen retrieval, sections were incubated overnight at 4 °C with primary CD31 and MCT4 antibodies diluted in Tris-buffered saline (TBS) containing 1% bull serum albumin and 0.1% tween 20. After pre-treatment with antigen retrieval sections were washed in TBS and incubated with secondary antibodies.

TUNEL staining was performed to detect the apoptosis of HUVECs. In situ cell death detection kit (Roche Applied Science, Mannheim, Germany) was used according to the manufacturer's instructions as previous described [16]. The apoptosis cells were recognized as a green fluorescent nucleus. Immunofluorescence images were taken under a fluorescence microscopy (Nikon, Japan) of at least five areas.

Lactate concentration measurement

Cells were wash with ice cold PBS three times and ultrasonicated. Lysates was centrifuged and the supernatant was analyzed by Amplite™ Colorimetric L-Lactate

Assay Kit (AAT Bioquest[®], CA, USA) following the manufacture's instruction. Lactate concentration was normalized to total protein content.

Intracellular pH measurement

HUVECs were cultured in 96-well assay plate (Corning Inc., NY, USA, black plate, clear bottom). After treatment, cells were loaded with BCECF AM (5 μ M) by incubating for 30 min at room temperature and exciting with 490 and 440 nm while monitoring the emission at 535 nm. Intracellular pH was measured by microplate reader (Infinite[®] M200, Tecan Group Ltd.). The calibration of pH was determined via a standard formula derived from the Henderson-Hasselbalch equation using minimum and maximum fluorescence ratios at pH=4 and pH=9 [17].

Quantitative real-time PCR and Reverse transcription PCR (RT-PCR)

Total RNA was isolated from HUVECs using standard procedure. The primer sequences were as follows: (1) MCT1, sense 5'- GTGGAATGCTGTCCTGTCCTC -3', antisense 5'- TCGATAATTGATGCCCATGCC -3', (2) MCT2, sense 5'- ATGCTGTCTTGAATGGTGTG -3', antisense 5'- TTATGGTTAAGGCGGGTTGC -3', (3) MCT3, sense 5'- CTGAGGCTGAAGGGGACTCT -3', antisense 5'- CGGCCTCGCCTCTATTTCTG -3', (4) MCT4, sense 5'- TTCGTCCCGCCCGTGTTTCGT -3', antisense 5'- TAGTCGCCCCGCCGTAGAGCC -3', (5) β -actin, sense 5'- AGAGCTACGAGCTGCCTGAC -3', antisense 5'- AGCACTGTGTTGGCGTACAG -3'. One μ g RNA was first reverse transcribed to cDNA with random primers using RT Master Mix kit (Takara Biotechnology, Dalian, China). RT-PCR was performed by

using the TaqMan Reverse Transcription kit (Takara) and the T100 Thermal Cycler (Bio-Rad, CA, USA) according to the manufacturer's instructions. PCR products were identified by electrophoresis with 1% agarose gels and photographed by the Gel Doc 1000 imaging system (Bio-Rad). Real-time PCR was performed using SYBR Premix Ex Taq II kit (Takara) and the 7300 real-time PCR system (ABI, CA, USA) as described in the manufacture's manual. The relative mRNA amounts of target genes were normalized to the values of β -actin. The results were expressed as fold changes of threshold cycle (Ct) value relative to the controls using the $2^{-\Delta\Delta C_t}$ method.

Western blot analysis

Cells were lysed in RIPA (Radio Immuno Precipitation Assay) buffer containing protease and phosphatase cocktails. Equal amounts of protein (50 μ g) were separated by 10% SDS-PAGE and electro-transferred to a PVDF membrane (Millipore). Membranes were blocked with 5% non-fat milk powder in TBST (0.1% Tween 20 in $1 \times$ TBS) for 1 h at room temperature, and then incubated with anti-MCT4 (1:100), anti-BAX (1:200), anti-Bcl2 (1:200) and anti- β -actin antibodies (1:1000) at 4 °C overnight. Finally, the membranes were incubated with the horseradish peroxidase-conjugated secondary antibody (Beijing TDY biotech company) (1:5000) for 1 h. Specific protein levels were normalized to β -actin for total protein analyses. The blot was detected with the ChemiDoc™ MP System detection system (Bio-Rad Laboratories, CA, USA).

Short Hairpin RNA (shRNA)-mediated protein knockdown

The recombinant plasmids pLKO.1/MCT4 shRNA targeting human MCT4 mRNA was kindly provided by Dr. Yuchun Gu (Institute of Molecular Medicine, Peking

University). The MCT4 shRNA (shMCT4) sequence was 5'-CCGGCGTCTACATGTACGTGTTTCATCTCGAGATGAACACGTACATGTAGACGTTTTTG -3'. HUVECs were transfected with pLKO.1/MCT4 shRNA or empty vector (Ctl shRNA) using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's protocol. After 48 hours, MCT4 was measured by Western blot.

Statistical analysis

All data are presented as means $\pm$ standard error (SE). Data were compared using an unpaired Student's t-tests or one-way analysis of variance (ANOVA) with Tukey post hoc test for multiple comparisons by SPSS 19.0 (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

Results

Morphology imaging of mice aortic endothelium

SEM images revealed that ECs were oval-shaped and linked closely in *C57BL/6* mice endothelium (Fig. 1A). Similar to SEM, SICM images also showed that the aortic ECs of *C57BL/6* mice had oval-shaped appearance and arranged densely (Fig. 1B and 1C). However, in *db/db* mice aortic endothelium, detachment of ECs was severe, showing a bumpy images (Fig. 1D and 1E). The endothelialised areas dramatically decreased in *db/db* mice (Fig. 1F). Immunofluorescence with CD31, a biomarker of ECs, also displayed the presence of de-endothelialized areas in aortic endothelium of *db/db* mice (Fig. 1G).

High glucose (HG) and IL-1 β induced endothelial cells apoptosis and intracellular lactate accumulation

HUVECs were labelled with an in situ TUNEL assay to detect apoptotic cells. Exposure to HG or IL-1 β resulted in a striking appearance of TUNEL-positive cells (Fig. 2A). Combining HG and IL-1 β exhibited an additive effect on apoptosis compared with HG or IL-1 β alone (Fig. 2A and 2B). Bcl2 mediates endothelial cells survival, whereas BAX promotes apoptosis [18]. As shown in Figure 2C, HG and IL-1 β decreased Bcl2 protein level but increased BAX protein level. Treatments with HG or IL-1 β did not affect the intracellular lactate. However, HG and IL-1 β induced a two-fold increase of intracellular lactate compared with normal glucose (Fig. 2D).

MCTs gene expression and the effect of HG and IL-1 β on MCT4 expression in HUVECs

MCT4 mediates lactate efflux in glycolytic cells. Thus, we first detected the expression of MCTs in HUVECs, and RT-PCR results showed that HUVECs highly expressed MCT1 and MCT4. It was therefore suspected that MCT4 would involve in HG and IL-1 β -induced intracellular lactate accumulation in HUVECs. As expected, HG did not affect MCT4 mRNA and protein level (Fig. 3B and 3C), as well as the location on cell membrane (Fig. 3D). However, HG plus IL-1 β induced a significant reduction of MCT4, as well as cell membrane location (Fig. 3B-D).

Effects of MCT4 on intracellular lactate concentration, pH and cell apoptosis in HUVECs

To assess the effect of MCT4 on apoptosis in HUVECs, specific shRNA targeting MCT4 was used to knockdown MCT4. Western blot result showed that a remarkable decrease of MCT4 protein appeared after transfection with shMCT4 for 48 hours (Fig. 4A). In contrast, transfection with Ctl shRNA did not interfere with MCT4 protein. After transfection with shRNA for 48 hours, HUVECs were incubated under fresh medium for 24 hours to evaluate the intracellular lactate, pH level and apoptosis. As shown in Fig. 4B-C, MCT4 knockdown resulted in observably increase in the intracellular lactate concentration and significant drop of intracellular pH compared with Ctl shRNA group. Furthermore, MCT4 silencing induced apoptosis in HUVECs (Fig. 4D). The untreated or treated with Ctl shRNA groups each had less than 5% cell apoptosis, treated with shMCT4 displayed about 15% cell apoptosis (Fig. 4E). Besides, BAX protein level strongly increased, but Bcl2 reduced after MCT4 knockdown (Fig. 4F).

Discussion

In the present study, we demonstrate that endothelium damages were severe in *db/db* mouse by using SCIM, and HG plus IL-1 β increased apoptosis and decreased MCT4 expression and location in HUVECs. Once MCT4 is reduced, lactate efflux is blocked to induce lactic acid accumulation and pH dropping, which is involved in triggering apoptosis.

SICM has been used for living cell surface imaging because of its non-contact and minimally damaging scanning advantage. The hopping scanning makes it possible to imagine more convoluted surfaces [19], such as vascular endothelium. This allows us to obtain images of vascular inner surface to show endothelium damage. Utilizing

SCIM technology, Potter et al. got the images of ECs under different patterns of shear stress [8]. In our study, two morphologies of aortic endothelium were identified: the regularly tightly connected surface (*C57BL/6* mice) and the large area defects (*db/db* mice). These morphologies were highly suggestive of crimple or exfoliation of ECs in diabetes. Immunofluorescence with CD31 is a traditional technique to show endothelium damage [20]. SCIM and immunofluorescence provided clear experimental evidence for endothelium damage in diabetes.

Hyperglycemia and inflammatory cytokines trigger vascular endothelial dysfunction and damage during the development of diabetes [3, 21]. IL-1 β regulates the development and clinical sequelae of atherosclerosis and stimulates other inflammatory cytokines [21]. ECs apoptosis has drawn much attention in the pathogenesis of the acceleration of atherosclerosis associated with diabetes. Previous works have indicated that HG and inflammatory cytokines, such as TNF- α and IL-1 β , lead to ECs apoptosis in diabetes. Inflammatory cells secrete IL-1 in the concentration range of 1–25 ng/ml range [22], so 25 ng/ml IL-1 β was used as final concentration. Our study also demonstrated that HUVECs underwent apoptosis in exposure to HG or IL-1 β for 24 h, and HG plus IL-1 β accelerated this process. Bax is an accelerator of apoptosis, while Bcl2 is a resister. We found that HG and IL-1 β up-regulated Bax, but down-regulated Bcl2, which provided another evidence for HG and IL-1 β -induced apoptosis in HUVECs. Recently, Bock et al. demonstrated that glycolysis is a major source of ATP in ECs. The glycolysis activity of ECs is closely related to cell function, such as proliferation [9], migration [9, 23] and adherence [24]. Lactate is the final product of glycolysis and regulates glycolysis and intracellular pH. Our results showed that HG and IL-1 β increased the intracellular lactate concentration. However, the intracellular lactate level did not rise after the only treatment of HG or IL-1 β . Thus, these clues hint

that hyperglycemia and inflammatory cytokines may interact and expand the adverse effects on endothelial cells through regulation of the intracellular lactate.

As it has been reported that MCT1 is responsible for the efflux of lactic acid in most cells and tissues, while MCT4 mediates lactate acid efflux in glycolytic cells [25]. Consequently, we examined whether MCT4 participates in HG and IL-1 β -induced lactate accumulation. Our results indicated that MCT4 expression and its location on plasma membrane were decreased under HG and IL-1 β condition in HUVECs. Nikooie et al. demonstrated that diabetes dramatically reduced MCT4 mRNA and their expression on sarcolemmal membrane [26]. Thus, the decrease of MCT4 may be involved in inflammation and metabolic changes (hyperglycemia). Tan et al. reported that MCT4 knockdown increased intracellular accumulation of lactate in macrophages [27]. As intracellular acidification and pH dropping are not only associated with glycolysis inhibition, but also with activation of apoptosis in many cells [28, 29], we propose that MCT4 reduction in HUVECs during HG and IL-1 β condition may lead to lactic acid acidification and pH dropping which trigger apoptosis. Specific shRNA targeting MCT4 was used to silence MCT4 in HUVECs, and we found that MCT4 silencing exhibited higher intracellular lactic acid acidification and lower pH in HUVECs. Furthermore, MCT4 knockdown resulted in dramatically apoptosis. In addition, MCT4-knockdown HUVECs showed a higher level of BAX and a lower level of Bcl2. Thus, our study indicated that MCT4 down-regulation is involved in HG and IL-1 β -induced apoptosis in HUVECs. We confirmed the reduction of MCT4 under HG and IL-1 β , but how MCT4 is modulated is still unknown. Further study should be carried out focusing on the underlying mechanism of MCT4 modulation by HG and IL-1 β in ECs.

In conclusion, the present study showed that SCIM is a distinct technology to display the morphology images of endothelium damage. Furthermore, HG and IL-1 β -induced apoptosis is related to down-regulation of MCT4 and subsequent lactate acid accumulation and pH dropping in HUVECs. The present data also suggest that MCT4 might be a novel treatment target for vascular complications of diabetes mellitus.

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Figure legends

Fig. 1 Morphology imaging of endothelium of mice aorta.

A, Representative topographical SEM images of *C57BL/6* mice aorta endothelium. Scar bar was 20 μm . B - D, Representative topographical SICM images of aorta endothelium from *C57BL/6* mice and *db/db* mice. 3D and 2D SCIM images were showed in B, D and C, E, respectively. The greyscale represents the height. F, The percentage of endothelialised area. Data are means $\pm$ SE (n=3, * $P < 0.05$ compared with *C57BL/6* mice). G, Representative immunofluorescence with CD31 from mice aortic endothelium. The red area represented endothelium and the nucleus was blue. Scar bar was 50 μm .

Fig. 2 Effects of high glucose (HG) and IL-1 β on apoptosis and intracellular lactate in HUVECs

A, HUVECs apoptosis was assessed using TUNEL staining in HG (25 mM) with or without IL-1 β (25 ng/ml) treating for 24 h. Apoptotic cells were labelled with green. Nucleus was labelled with blue. Scar bar was 50 μm . B, The percentage of TUNEL positive cells. Values are means $\pm$ SE (n=3, * $P < 0.05$ compared with normal glucose (NG), ** $P < 0.01$ compared with HG). C, BAX and Bcl2 were detected by Western blot. Data represent means $\pm$ SE (n=3, * $P < 0.05$ compared with NG, ** $P < 0.01$ compared with NG). D, Intracellular lactate acid concentration at different interventions for 48 h. Values are means $\pm$ SE (n=3, * $P < 0.05$ compared with NG).

Fig. 3 Monocarboxylate transporters (MCTs) mRNA expression and the effect of high glucose (HG) and IL-1 β on MCT4 expression in HUVECs

A, The mRNA expression of MCT 1 - 4 in cultured HUVECs were analysed by RT-PCR. B and C, The mRNA and protein levels of MCT4 in HUVECs under HG (25 mM) with or without IL-1 β (25 ng/mL) were determined using real-time PCR and Western blot, respectively. β -actin was used for normalization. Data are means $\pm$ SE (n=3, * P < 0.05 compared with normal glucose (NG)). D, Location of MCT4 was detected by immunofluorescence. MCT4 was labelled with green and CD31 was labelled with red. Nucleus was labelled with blue. Scar bar was 20 μ m.

Fig. 4 Effects of MCT4 silencing on intracellular lactate, pH and cell apoptosis in HUVECs.

A, The efficiency of pLKO.1/MCT4 shRNA were verified using Western blot. β -actin was used for normalization. Data are means $\pm$ SE (n=3, ** P < 0.01 compared with Ctl shRNA). B, Intracellular lactate acid concentration at different interventions. Lactate acid concentration in each well was normalized to total protein content. Data are means $\pm$ SE (n=3, ** P < 0.01 compared with Ctl shRNA). C, Intracellular pH value was measured using BCECF probe at different interventions. Data are means $\pm$ SE (n=5, ** P < 0.01 compared with NG). D, Apoptosis was detected by TUNEL assay at different interventions. Apoptotic cells were labelled with green. Nucleus was labelled with blue. Scar bar was 50 μ m. E, The percentage of TUNEL positive cells. Values are means $\pm$ SE (n=3, ** P < 0.01 compared with Ctl shRNA). F, BAX and Bcl2 were detected by Western blot. Data represent means $\pm$ SE (n=3, * P < 0.05 compared with Ctl shRNA, ** P < 0.01 compared with Ctl shRNA).

Figure1

Figure2

Figure3

Figure4