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HUMAN ENDOTHELIAL CELLS IN HIGH GLUCOSE: NEW CLUES FROM CULTURE IN 3D MICROFLUIDIC CHIPS

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ABSTRACT

Several studies have demonstrated the role of high glucose in promoting endothelial dysfunction utilizing traditional two-dimensional (2D) culture systems, which, however, do not replicate the complex organization of the endothelium within a vessel constantly exposed to flow. Here we describe the response to high glucose of micro- and macro-vascular human endothelial cells cultured in biomimetic microchannels fabricated through soft lithography and perfused to generate shear stress. In 3D macrovascular endothelial cells exposed to a shear stress of 0.4 Pa respond to high glucose with cytoskeletal remodeling and alterations in cell shape. Under the same experimental conditions, these effects are more pronounced in microvascular cells that show massive cytoskeletal disassembly and apoptosis after culture in high glucose. However, when exposed to a shear stress of 4 Pa, which represents the physiological shear stress in the microvasculature, HDMEC show alterations of the cytoskeleton but no apoptosis. This result emphasizes the sensitivity of HMEC to flow. No significant differences in the thickness of glycocalyx was detected in HUVEC and HDMEC exposed to high glucose.

We conclude that culture in microfluidic microchannels unveils unique insights into endothelial dysfunction by high glucose.

KEYWORDS

Endothelium, microfluidics, microvasculature on a chip, diabetes, glucose, cytoskeleton

INTRODUCTION

Vascular endothelial cells (EC), which are located at the innermost layer of the blood vessel wall, preserve vascular integrity, guarantee the adequate perfusion of all the tissues and regulate metabolic homeostasis (Circ Res (2018)123:477). Beyond their role as a barrier between the blood and the surrounding tissues, EC serve fundamental functions in maintaining blood fluidity, regulating vascular tone, and contributing to immunity and inflammation. EC display a high degree of plasticity and modulate their activities in response to various mechanical and biochemical stimuli to maintain tissues homeostasis [1]. Indeed, being at the interface with the blood, they sense and respond to any variation of blood composition. In addition, EC are constantly subjected to shear stress, generated by blood flow and tangential to the endothelial surface [2], and hydrostatic pressure, due to blood pressure which originates a circumferential stretch [3]. These mechanical forces model endothelial shape, cytoskeleton and function [4–7].

Among others, high concentrations of glucose impair endothelial function. Accordingly, diabetes, a complex metabolic disease characterized by hyperglycemia, is associated with endothelial dysfunction leading to macro- and micro-vascular complications that are responsible for high morbidity and mortality [8–11]. Diabetes affects more than 300 million people worldwide and causes the death of about 1.6 million people per year [12]. Experiments performed on human EC in culture have demonstrated that high glucose induces oxidative stress and glycation-driven modifications of proteins, thus promoting a pro-inflammatory and pro-thrombotic phenotype [13–16]. These results were obtained in monolayers of EC in two-dimensional (2D) culture systems, in which the cells are grown on a flat substrate in the absence of flow. Undoubtedly, this experimental approach has yielded important hints in cell biology, biochemistry and drug discovery. However, 2D cell culture systems present a number of limitations, since they do not replicate the dynamic and complex structural organization of the endothelium. Consequently, alternative experimental models have been developed to better mimic the complexity of the vascular system and provide new insights in various fields, from

basic research to the clinics, including the screening of new drugs and the development of new tools for regenerative medicine [17]. Thanks to the merge of microfluidic technology and cell biology, it is now possible to culture EC in perfusable networks of microchannels and follow their behavior upon exposure to various stimuli.

We compared the effects of high concentrations of glucose on ECs in the classical 2D static culture vs a microfluidic 3D-flow culture system. We utilized the previously described “microvasculature on a chip” [18]. Since EC display a significant heterogeneity at the structural and functional levels according to their position in the vascular tree, we compared human EC isolated from the umbilical vein (HUVEC), a consolidated model of macrovasculature, and human dermal microvascular EC (HDMEC). To this purpose, it is noteworthy that microvascular EC represent the majority of the endothelium since they line a surface about 50 times larger than macrovascular EC [19].

We focused on the cytoskeleton and the glycocalyx, both targets of mechanical and biochemical signals arising from the microenvironment and both involved in mechanosensing and mechanotransduction [20,21]. The cytoskeleton is a dynamic structure which maintains the shape and the organization of the cell, and contributes to governing micro and macro-vascular EC response [Frontiers 2021]. The glycocalyx is a glycoproteic complex which coats the luminal surface of vascular endothelium and controls permeability as well as blood cell adhesion to the endothelium (Butler, JM, Am J Pathol 2020). In 3D cultures exposed to 0.4 Pa shear stress high glucose induced alterations of cytoskeletal organization in HUVEC without exerting any effect on the glycocalyx. Under the same experimental conditions, pronounced cytoskeletal rearrangements occurred in HDMEC and this was associated with increased apoptosis. However, because shear stress levels are higher in capillaries than in arteries or veins (Front. Physiol. 11:861.doi: 10.3389/fphys.2020.00861), we also cultured HDMEC in 3D with a shear stress of 4 Pa and found that high glucose induces marked alterations of the cytoskeleton but no apoptosis, suggesting that the rate of flow is fundamental for a correct interpretation of microvascular cell behavior in 3D microfluidic systems.

Interestingly, in HUVEC and HMEC cultured in the microfluidic devices high glucose does not alter glycocalix thickness.

MATERIALS AND METHODS

2D Static Cell culture

HUVEC and HDMEC from Lonza (Basel, Switzerland) were cultured on 2% fibronectin coated dishes using EBM-2 and EGM-2 MV, respectively, and used up to passage 4 according to manufacturer's instructions. HUVEC were used as a model for macrovascular EC, while HDMEC for microvascular EC. In some experiments, D-Glucose (Sigma Aldrich, St. Louis, Missouri, USA) was added to normal medium, in which the concentration of glucose is 1 mg/mL (5.5 mM, CTR), to obtain a final concentration of 5.4 mg/mL (30 mM, D-glu). L-glucose (30 mM, L-glu) was used as a control of osmolarity. The experiments were repeated four times.

3D-Flow Cell culture

In 3D experiments, HUVEC and HDMEC were cultured in microfluidic devices fabricated using a standard soft-lithography technique [18]. After coating with fibronectin (2%), the cells were seeded and subjected to a constant flow of 1 $\mu\text{L}/\text{min}$ of their growth medium so that the cells in the innermost channels of the microfluidic network (section $30 \times 30 \mu\text{m}$) experience a wall shear stress close to the physiological post capillary vein condition (about 0.4 Pa). HDMEC were also exposed to a constant flow of 10 $\mu\text{L}/\text{min}$ to generate a ~ 4 Pa shear stress, a value reflecting the one occurring in the microvasculature. Cells were cultured in the microfluidic devices for 7 days before beginning the experiment and then they were fixed for immunofluorescence. The experiments were repeated three times.

Western blot

Cells in 2D static conditions were lysed in 10 mM Tris-HCl (pH 7.4) containing 3 mM MgCl₂, 10 mM NaCl, 0.1% SDS, 0.1% Triton X-100, 0.5 mM EDTA and protein inhibitors. Equal amounts of proteins were separated by SDS-PAGE on 4–20% Mini-PROTEANTGX Stain-free Gels (Bio-Rad, Hercules, CA, USA) and transferred to nitrocellulose membranes by using Trans-Blot® Turbo™ Transfer Pack (Bio-Rad, Hercules, CA, USA). Western blot was performed using antibodies against Actin (Tebu Bio-Santa Cruz, Magenta, Italy) and nitrocellulose sheets of these Stain-free Gels were used as control loading. Secondary antibodies labelled with horseradish peroxidase (Amersham Pharmacia Biotech Italia, Cologno Monzese, Italy) were used and immunoreactive proteins were detected with the Clarity™ Western ECL substrate (Bio-Rad, Hercules, CA, USA). Images were captured with a ChemiDocMP Imaging System (Bio-Rad, Hercules, CA, USA). The experiment was repeated at least three times. A representative blot is shown.

Confocal imaging

Cells were fixed in phosphate-buffered saline containing 4% paraformaldehyde and 2% sucrose pH 7.6, permeabilized with Triton 0.3%, and incubated with phalloidin-TRITC (Thermo Fisher Scientific, Waltham, Massachusetts, USA) for 1 h at 4°C [21]. Nuclei were stained using Hoechst 33342 (Molecular Probes). Wheat Germ Agglutinin (WGA) Alexa Fluor 488 Conjugate (5 µg/ml, Molecular Probes) was utilized to stain the glycocalyx. Cells were then mounted with ProLong™ Gold Antifade Mountant (Invitrogen, Carlsbad, California, USA) and images were acquired using a 40X objective in oil by a ZEISS 710 confocal microscope. Images were analyzed using ImageJ.

Actin alignment quantification

Images derived from fluorescence microscopy for F-actin on HDMEC and HUVEC in control medium and in the presence of 5.4 mg/mL of D- or L- glucose were analyzed using the OrientationJ plugin of ImageJ, as described in detail in [23] to obtain orientation maps in which an angle theta is

attributed to every pixels involved in oriented actin structures. For 2D images, an ideal horizontal axis was assumed as $\theta=0$; in 3D the direction of flow within the $30 \times 30 \mu\text{m}$ channel was assumed as $\theta=0$. Angular distributions of actin fibers were calculated over 30 angular bins of width 3° , spanning the range from 0 to 90° . Pixels displaying an angle θ or $-\theta$ were counted in the same angular bin centered on θ . Distributions were plotted as the fraction of image pixels (called "frequency" in the following figures) whose angle falls within a given bin.

Apoptosis quantification

The Click-iT® TUNEL Alexa Fluor® 488 Imaging Assay (Thermofisher Scientific) was performed on HDMEC culture in 2D or 3D according to manufacturer's instruction.

Statistical analysis

Data are reported as means \pm SD. The data were normally distributed, and they were analyzed using one-way repeated-measures ANOVA. The p-values deriving from multiple pairwise comparisons were corrected by the Bonferroni method. Statistical significance was defined for a p-value ≤ 0.05 .

Regarding the figures, * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$.

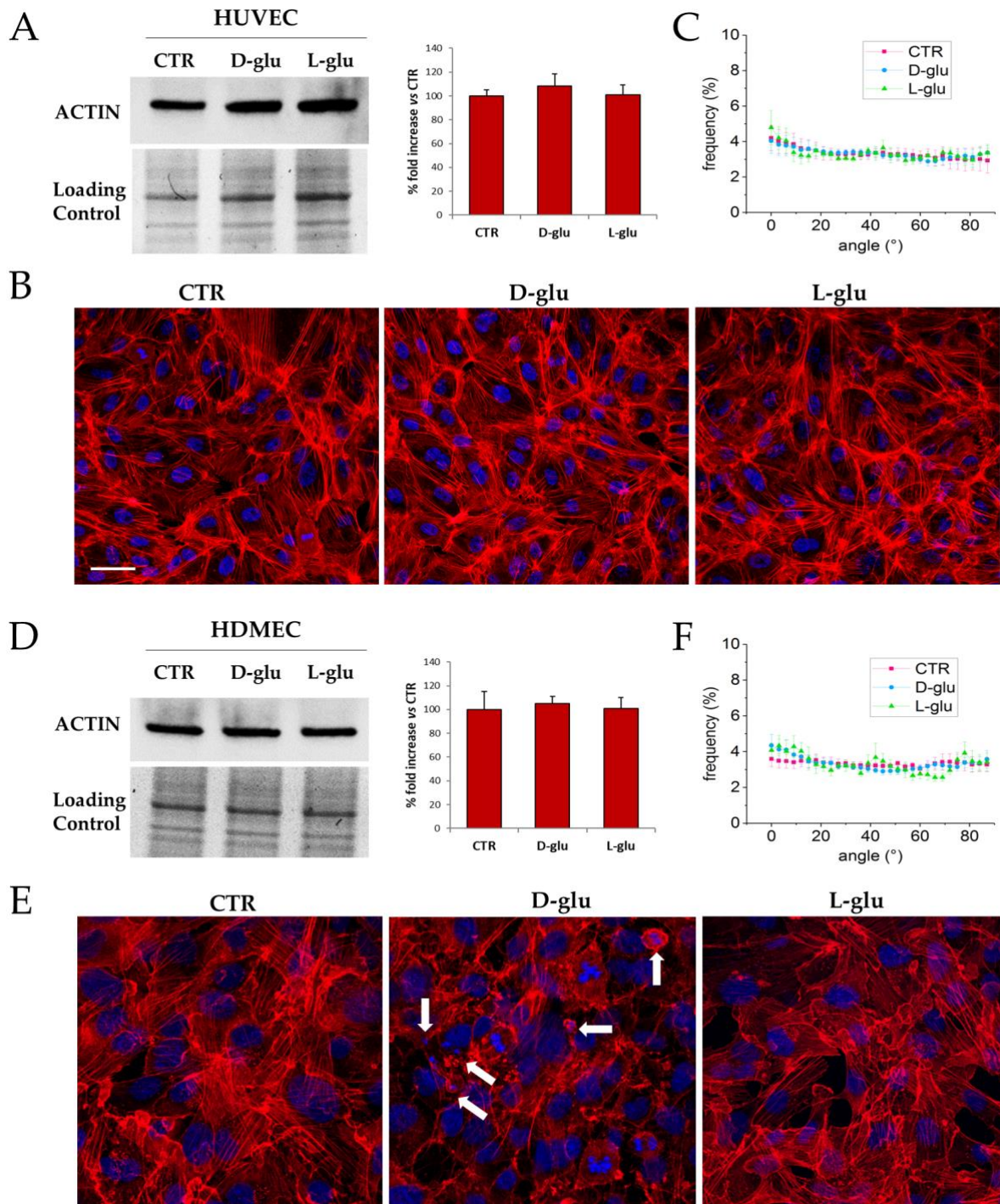
RESULTS

The effect of glucose on the actin cytoskeleton in HUVEC and HDMEC in 2D culture

We investigated the effect of high D-glucose containing medium on the actin cytoskeleton in HUVEC and HDMEC cultured in 2D in a petri dish under static conditions. The cells were exposed to high (30 mM) concentration of extracellular D-glucose for 24 h. Culture medium (CTR) and L-glucose 30 mM (L-glu) were used as controls. We then analyzed the total amounts of actin by western blot as well as its organization after staining with fluorescent phalloidin (Figure 1). In HUVEC we found that D-glucose has no significant effect on total actin content (Figure 1A) and its organization in fibers (Figure 1B) as evaluated by western blot and confocal microscopy, respectively. In addition, we quantified the orientation of actin fibers in several images of HUVEC cultured for 24 h with normal and high glucose-containing medium. HUVEC display many actin fibers both in CTR and D- and L- glucose conditions and no differences in the total orientation of the fibers was found (Figure 1C).

Also in HDMEC western blot shows no differences of actin content upon exposure to high glucose vs their controls (Figure 1D). Confocal microscopy after staining with fluorescent phalloidin shows that in basal condition (CTR) HDMEC display less fibers than HUVEC together with an enrichment of cortical actin. After culture in D-glu for 24h, F-actin resulted disorganized, with a lower amount of stress fibers and the presence of short or globular structures. No modulation of the orientation was found (Figure 1F). Nuclear staining highlights the presence of condensed and fragmented nuclei, hallmark of apoptosis, in D-glu treated HDMEC (Figure 1E). L-glucose has no effects.

In HUVEC and HDMEC glycocalyx was hard to detect and discontinuous, and no differences were observed in treated or untreated cells (data not shown). These results are in keeping with data from the literature indicating that shear stress is important for the development and maintenance of the glycocalyx (33).

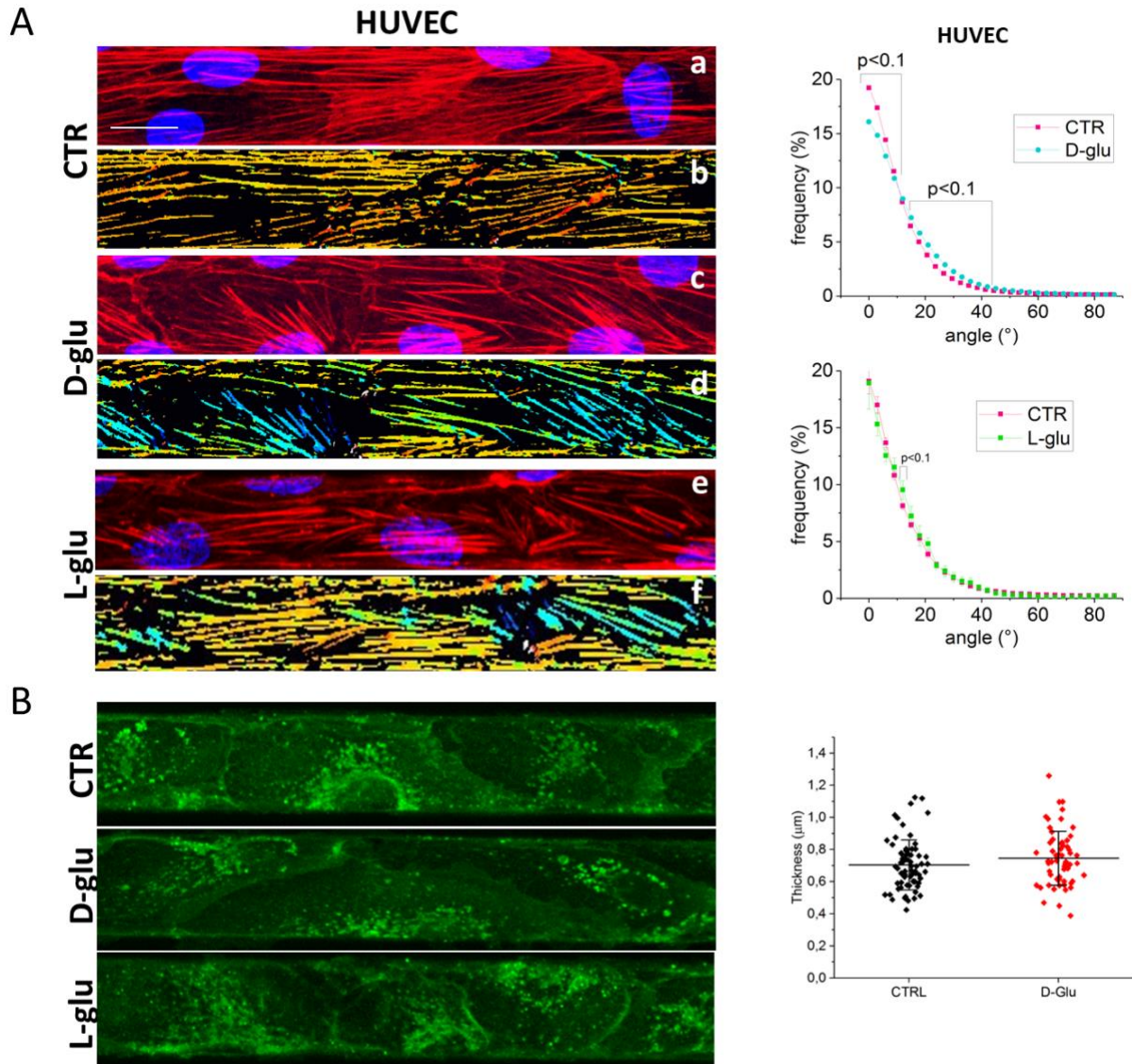


The effect of glucose on actin cytoskeleton in HUVEC cultured in 3D-microfluidic devices

HUVEC were cultured in microfluidic channels that mimic a vascular network. Specifically, we utilized the device described in [18], which has previously been used for HUVEC.

After seeding, HUVEC were grown to confluence in the presence of a steady flow of culture medium. Channels of nominal section $30 \times 30 \mu\text{m}$ were subjected for 7 days to a wall shear stress of about 0.4 Pa, which represents the conditions occurring in veins, and then perfused with medium containing either physiological or high concentrations of glucose (5.5 or 30 mM, respectively). 24 h later the cells were stained with phalloidin. As shown in figure 2A (left panel), HUVEC CTR and L-glucose treated cells show spindle-like morphology in response to flow, whereas HUVEC in D-glucose are less elongated. Stress fibers are clearly visible, but they tend to be shorter in HUVEC exposed to high D-glucose. While actin fibers in CTR and L-glucose HUVEC align to the direction of flow, they tend to be less flow-aligned in D-glucose treated cells. The orientation of F-actin was studied and the angle formed by actin fibers in relation to flow direction was plotted as described above. No alterations were detected (Figure 2A, right panels). The nuclei were conserved.

We then stained the glycocalyx using fluorescent WGA and analyzed the thickness. CTR, D- and L-glucose samples reveal comparable glycocalyx staining (Figure 2B left panel) and thickness (Figure 2B right panel).



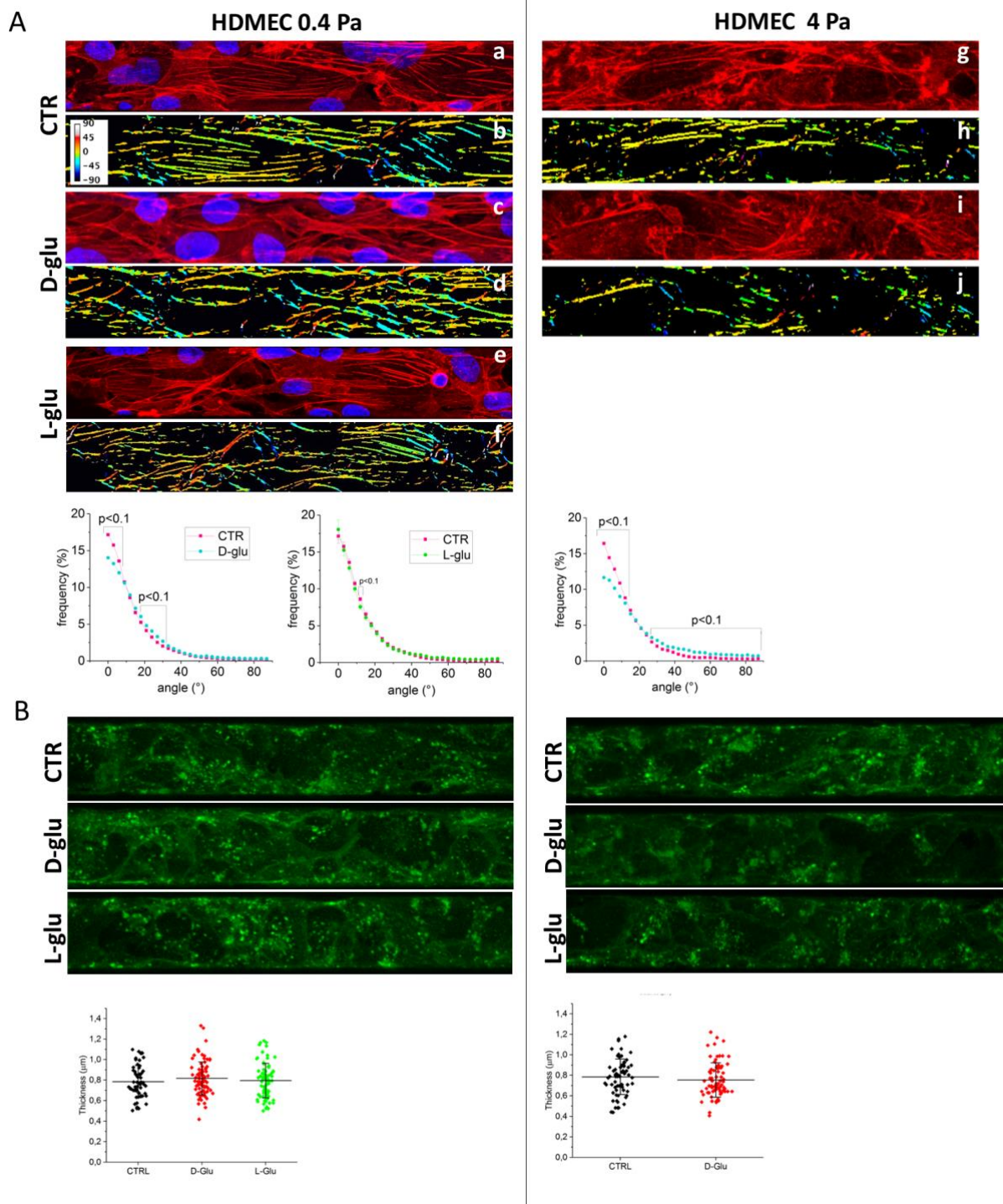
The effect of glucose on actin cytoskeleton in HDMEC cultured in 3D-microfluidic devices

We then turned our attention to HDMEC, which have never been cultured in 3D microfluidic systems. Initially, our aim was to compare HUVEC and HDMEC and, therefore, HDMEC were exposed to a shear stress of 0.4 Pa in medium containing normal or high glucose. After 24 h in the chip in the presence of high D-glucose, HDMEC presented many apoptotic/fragmented nuclei and most of the channels in the microfluidic device were blocked by cells detached from the wall of the channel (data not shown), making the analysis of actin cytoskeleton impossible. Therefore, we reduced the time of

exposure to high glucose to 16 h. In HDMEC cultured in CTR and L-glucose medium, actin fibers aligned with the direction of the flow, although they were less orderly organized than in HUVEC, and the nuclei appeared intact. In HUVEC treated with high D-glucose, stress fibers were disassembled and fragmented and tended to accumulate in the periphery of the cell (Figure 3A).

We then exposed HDMEC to 4 Pa shear stress for 16 and 24 and performed the same analysis (Figure 3A, right panels). Since comparable results were obtained after culture in the presence of high D-glucose for 16 or 24 h, we only show the data captured at XX h. Increased shear stress impacts on the structure of the cytoskeleton. Stress fibers are less organized and their orientation is more chaotic than in cells exposed to low shear stress. High D-glucose massively disassembles and fragments actin fibers, while L-glucose has no effect. Nuclei appear intact.

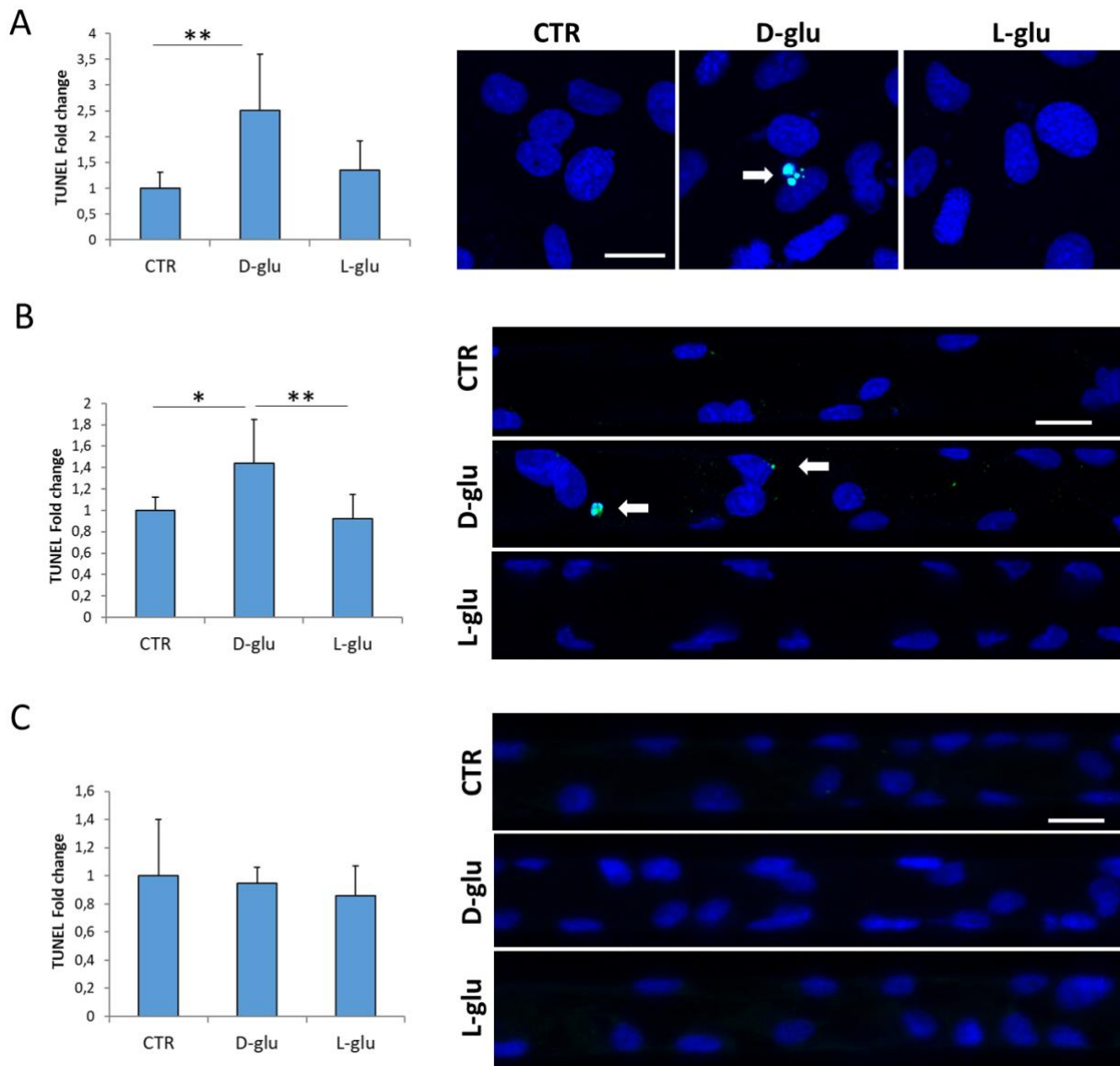
We also stained the glycocalyx and analyze its thickness in both the experimental conditions. Also in HDMEC, glycocalyx staining (upper panels) and thickness (lower panels) were not affected in all the conditions tested (Figure 3B).



The induction of apoptosis in D-glucose treated HDMEC cultured in 2D static condition and in 3D-microfluidic device

As previously shown, HDMEC treated with high D-glucose display many fragmented nuclei, suggesting that D-glucose induces apoptosis in these cells. To assess this hypothesis, we used the

TUNEL assay, a method for detecting DNA fragmentation by labeling the 3'-OH end in the double-strand DNA breaks generated by internucleosomal cleavage during apoptosis. Figure 4 shows that high D-glucose induces DNA damage in HDMEC grown in 2D (Figure 4A) or in 3D only under low shear stress (Figure 4B), while 4Pa shear stress prevents D-glucose induced apoptosis (Figure 4C). These results were confirmed by the analysis of nuclear parameters, i.e. nuclear area, nuclear perimeter and the brightness of each nuclear staining, which, as described in (25), represent a direct correlation with the induction of apoptosis (Supplementary figure).



DISCUSSION

Hyperglycemia is responsible for macro- and micro-vascular complications that account for most of the morbidity and mortality of diabetic patients [26]. As the endothelium is fundamental for vascular integrity [27], it is relevant that *in vitro* studies have demonstrated the detrimental effects of high extracellular glucose on endothelial function [28]. These studies have been performed in the absence of shear stress in 2D conditions. It is becoming clear that 2D culture, while significantly advanced our knowledge on endothelial cells, can generate results which deviate the *in vivo* behavior. To overcome these restraints, bio-inspired microfluidic devices have been developed to emulate the 3D structure of the vessel and to include fluid perfusion so that also mechanobiological cues are taken into account (Duval 2017). In this study we utilized the “vasculature on a chip”, which has previously been exploited for studying HUVEC (), because it mimics the geometrical features of the vasculature with sequential bifurcations and microvessels of different caliber [18]. Here we compare the response to high glucose of primary human EC cultured in 2D static conditions or in 3D perfused channels. Moreover, since significant differences are reported in EC from different districts in terms of phenotype, gene expression and function [29], we used both macro- and micro-vascular human EC. In 2D culture conditions, high glucose for 24 h did not alter HUVEC cytoskeletal organization and actin content. Moreover, there is no evidence of nuclear fragmentation, a hallmark of apoptosis. This result is in agreement with previous reports, showing the occurrence of apoptosis only after long times of exposure to high glucose [30–32]. In particular, Ido et al [32] detected no differences in morphology and a similar percentage of apoptotic cells (3–4%) in HUVEC incubated for 24 h in media containing 5 or 30 mmol/l glucose, while by 72 h about 18% of cells exposed to high glucose are apoptotic [32]. When cultured in 3D microfluidic chips, no signs of apoptosis were detected, but differences in the alignment of actin fibers emerged in HUVEC exposed to high glucose. The glycocalyx, is the primary structure exposed to blood flow and acts as a sensor that transmits the mechanical stimulus to the actin cytoskeleton of the cells.13,18. Since shedding of the glycocalyx

contributes to diabetes-induced vascular dysfunction [33], we hypothesized that cytoskeletal alterations could be due to alterations of the glycocalyx [34]. Alterations of glycocalyx, composition and/or structure, as observed for example in *db/db* transgenic diabetic mice [35], render the cells less sensitive to flow and, consequently, impair the shear stress response, because less force is transmitted to the cell interior. However, we did not detect alteration in the thickness of the glycocalyx.

It is believed that stress fiber alignment in response to shear stress is a feature of all endothelial cells. However, it has been reported that brain microvascular EC respond differently to physical stimuli when compared to macrovascular EC and do not align with flow [36]. We obtained similar results with HDMEC, since the cells show randomly oriented actin fibers, which are not completely aligned with the flow. These results underscore significant differences in the morphology and flow-driven alignment between HUVEC and HDMEC.

Our experiments also demonstrate that HDMEC are more sensitive than HUVEC to high glucose. Indeed, in 2D culture conditions, we found cytoskeletal disorganization and clear evidence of apoptosis in HDMEC after exposure to 30 mM of D-glucose for 24 h. These events are even more pronounced when the cells are cultured in 3D microfluidic chips. Cytoskeletal disassembly is clear after 16 h of culture in 30 mM D-glucose. Stress fibers are fragmented and tend to accumulate at the periphery of the cells. After 24 h of culture in high glucose in 3D, nuclear fragmentation is conspicuous and most of the cells detach from the vessel wall. As mentioned above for HUVEC, we hypothesize that high glucose might jeopardize the integrity of the glycocalyx. In addition, excessive accumulation of reactive oxygen species might impair cell viability and lead to the onset of apoptosis [37].

In summary, our results demonstrate that i) the detrimental effect of high glucose is more pronounced in HDMEC than in HUVEC and this might have important clinical implications; and ii) the presence of flow sensitizes the cells to high glucose, thus reinforcing the concept that EC integrate biochemical and mechanical signals through complex circuitries to evoke comprehensive functional response.

All these *in vitro* studies, EC are either (i) cultured under “no flow” conditions until confluence, and then placed under hydrodynamic shear, or (ii) cultured under flow from the moment they are plated into the flow device, but experiencing the same level (high or low) or pattern (laminar, recirculating, graded, or pulsating) of shear stress *from the start* of the culture. Most studies, therefore, investigated the response of EC when the flow is “switched on” or of EC submitted to specific shear conditions applied steadily from “time zero.” Strikingly enough, only a very limited number of works have addressed the question of the response of already shear-adapted EC to a loss of shear stress stimulation,^{26,27,39-42} as would occur, for instance, in occlusive microvascular events, or as a result of altered hemodynamics such as encountered in space flights^{43,44} or in ground simulations of microgravity conditions.⁴⁵

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No potential conflict of interest was reported by the authors.

AUTHOR CONTRIBUTIONS

L Locatelli performed most of the experiments; M Inglebert and P Kumari Sinha helped with culture in the microfluidic devices and R Scrimieri with 2D cell culture; GV Zuccotti, L Bureau, P Milani, C Misbah and JAM Maier designed research; L Locatelli and JAM Maier wrote the manuscript; and all authors analyzed the data.

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LEGENDS TO THE FIGURES

Figure 1. *The effect of high glucose on HUVEC cytoskeleton in 2D culture conditions.*

HUVEC were cultured for 24h in medium containing normal (CTR) or high D- or L-glucose.

(A) Western blot was performed with anti-actin antibodies. Nitrocellulose sheets were used to show that equal amounts of protein were loaded. Lower panel shows the result of densitometry.

(B) Confocal microscopy was performed on HUVEC stained with phalloidin-TRITC and Hoechst 33342. The images of actin staining were processed and analyzed using ImageJ software. Scale bar = 30 μ m.

(C) The plot was obtained by the quantification of the angle assumed by fibers in comparison to an ideal horizontal axis (named theta=0) during the orientation analysis. For each condition and for each angular bin of the distribution, we have plotted the mean \pm 1 standard error computed over n=6 images.

Figure 2. *The effect of high glucose on HDMEC cytoskeleton and apoptosis in 2D culture conditions.*

HDMEC were cultured for 24h in medium containing normal (CTR) or high D- or L-glucose.

(A) Western analysis was performed using anti-actin antibodies. Nitrocellulose sheets were used as a control of loading. Densitometry is shown in the lower panel.

(B) Confocal microscopy was performed after staining with phalloidin-TRITC and Hoechst 33342. Scale bar = 30 μ m.

(C) The plot was obtained by the quantification of the previous orientation analysis. For each condition and for each angular bin of the distribution, we have plotted the mean \pm 1 standard error computed over n=6 images.

Figure 3. *The effect of high glucose on actin cytoskeleton and nuclei in HUVEC and HDMEC cultured in 3D-microfluidic channels.*

- (A) HUVEC were cultured in medium containing normal or high D- or L- glucose for 24h, while HDMEC for 16 h. HUVEC (left panel) and HDMEC (right) were observed by confocal microscopy. The respective orientation analysis was performed using ImageJ (b, f, d for HUVEC and h, i, l for HDMEC). Scale bar = 20 μ m
- (B) Considering the flow direction as $\theta=0$, angle distributions are plotted as the mean value computed for each angular bin, over n=14 samples (HUVEC CTR), n=31 (HUVEC D-glu), n=7 (HUVEC L-glu), n=27 (HMEC CTR), n=6 (HMEC D-glu), n=9 (HDMEC L-glu). Instead of plotting \pm standard error as for 2D static, we provide brackets indicating the angular ranges over which the CTR and D- L- glucose distributions are statistically different, based on a bin-by-bin t test performed at a threshold of 0.1.
- (C) HDMEC were cultured in medium containing high D-Glucose for 24h. After staining with Hoechst 33342, confocal microscopy was performed. A representative image from microvessels with different caliber is shown. Scale bar = 10 μ m

Figure 4. *The effect of high glucose on nuclei in HDMEC cultured in 2D culture condition or in 3D-microfluidic channels.*

- (A) HDMEC were cultured in medium containing normal or high D- or L- glucose for 24h in 2D condition, while in 3D condition for 16 h. HDMEC were observed by confocal microscopy for nuclear staining using Hoechst 33342 and the quantification of Area, Perimeter and Brightness of each nucleus of different images was performed using ImageJ and plotted. Statistical analysis was performed using ANOVA.
- (B) HDMEC were cultured in medium containing normal or high D- or L- glucose for 24h in 2D condition, while in 3D condition for 16 h. Click-iT® TUNEL Alexa Fluor® 488 Imaging Assay was performed and shows green fluorescence in the presence of DNA fragmentation. The quantification of green fluorescence of different images was performed using imageJ and the mean of each sample was plotted. Statistical analysis was performed using ANOVA.

Supplemental data

Supplementary figure: The effect of high glucose on nuclear morphology in HDMEC cultured in 2D culture condition or in 3D-microfluidic channels.

HDMEC were cultured in medium containing normal or high D- or L- glucose for 16h in 2D and in 3D condition. HDMEC were observed by confocal microscopy for nuclear staining using Hoechst 33342 and the quantification of Area, Perimeter and Brightness of each nucleus of different images was performed using ImageJ and plotted. Statistical analysis was performed using ANOVA.

