Matrix metalloproteinase-9 mediated shedding of syndecan-4 in glomerular endothelial cells

Trine M Reine[1,4,5], Francesca Lanzalaco[1,2], Oddrun Kristiansen[1,7], Anne Randi Enget[1], Simon Satchell[3], Trond G Jenssen[4,6], Svein O Kolset[1]

[1]Department of Nutrition, Institute of Basic Medical Sciences, University of Oslo, Oslo, Norway, [2]Sciences and Technology, University of Milan, Milan, Italy, [3]Academic Renal Unit, University of Bristol, UK, [4]Section of Renal Diseases, Department of Organ transplantation, OUS, Oslo, Norway, [5]Institute for Cancer Genetics and Informatics, Oslo University Hospital, Oslo, Norway, [6]Metabolic and Renal Research group, UiT The Artic University of Norway, Tromsø, Norway [7]Department of Obstetrics, Division of Obstetrics and Gynecology, Oslo University Hospital, Oslo, Norway

Corresponding author: Trine M Reine, t.m.reine@medisin.uio.no

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Abstract

Background:

Diabetic nephropathy is the most common cause of end-stage renal failure in the western world and Asia. The mechanisms are not fully elucidated, but disruption of glomerular endothelial glycocalyx and shedding of its components including syndecans has been implicated.

Aims:

We hypothesize that reduced glomerular filtration in diabetes is caused by disruption of endothelial glycocalyx in glomeruli, including increased shedding of syndecan-4. The aim of this study was to determine the effects of experimental diabetic conditions by means of hyperglycemia and IL-1β exposure on syndecan-4 shedding in glomerular endothelial cells (GEnC), and to investigate regulation of shedding by sheddases.

Results:

We found that in GEnC the expression of syndecan-4 is higher than that of the other syndecans. In polarized GEnC, apical shedding of syndecan-4 and syndecan-4 gene expression was increased by 60% after IL-1β stimulation, but not affected by hyperglycemic conditions. This was accompanied by a 50% increase in Matrix Metalloproteinase-9 (MMP9) gene expression in IL-1β stimulated cells but not hyperglycemia. MMP9 knockdown reduced syndecan-4 shedding by 50%.

Conclusion:

IL-1β but not hyperglycemia increases the shedding of syndecan-4 from GEnC in an MMP9-dependent manner. This provides a potential mechanism of GEnC damage in diabetes and other inflammatory conditions.

Keywords: Diabetic nephropathy, Endothelial glycocalyx, Glomerular endothelial cells, MMP-9, Syndecan-4.

Introduction

All healthy vasculature is covered by an endothelial glycocalyx. This is a hydrated polyanionic gel consisting of cell-surface anchored proteoglycans including syndecans, as well as glycoproteins and glycolipids, supplemented with adsorbed soluble proteoglycans, glycosaminoglycans and plasma constituents (1). In the glomeruli, the basement membrane, podocytes and glomerular endothelium with its glycocalyx are all part of the filtration barrier (2). During pathological conditions components of the glycocalyx are shed, decreasing the size of the glycocalyx, increasing the endothelial permeability and reducing filtration capacity of the glomeruli, ultimately resulting in proteinuria (3). These components are released into the blood and can be detected in human serum.

Diabetes mellitus (DM) is a state of both chronic and acute hyperglycemia, and low-grade inflammation (4, 5), and it is known that short-term hyperglycemia induces insulin resistance, endothelial dysfunction and increase the vascular permeability (6-8) Diabetic nephropathy is the most common cause of end-stage renal failure in the western world and Asia (9, 10). The mechanisms behind the impaired filtration capacity of the kidneys are not fully elucidated, although hyperglycemia is recognized as an important risk factor for the development of diabetic nephropathy. Glucose-induced increased levels of interleukin 1β (IL-1β) in the circulation are seen both in Type 1 (T1)DM and T2DM (11, 12). IL-1β levels could reflect severity of the disease, and in a recent study we measured higher levels of IL-1β together with higher glycated hemoglobin A1c (HbA1c) in patients with T1DM and kidney failure compared to those with T1DM but with normal renal function (13).

The syndecans are a group of four transmembrane proteoglycans that are part of the endothelial glycocalyx and constituents of focal adhesion sites facing the basement membrane as well as components of the basement membrane itself (14, 15). Syndecan-1 and -3 are substituted with both heparan sulfate and chondroitin sulfate chains, while syndecan-2 and -4 are decorated with heparan sulfate chains only. The functionality of the syndecans is contributed by these glycosaminoglycans through their interaction with different extracellular ligands. Such ligands include anticoagulants such as antithrombin and growth factors like fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF) and transforming growth factor-β (TGFβ), in addition to different proteases, protease inhibitors and cytokines. In line with this, we have previously demonstrated a major role for syndecan-4 in angiogenesis (16).

All syndecans can be shed from the cell surface of endothelial cells by the action of sheddases (17), resulting in increased syndecan serum levels. Soluble syndecan ectodomains can function as paracrine and autocrine effectors or competitors, and may have important functions in influencing coagulation pathways, angiogenesis or inflammation. Syndecan shedding is increased as a response to acute vascular trauma, as we have demonstrated for syndecan-1 (18). We have also documented that increased serum syndecan-1 is related to early diabetic nephropathy (19), and we observed a trend for increased syndecan-1 serum levels in the hyperglycemic group compared to normoglycemic kidney transplanted patients together with increased levels of the syndecan-1 sheddase matrix metalloproteinase-9 (MMP-9) (20). This promising potential for syndecans as markers for endothelial dysfunction is further strengthened by our most recent results generated. In a case-cohort study including 1500 participants from the Tromsø 5 study, we found that syndecan-4 shedding is an independent

predictor of myocardial infarction and correlated with baseline estimated glomerular filtration rate (eGFR) (21).

The different syndecans have different cell- and tissue- specific expressions and functions. It is commonly reported that syndecan-3 is mainly expressed in neural cells, while syndecan-4 is high in epithelium and fibroblasts, and low in neural and endothelial cells (22) (23). In contrast to this, we have published data demonstrating high expression of both syndecan-4 and syndecan-3 in human umbilical vein endothelial cells (HUVEC) but low expression of syndecan-1 and -2 (16). The high endothelial expression of syndecan-3 and -4 is also supported by other recent publications (24-26). Conditionally immortalized glomerular endothelial cells (ciGEnC) are the cells of choice to study endothelial glycocalyx in glomeruli. These cells are covered by an extensive glycocalyx of up to 200 nm (2), and also express several cell surface proteoglycans including syndecan-4 (27), likely to contribute to the integrity of the glomerular filtration barrier.

We hypothesize that impaired regulation of glomerular filtration in diabetes is caused by disruption of the endothelial glycocalyx in the glomeruli, including increased shedding of syndecan-4. The aim of this study was to determine the effects of experimental diabetic conditions on syndecan-4 shedding in glomerular endothelial cells, and to investigate how the shedding is regulated by targeting the possible sheddases involved.

Materials & Methods

ciGEnC culture

The human ciGEnC have previously been characterized in detail (2, 28). In short, primary culture GEnC were transduced with temperature-sensitive (ts) simian virus 40 large tumor (SV40LT) antigen and telomerase using retroviral vectors. At the permissive temperature (33^{o}C) the tsSV40LT transgene is activated causing cell proliferation without telomere shortening. At 37 ^{o}C the transgene is inactivated and the cells retain their properties as primary cells. Cells were cultured in MCDB 131 medium (Sigma) containing 5 mM glucose and supplemented with 7% heat-inactivated FCS (Sigma), basicFGF (1 ng/ml, R&D), hydrocortisone (1 µg/ml, Sigma), EGF (10 ng/ml, R&D), gentamicine (50 µg/ml, GIBCO Invitrogen) and fungizone (250 ng/ml, GIBCO Invitrogen) at 5% CO_2. The cells were subcultured at the permissive temperature and used for experiments after 5 days at 37 ^{o}C. Incubations with IL-1β (R&D systems) were performed for 24 hours using a concentration of 0.5 ng/ml; determined by dose-response and time-response experiments (not shown).

Polarized cells were obtained as described in Meen et. al. (29). GEnC were cultured for 5 days at the non-permissive temperature, and then seeded on semipermeable filters; Costar Transwell clear polyester membrane inserts with a pore size of 0.4 μm (Sigma-Aldrich) in 12-well plates. The cells were seeded at a density of 1 × 10^5 cells/cm^2 with 1.5 ml of culture medium in the basolateral compartment and 0.5 ml in the apical compartment and cultured for several days to obtain a confluent monolayer and allow the cells to polarize. The medium was changed every second day until the start of the experiment, when a tight monolayer had been established. The polarized ciGEnCells were subjected to experimental diabetic

conditions in the form of hyperglycemia (25 mM glucose) and / or 0.5 ng/ml IL-1β at 33°C and 5% CO_2 for 24 hours, and compared to control cells cultured in 5 mM glucose.

Cytotoxicity assay

The possible cytotoxic effect of exposure to IL-1β and hyperglycemia was investigated by measurement of lactate dehydrogenase (LDH) activity released from damaged cells, using the Cytotoxicity Detection Kit (11 644 793 001, Roche) according to the manufacturer's instructions. In brief, conditioned media from cells cultured under the different conditions was harvested; assay reagent was added and incubated at 20°C for up to 30 min before recording the absorbance at 490 nm. Conditioned media from Triton X-100 treated cells were included as positive control, and non-conditioned medium was included as negative control. % of maximum cytotoxicity (positive control) was calculated for each sample.

ELISA-assays

Conditioned medium was collected and centrifuged to remove cell debris, and shedded syndecan-4 and soluble intracellular adhesion molecule-1 (sICAM-1) were detected according to the manufacturer's instructions using the Human Syndecan-4 Assay Kit (JP27188, IBL) and Human ICAM-1/CD54 Duoset-1 (DY720-05, R&D). All standards were within the limits of detection and inter- and intra-assay coefficient of variation (CV) was < 10%.

Gene expression analysis

Total RNA was isolated from cultured GEnC using the E.Z.N.A. Total RNA kit 1 (R6834-02, Omega Bio-Tek) according to the manufacturer's instructions. RNA quantity measurements were performed using the ND1000 Spectrophotometer (Saveen Werner) and RNA was stored at -20°C until further analysis. A quantity of 250 ng RNA was reversely transcribed in a total

volume of 20 µl using the "High capacity RNA-to-cDNA kit" (4387406, Applied Biosystems). Quantitative Real-time PCR (qRT-PCR) was performed on a CFX96 Real-Time System c1000 Touch (BIO-RAD) using TaqMan Gene Expression Master Mix (4369016, Applied Biosystems) and predesigned TaqMan Gene Expression Assays as listed in Table 1. 60S ribosomal protein L30 (RPL30) or beta actin (ACTB) was used as endogenous control. The cycle threshold (Ct) cutoff value was set to 40 cycles, and the relative mRNA level for each transcript was calculated by the ΔΔCt method (30). Briefly, the Ct values for each gene was normalized against the Ct values of the reference gene (= ΔCt). For comparison of gene expression in treated versus control cells, ΔΔCt was calculated as ΔCt in treated cells subtracted the ΔCt for control cells. The fold change in mRNA expression was calculated as $2^{-\Delta\Delta Ct}$.

Gene knockdown

The expression of human MMP9 was reduced using siRNA (sc-29400, Santa Cruz) or the silencer negative control siRNA (scramble, AM4635, Ambion) at 0.02 µM. ciGEnCells were reversly transfected with Lipofectamine RNAiMAX transfection reagent (13778030, Thermo Fisher Sceintific) in Opti-MEM (Invitrogen) at a cell density of 200 000 cells per ml (25 000 cells / cm^2), allowed to adhere and incubated for 20 hours. Then, the transfection medium was replaced with MCDB growth medium and experiment initiated. The silencing efficiency was quantified by qRT-PCR.

Statistical analysis

The data were analyzed using Graph Pad Prism 5.03. Data are presented as mean ± SEM of the indicated number (n) of experiments. Normality tests were conducted only for those experiments with n>6. For all other experiments, a normal distribution was assumed. Comparative analysis of the data was carried out using the Student's paired t-test, One-way

ANOVA or Two-way ANOVA or Friedmans test as appropriate. Differences of $p<0.05$ were considered significant.

Results

Polarized ciGEnCells were subjected to experimental diabetic conditions in the form of hyperglycemia (25 mM glucose) and / or 0.5 ng/ml IL-1β for 24 hours, and compared to control cells cultured under normoglycemic conditions of 5 mM glucose. Both morphological inspection and von Willebrand Factor (vWF) expression indicated that the cells retained the characteristics of primary endothelial cells (Fig 1A and B). Cytotoxicity determined by LDH-leakage showed no reduced viability as a result of the treatments (Fig 1C).

Increased expression of ICAM-1 and shedding of ICAM-1 from the endothelial cell surface are signs of endothelial cell activation or dysfunction. We observed a constitutive shedding of sICAM-1 to the apical side of the cells, while the levels retrieved at the basolateral side were comparably very low. Upon IL-1β stimulation, the levels of sICAM-1 were increased 1.8 fold at the apical side. The basolateral levels were increased 20 fold, but were still at very low levels compared to the apical levels. Hyperglycemia on the other hand, had no effect on the levels of sICAM-1 (Fig 2A). Also; ICAM-1 gene expression was increased 2.7 fold by IL-1β-stimulation while unaffected by hyperglycemia (Fig 2B).

We found that ciGEnCells express all four syndecans. (Fig 2C). Syndecan -4 expression was significantly increased by IL-1β but not glucose (Fig 2D). The levels of shedded syndecan-1 were below the limit of detection, and we lacked satisfying tools for detection of soluble syndecan-2 and -3. However, we observed a constitutive shedding of syndecan-4 to the apical side of the cells. This was increased 1.6 fold to the apical side and 1.2 fold to the basolateral

side as a response to IL-1β stimulation (Fig 2E), but remained unaffected by hyperglycemia. In line with this, gene expression was unaffected by hyperglycemia, but increased 1.6 fold by IL-1β-stimulation (Fig 2F).

Several sheddases are candidates for the regulation of syndecan-4 shedding (17). The relative gene expression of these sheddases (MMP2, MMP9, ADAM17, a disintegrin and metalloproteinase (ADAMST)1, ADAMST 4) and some of their inhibitors (tissue inhibitor of metalloproteinases (TIMP)-1 and TIMP-2) was determined (Fig 3). Among these targets, only MMP9 was increased by IL-1β (Fig 3). Hyperglycemia did not affect gene expression of any of these targets. This observation led us to hypothesize that MMP9 is involved in the inflammation-driven increase in syndecan-4 shedding in these cells.

To investigate further the possible role of MMP9 in syndecan-4 shedding, we made use of siRNA gene silencing (Fig 4A). Gene silencing reduced MMP9 gene expression by 84 % in unstimulated cells and with 65 % in IL-1β stimulated cells. This difference reached statistical significance when applying a parametric test only. This resulted in a 47 % reduction in syndecan-4 shedding in unstimulated cells and a 51 % reduction in stimulated cells (Fig 4B). SDC4 gene expression was not affected by MMP9 knockdown (Fig 4C). Comparison of siMMP9 to scrambled RNA gave similar results, indicating no effect of siRNA transfection per se. In comparison, soluble levels and gene expression of s-ICAM-1 was not reduced by the reduced activity of MMP9 (Fig 4E, D).

Discussion

In this study, in human glomerular endothelial cells we found that shedding of both syndecan-4 and ICAM-1 was increased by the pro-inflammatory cytokine IL-1β, but not by hyperglycemia.

Furthermore, MMP9 was involved in shedding of syndecan-4, but not ICAM-1. The main results illustrating that IL-1β but not hyperglycemia increase the shedding of syndecan-4 from GEnC in an MMP9-dependent manner are illustrated in Fig. 5.

These results are comparable to the findings of Ramnath R et. al. They showed that treatment with the pro-inflammatory cytokine tissue necrosis factor (TNF)α induced MMP9-mediated shedding of syndecan-4 in the same cell type as in our study. Importantly, they demonstrated that syndecan-4 shedding resulted in increased permeability of ciGEnC monolayers (27). This translates to a role of syndecan-4 in glycocalyx integrity.

The components of the glomerular filtration barrier are the specialized fenestrated glomerular endothelial cells covered with its glycocalyx, the glomerular basement membrane, and the podocytes with their slits. The endothelial glycocalyx has emerged as an important component of the renal filtration barrier. Structural alterations and shedding of glycocalyx components is seen in diabetic nephropathy (31, 32), and disruption of the glycocalyx leads to proteinuria in various experimental in vivo studies (33, 34). The syndecans are important constituent of the glycocalyx, and in line with other publications we found that syndecan-4 is highly expressed by GEnC and an important component of the glycocalyx (27). We hypothesized that hyperglycemia and IL-1β contribute to impaired renal filtration in diabetes trough increasing the shedding of syndecan-4 from the surface of glomerular endothelial cells.

Increased expression of ICAM-1 and release of sICAM-1 from the endothelial cell surface are signs of endothelial activation or dysfunction. In our hands, hyperglycemia had no effect on ICAM-1 gene expression or sICAM-1 levels in GEnC. Furthermore, we found no effect of hyperglycemia on syndecan-4 shedding or gene expression. This indicates that hyperglycemic conditions are tolerated well by these cells, not resulting in cellular dysfunction or stress.

These observations are in contrast to Singh A et.al., who demonstrated decreased synthesis of glycosaminoglycans (the sugar side chains of proteoglycans including syndecans) and increased permeability as a result of high glucose treatment in GEnC (35). However, the discrepancy between these two studies could be explained by the different experimental conditions used. Our exposure to hyperglycemia was short term (24 hours), while Sing et al stimulated the cells for 2-14 days. We chose to perform a more short term exposure to high glucose, in line with what is practically feasible and as a commonly used time point.

In clear contrast to the lack of effects seen after hyperglycemic treatment, we found that the pro-inflammatory cytokine IL-1β lead to activation of the endothelial cells. Both gene expression and the constitutive release of ICAM-1 from the apical surface were increased as a response to this treatment. This was accompanied by an increase in apical shedding and gene expression of the glycocalyx component syndecan-4.

These results suggest that the detrimental effects of the diabetic condition on glomerular endothelium are not caused by direct effects of glucose on the endothelium, but rather mediated trough secondary downstream effects of hyperglycemia or other aspects of the diabetic state. Several mechanisms are proposed for how glucose levels through altered flux trough different metabolic pathways affect expression of cytokines. These include effects on advanced glycated end products (AGE)-formation and receptor for AGE (RAGE)-binding, activation of protein kinese C (PKC) and increased O-linked N-acetylglucosamine (O-GlcNAc) signaling (36). Accordingly, increased levels of proinflammatory cytokines including IL-1β are seen in hyperglycemia and diabetes (11, 12). Endothelial cells respond to several cytokines released systemically, including the classical proinflammatory IL-1 cytokines. IL-1β signals via IL-1 Receptor-1. This results in activation of transcription factors including nuclear factor κB

(NFκB), inducing the mRNA expression of a series of genes to exert biological effects (37). The activation of the IL-1 pathway is linked to atherogenesis and CVD in diabetes (11). In recent years, new pathways involved in the development and progression of diabetic kidney disease have been elucidated; and accumulated data have emphasized the critical role of inflammation in the pathogenesis of diabetic nephropathy, triggered by factors such as increased intraglomerular pressure and hyperfiltration (38).

We investigated the regulation of IL-1β-induced syndecan-4 shedding in the glomerular endothelial cells. According to the literature, there are several candidate sheddases of syndecan-4 (17), including MMP2, MMP9, ADAM17, ADAMTS1, ADAMTS4 and the inhibitors TIMP-1 and TIMP-2. However, only expression of MMP9 was increased by IL-1β. Silencing the MMP9 gene we showed that syndecan-4 shedding was regulated by MMP-9. ICAM-1 release from the cell surface, however, was not affected in the MMP9 knockdown, indicating that the shedding of this component is differently regulated.

A major part of the functionality of the syndecans is mediated by their glycosaminoglycan side chains interacting with different partner molecules. The functional consequences of shedding of glycocalyx components are under intensive study. Increased shedding could merely be a mark of a dysfunctional endothelium where the balance between synthesis and degradation and shedding is dysregulated. Alternatively, shedding could be a protective mechanism, by reducing intracellular signaling and intracellular events, to protect partner molecules from degradation or to present them to other cells, to create chemotactic gradients, or to clear the components in the liver. Importantly, it is reasonable to assume that shedded syndecans and syndecans present at the cell surface have very different functions.

Strengths and limitations

Some limitations of this study warrant discussion. In this study, we chose to study the effects of short-term hyperglycemia, although long term exposure is also of obvious interest in diabetes. The disorder of glucose levels in diabetes has two main components: long-term chronic hyperglycemia and acute glycemic fluctuations between high and low values. Acute hyperglycemia has been associated with increased endothelial dysfunction and permeability (8). However, the impacts of chronic hyperglycemia are more extensively studied (39, 40).

A limitation of this study is the relatively low number of repetitions. Normally distribution of the data was assumed but could not be calculated for all experiments. Thus, the results of the statistical tests should be interpreted with caution and with the kind of statistical test employed in mind.

As a simplified *in vitro* study, this model do not reflect all parameters present *in vivo*, including all components of the blood, sheer stress etc. However the simplified *in vitro* approach has advantages when studying the contribution of individual components. Another strength of the study is the more *in vivo* relevant system of polarized cells obtained by culturing the on semipermeable filters.

Perspectives

The glomerular endothelium is emerging as a key player in diabetic nephropathy. IL-1β increases the shedding of syndecan-4 from these cells in an MMP9-dependent manner. This provides a potential mechanism of GEnC damage in diabetes and other inflammatory conditions. The glycocalyx is present at the apical surface of all endothelial cells, and damage of the glycocalyx has been implicated in several diseases. Thus, the implications of this work are not limited to glomerular endothelium, but extend to vascular function and disease throughout the circulation.

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Tables

Table 1 – TaqMan gene expression assays used in qRT-PCR analysis

Gene	Protein	Assay ID
RPL30	60S ribosomal protein L30	Hs00265497_m1
ACTB	Beta actin	Hs99999903_m1
VWF	Von Willebrand Factor	Hs01109446_m1
MMP9	Matrix metalloproteinase-9	Hs00234579_m1
MMP2	Matrix metalloproteinase-2	Hs01548727_m1
TIMP1	Tissue inhibitor of metalloproteinases-1	Hs00171558_m1
TIMP2	Tissue inhibitor of metalloproteinases-2	Hs00234278_m1
ADAM17	ADAM-17	Hs01041915_m1
ADAMTS1	ADAMTS1	Hs00199608_m1
ADAMTS4	ADAMTS4	Hs00192708_m1
SDC1	Syndecan-1	Hs00896423_m1
SDC2	Syndecan-2	Hs00299807_m1
SDC3	Syndecan-3	Hs00206320_m1
SDC4	Syndecan-4	Hs00161617_m1
ICAM-1	Intercellular Adhesion Molecule 1	Hs00164932_m1

Figure legends

Fig 1. (A) Normal endothelial cobblestone morphology of a confluent layer of ciGEnCells at 37^{o}C, both control cells (Ctr) and IL-1β stimulated cells (IL-1β).. (B) Gene expression of the endothelial marker vWF in control cells at 37^{o}C, expressed as ΔCt values (Ct of target gene – Ct of reference gene), n=4. (C) Cytotoxicity of IL-1β, hyperglycemia or the combination when exposed to ciGEnCells at 37^{o}C for 24 hours and compared to untreated control cells (Ctr), n=6. Only statistical significant differences related to the ctr are marked in the figures. * $p<0.05$, **$p<0.01$, ***$p<0.001$

Fig 2. Effect of hyperglycemia and IL-1β on ICAM-1 and syndecan shedding and gene expression (A) Soluble ICAM-1 levels detected in the apical and basolateral compartements, compared to the apical control, n=8. (B) Gene expression of ICAM-1 expressed as fold change and related to the untreated Ctr, n=6. (C) Comparison of gene expression levels of the syndecans, presented as ΔCt values (Ct of target gene – Ct of reference gene). Note that the lower the Ct value, the higher gene expression, n=3. (D) Effect of experimental diabetic conditions on syndecan gene expression levels, expressed as fold change related to the untreated control, n=3. (E) Levels of shedded syndecan-4 in the apical and basolateral compartments, compared to the apical control, n=7. (F) Gene expression of SDC4 expressed as fold change and related to the untreated Ctr, n=6. Only statistical significant differences related to the ctr are marked in the figures. * $p<0.05$, **$p<0.01$, ***$p<0.001$

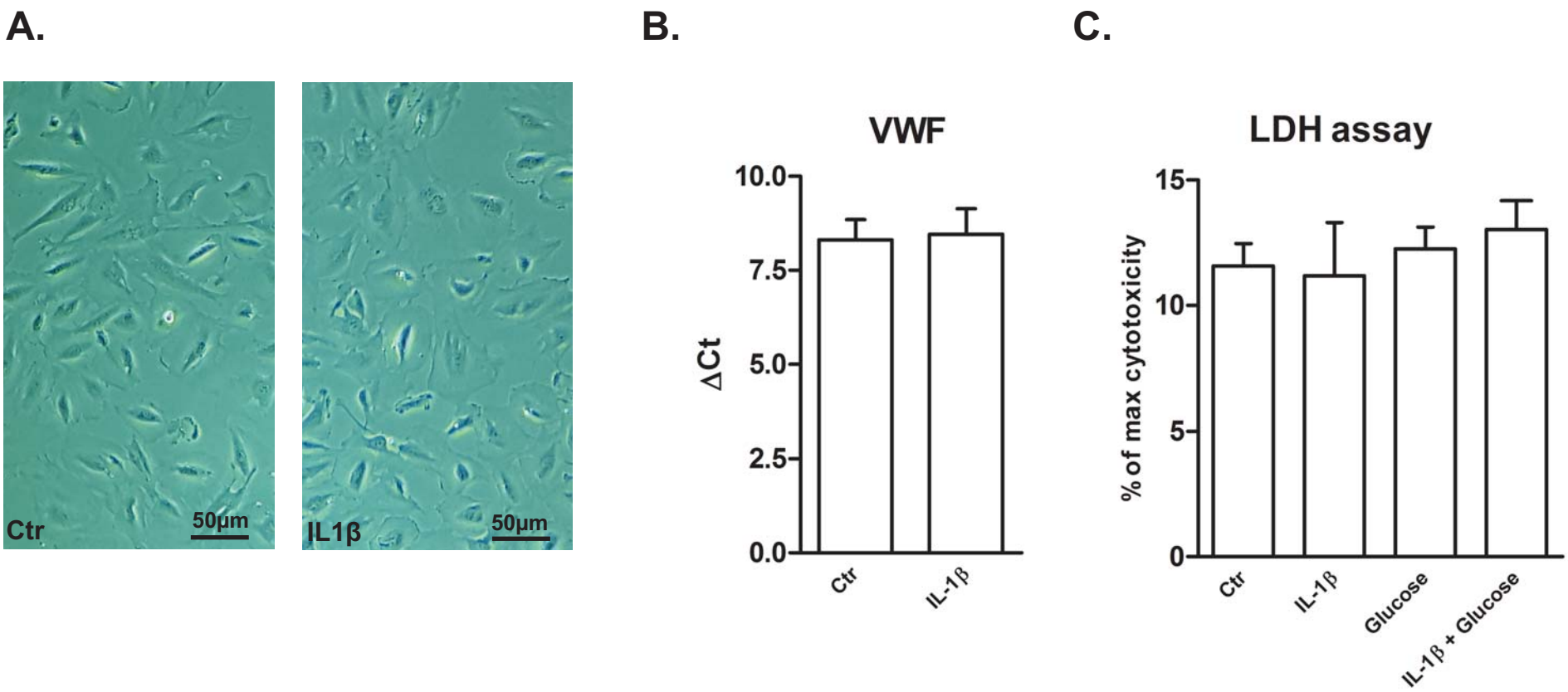
Fig 3. Effect of hyperglycemia and IL-1β on sheddase gene expression. Effect of IL-1β, 25 mM of glucose or the combination on gene expression of sheddases, expressed as fold change and related to the untreated control (Ctr), n=6. Only statistical significant differences related to

the ctr are marked in the figures. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, and each target is evaluated independently.

Fig. 4. Effects of MMP-9 gene silencing on syndecan-4 and ICAM-1 shedding and gene expression. (A) MMP9 expression in MMP9 knockdown (siMMP9) compared to control (Ctr) both in the absence (n=3) and presence (n=7) of IL-1β stimulation.(B, C) Levels of shedded syndecan-4, n=6 (B) and SDC4 gene expression, n=3 (C) in unstimulated and IL-1β stimulated MMP9 knockdown cells compared to unstimulated and IL-1β stimulated control cells. (D, E) Levels of soluble ICAM-1 (D) and ICAM-1 gene expression (E) in unstimulated and IL-1β stimulated MMP9 knockdown cells compared to unstimulated and IL-1β stimulated control cells, n=3. Gene expressions were presented as fold change relative to untreated control (Ctr). It is indicated whether or not siMMP9 data are significantly different from ctr, and whether or not siMMP9 + IL1β data are significantly different from IL-1β. ns $p>0.05$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$ when using parametric tests.

Fig 5. Inflammation but not hyperglycemia increase endothelial syndecan-4 shedding in an MMP9-dependent manner. (A) In control cells, syndecan-4 is presented mainly at the apical surface of the endothelium with a constitutive but low degree of shedding. (B) Hyperglycemia alone did not affect syndecan-4 shedding from the cell surface. (C) In contrast, the pro-inflammatory cytokine IL-1β increased gene expression of syndecan-4 and MMP-9, increasing the MMP-9 mediated shedding of syndecan-4 and levels of soluble syndecan-4. AP: apical side (vessel lumen), BL: basolateral side.

Figure 1


A.
Ctr
50µm
IL1β
50µm
B.
VWF
ΔCt
10.0
7.5
5.0
2.5
0.0
Ctr
IL-1β
C.
LDH assay
% of max cytotoxicity
15
10
5
0
Ctr
IL-1β
Glucose
IL-1β + Glucose

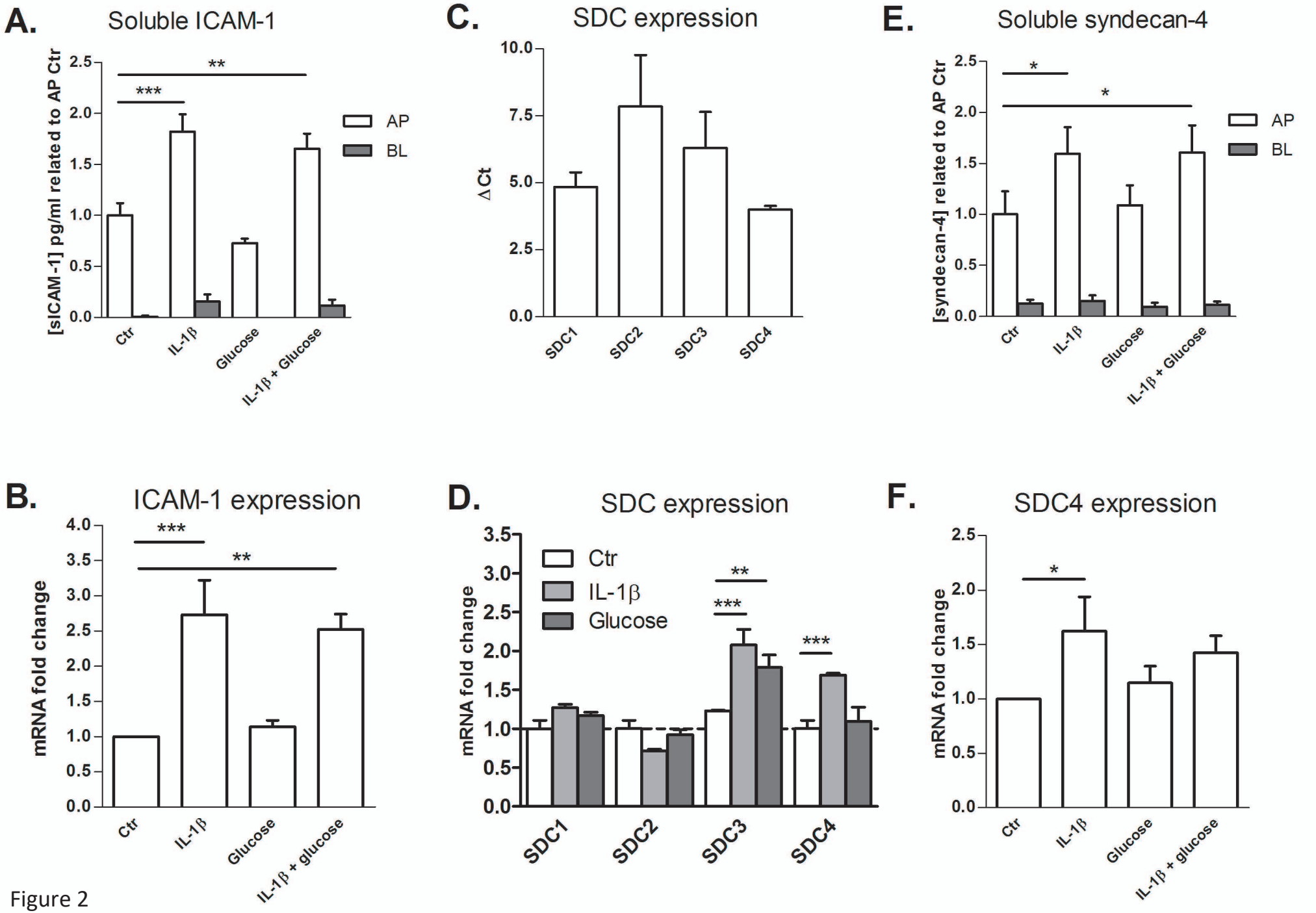
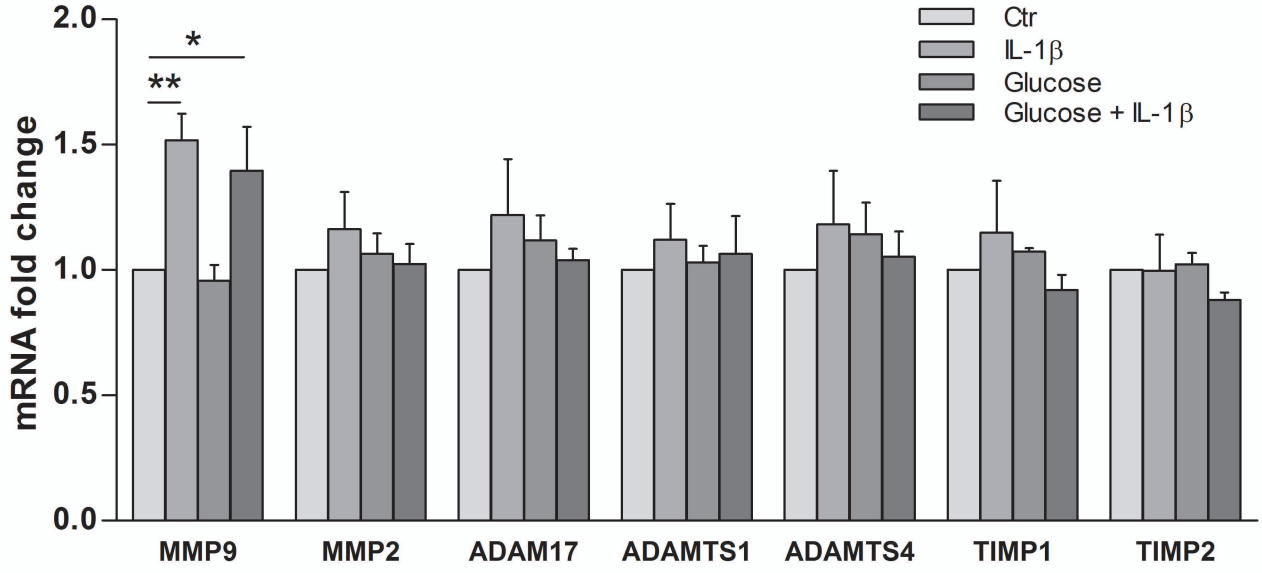

A.
Soluble ICAM-1
[sICAM-1] pg/ml related to AP Ctr
AP
BL
Ctr
IL-1β
Glucose
IL-1β + Glucose
C.
SDC expression
ΔCt
SDC1
SDC2
SDC3
SDC4
E.
Soluble syndecan-4
[syndecan-4] related to AP Ctr
B.
ICAM-1 expression
mRNA fold change
D.
SDC expression
Ctr
IL-1β
Glucose
F.
SDC4 expression

Figure 2

Figure 3


2.0
1.5
1.0
0.5
0.0
mRNA fold change
*
**
Ctr
IL-1β
Glucose
Glucose + IL-1β
MMP9
MMP2
ADAM17
ADAMTS1
ADAMTS4
TIMP1
TIMP2

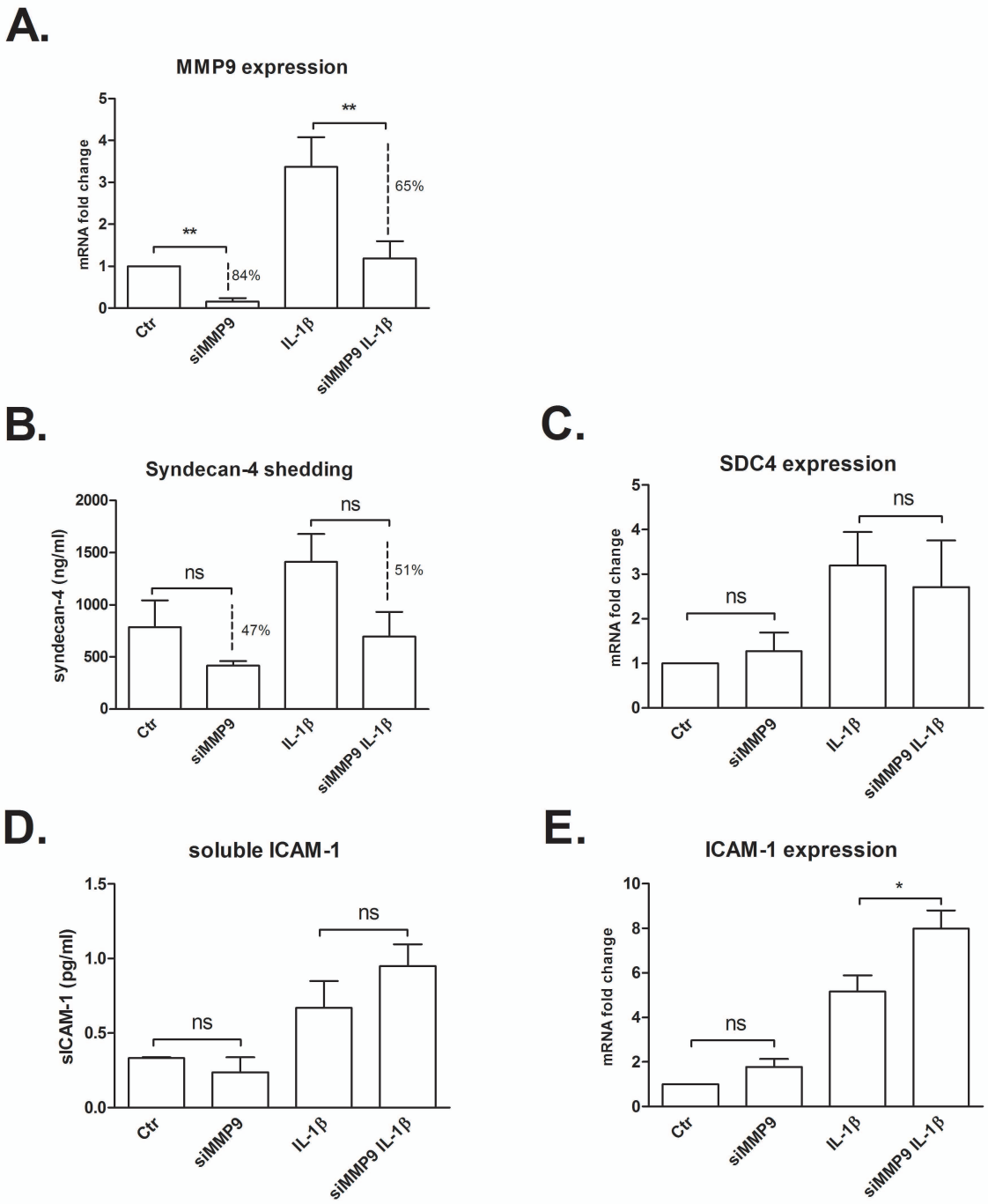
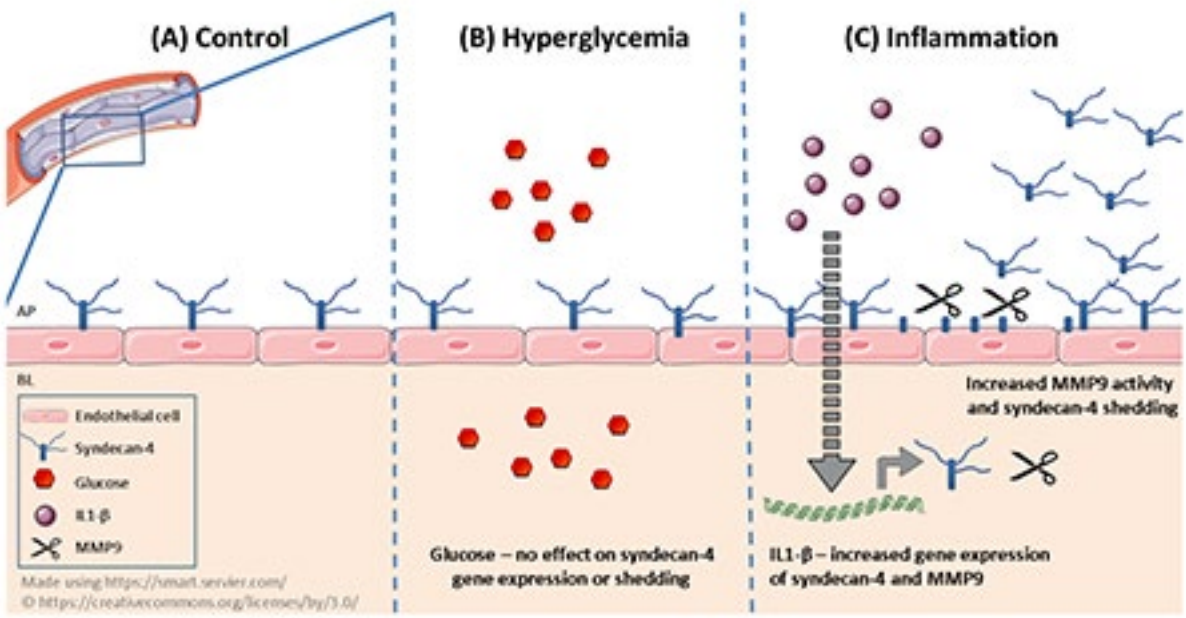

A.
MMP9 expression
mRNA fold change
5
4
3
2
1
0
**
**
84%
65%
Ctr
siMMP9
IL-1β
siMMP9 IL-1β
B.
Syndecan-4 shedding
syndecan-4 (ng/ml)
2000
1500
1000
500
0
ns
ns
47%
51%
Ctr
siMMP9
IL-1β
siMMP9 IL-1β
C.
SDC4 expression
mRNA fold change
5
4
3
2
1
0
ns
ns
Ctr
siMMP9
IL-1β
siMMP9 IL-1β
D.
soluble ICAM-1
sICAM-1 (pg/ml)
1.5
1.0
0.5
0.0
ns
ns
Ctr
siMMP9
IL-1β
siMMP9 IL-1β
E.
ICAM-1 expression
mRNA fold change
10
8
6
4
2
0
*
ns
Ctr
siMMP9
IL-1β
siMMP9 IL-1β

Figure 4

Figure 5


(A) Control
(B) Hyperglycemia
(C) Inflammation
AP
BL
Endothelial cell
Syndecan-4
Glucose
IL1-β
MMP9
Increased MMP9 activity and syndecan-4 shedding
Glucose – no effect on syndecan-4 gene expression or shedding
IL1-β – increased gene expression of syndecan-4 and MMP9