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Hyperglycemia-Induced Modulation of the Physiognomy and Angiogenic Potential of Fibroblasts Mediated by Matrix Metalloproteinase-2: Implications for Venous Stenosis Formation Associated with Hemodialysis Vascular Access in Diabetic Milieu

Rajiv Janardhanan[a], **Sreenivasulu Kilari**[b], **Edward B. Leof**[c], **Sanjay Misra**[b,c]

[a]Amity Institute of Public Health, Amity University Uttar Pradesh, Noida, Uttar Pradesh, India

[b]Vascular and Interventional Radiology Translational Laboratory, Department of Radiology, Mayo Clinic, Rochester, Minn., USA

[c]Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, Minn., USA

Abstract

Purpose: It is hypothesized that venous stenosis formation associated with hemodialysis vascular-access failure is caused by hypoxia-mediated fibroblast-to-myofibroblast differentiation accompanied by proliferation and migration, and that diabetic patients have worse clinical outcomes. The aim of this study was to determine the functional and gene expression outcomes of matrix metalloproteinase-2 (*Mmp-2*) silencing in fibroblasts cultured under hyperglycemia and euglycemia with hypoxic and normoxic stimuli.

Materials and Methods: AKR-2B fibroblasts were stably transduced using lentivirus-mediated shRNA-*Mmp-2* or scrambled controls and subjected to hypoxia or normoxia under hyperglycemic or euglycemic conditions for 24 and 72 h. Gene expression of vascular endothelial growth factor-A (*Vegf-A), Vegfr-1, Mmp-2, Mmp-9* and tissue inhibitors of matrix metalloproteinases (*Timps*) were determined by RT-PCR. Collagen I and IV secretion and cellular proliferation and migration were determined.

Results: Under hyperglycemic conditions, there is a significant reduction in the average gene expression of *Vegf-A* and *Mmp-9*, with an increase in *Timp-1* at 24 h of hypoxia ($p < 0.05$) in *Mmp-2*-silenced fibroblasts when compared to controls. In addition, there is a decrease in collagen I and IV secretion and cellular migration. The euglycemic cells were able to reverse these findings.

Conclusion: These findings demonstrate the rationale for using anti-*Mmp-2* therapy in dialysis patients with hemodialysis vascular access in helping to reduce stenosis formation.

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Disclosure Statement

The authors have no conflict of interest to disclose.

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Keywords

Fibroblasts; Glycemic variability; Diabetes; Vascular access; Extracellular matrix

Introduction

More than 645,697 patients in the USA require hemodialysis for the purification of their blood due to end-stage renal disease [1]. The arteriovenous fistula (AVF) has become the first-line therapy for patients requiring hemodialysis vascular access. AVFs have been used for more than 50 years and are prone to venous stenosis formation [2]. Although the exact mechanism for venous stenosis is not yet clear, several major factors have been hypothesized including hypoxia, shear stress, oxidative stress and inflammation [3, 4]. As a consequence, these factors lead to an accumulation of macrophages, fibroblasts and smooth-muscle cells in the vessel wall, that lead to stenosis formation [5]. Furthermore, hypoxia contributes to an increase in growth factors that induce fibroblast proliferation, migration and differentiation to myofibroblasts and result in fibrosis [6-8]. In addition to all of the above factors, hyperglycemia associated with diabetes exacerbates the formation of venous stenosis [9].

Our laboratory has used a hypoxic fibroblast cell culture system to understand the mechanisms responsible for venous stenosis formation. Previous results from our laboratory indicate that with a hypoxic stimulus, there is an increased expression of matrix metalloproteinase-2 (*Mmp-2*) accompanied by fibroblast-to-myofibroblast differentiation [10]. Moreover, inhibiting vascular endothelial growth factor-A (*Vegf-A*) gene expression in this model has been associated with a decrease in the differentiation of fibroblasts to myofibroblasts, which is accompanied by a reduction in the expression of *Mmp-2* gene and MMP-2 protein [3]. In line with these results, the implantation of autologous blood outgrowth endothelial cells to the adventitia of the arteriovenous grafts also decreases MMP-2 protein activity, accompanied by a decrease in venous stenosis formation [11]. However, under hypoxic stress, the effect of *Mmp-2* gene knockdown in hyperglycemic cells and in euglycemic cells previously cultured in hyperglycemic milieu is unknown. This holds implications for patients with diabetes.

Experimental studies have shown that under hypoxic stress, fibroblasts will secrete VEGF-A, which is necessary for the coordination of multiple cellular functions [12-14]. Fibroblasts have a synthetic function (to secrete collagens I and IV for tissue remodeling to occur) and contain elaborate cytokines [MMPs, VEGF-A, platelet-derived growth factor (PDGF) and others] responsible for cell proliferation and migration [15, 16].

We used an in vitro fibroblast cell culture model using both hyperglycemia and hypoxia to simulate diabetic injury in hemodialysis vascular access. The goal of the study was to evaluate the loss of *Mmp-2* function on fibroblasts acclimatized to hyperglycemia and then to euglycemia during hypoxia and normoxia. We investigated the gene expression of *Vegf-A, Vegfr-1, Mmp-9* and tissue inhibitors of matrix metalloproteinases (*Timps*). Functional analysis was performed to evaluate collagen I and IV synthesis and cellular migration and proliferation.

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Materials and Methods

Monoclonal antibodies against β-actin (clone AC-15) and α-smooth-muscle actin (α-SMA) were purchased from Sigma-Aldrich, St. Louis, Mo., USA. Collagen I and IV were purchased from Rockland Immunochemicals Inc., Limerick, Pa., USA. DMEM and all other chemicals for cell culture were obtained from Invitrogen Inc., Waltham, Mass., USA, unless otherwise specified.

Cell Culture and Treatments

The experimental design is depicted in figure 1. AKR-2B murine fibroblast cell line was obtained from Dr. Edward Leof, Mayo Clinic, Rochester, Minn., USA. Cells were cultured in DMEM supplemented with glucose to a final concentration of 30 mM, plus 10% fetal bovine serum (FBS), 1% penicillin and streptomycin. Cells were maintained at 37 ° C in a humidified CO_2 incubator with 5% CO_2 and 95% air [3].

Culture of AKR-2B Cells under Varying Glycemic Conditions

AKR-2B cells were preconditioned in hyperglycemic milieu (media containing 30 mM glucose). All experiments were performed with these preconditioned cells and considered as a hyperglycemic condition. A subculture of the hyperglycemia-preconditioned cells was grown in regular DMEM containing 5 mM glucose supplemented with 10% FBS, 1% penicillin and streptomycin, i.e. euglycemia-acclimatized cells.

Lentivirus-Mediated Mmp-2 Gene Silencing

To assess the efficacy of glycemic changes upon the invasiveness of fibroblasts and myofibroblasts, the expression of the constitutive matrix regulatory and signaling moiety of *Mmp-2* was silenced with lentivirus (LV)-mediated shRNA transduction [4, 17]. Briefly, AKR-2B cells were seeded into 10-cm dishes and grown to about 50–60% of confluency. One milliliter of LV solution (approx. 2×10^7 plaque-forming units/ml) carrying *Mmp-2* shRNA or control shRNA (Dharmacon, GE Healthcare, Lafayette, Colo., USA; accession Nos. NM_008610 and XM_006530751) and 5 ml of fresh medium were added to cells with 10 μg/ml polybrene and incubated for 16 h. The transduced cells were selected by subculturing in DMEM supplemented with 10% FBS and 1 μg/ml puromycin. All experiments were performed with the above puromycin-resistant cells.

Hypoxia Induces Physiognomic and Biochemical Changes

Lentiviral-transduced AKR-2B cells were maintained under both euglycemic and hyperglycemic conditions, seeded at a density of 1×10^5 cells/cm^2 and allowed to grow to 50–60% confluency. Cells (under both euglycemic and hyperglycemic conditions) were serum-starved overnight with DMEM, supplemented with 0.1% FBS and then exposed to normoxic (21% O_2, 5% CO_2 and a balance of nitrogen) or hypoxic conditions (3% O_2, 5% CO_2 and a balance of nitrogen) at 37 ° C.

AKR-2B cells grown under euglycemic or hyperglycemic conditions were subjected to normoxic or hypoxic conditions and evaluated for morphological and biochemical

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features of myofibroblast transdifferentiation, proliferation, migration and the modulation of extracellular matrix moieties and MMP activity assessed by zymography.

Actin Dynamics in Fibroblasts

AKR-2B cells maintained under euglycemic or hyperglycemic conditions were subjected to hypoxic stress for 24 or 72 h, as were the respective normoxic controls. After the incubation period, cells were washed 3 times with prewarmed (37 ° C) PBS and fixed in paraformaldehyde (4% PFA) in PBS for 30 min. After fixation, cells were washed twice in PBS and permeabilized with 0.1% Triton X-100 in PBS for 1 min. After washing 3 times with PBS, the cells were incubated in rhodamine-phalloidin (1:1,000 dilution, Sigma-Aldrich) in PBS for 30 min at room temperature. The cells were washed 5 times in PBS and mounted with mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI; Vector Labs, Burlingame, Calif., USA). Images were acquired using a confocal microscope (excitation: 550 nm and emission: 580 nm) with –40 and ×63 objectives as described previously [3, 4].

Cell Proliferation Assay

LV-transduced AKR-2B cells acclimatized to both euglycemic or hyperglycemic conditions were seeded in 24-well plates at a density of 5×10^4 cells/ml and cultured for 48 h in their respective glycemic conditions. Cells were serum-starved (0.1% serum) overnight and subjected to hypoxic or normoxic conditions for 20 h and then 1 μCi of ^{3}H-thymidine was added to the media and incubated for another 4 h under their respective incubating conditions. After 4 h, cells were washed with ice-cold PBS, fixed with 100% cold methanol and collected for the measurement of trichloroacetic acid-precipitated radioactivity [18]. Experiments were repeated at least 3 times.

Cell Migration Assay

Chemotaxis was measured using a modified Boyden chamber migration assays (BD) with the use of 8-μm pore-size, polycarbonated transwell inserts coated with collagen as described previously [3, 4]. Briefly, *Mmp-2*-silenced AKR-2B cells were acclimatized to euglycemic or hyperglycemic conditions and suspended in serum-free medium and then seeded in the upper well (20,000 cells/well). The cells were then incubated for 4 h at 37 ° C in a CO_2 incubator or hypoxia chamber, before being fixed with 4% PFA and stained with 0.2% crystal violet dissolved in 2% ethanol. Migration was quantified by counting the number of cells on the filter using bright-field optics with a Nikon Diaphot microscope (Nikon Instruments Inc., Melville, Ky., USA) equipped with a 16-square reticule (1 mm^2). Cells that passed through the filter were quantified from random microscopic fields. Each assay was carried out in triplicate, with 6 replicates of each group per assay.

Assessing Collagen Activity from Conditioned Media

One of the characteristic features of the proliferating and invasive cells is having the ability to significantly influence the turnover rates of the surrounding extracellular matrix rich in collagen polymers through the activation of matrix-regulating signaling pathways. In order to ascertain the effect of glycemic changes as well as the silencing of *Mmp-2* on the invasive

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nature of the murine fibroblast cells, the activity of collagen type I and IV were assessed from the conditioned media collected at the end of the incubation periods. The conditioned media were then concentrated using Amicon Ultra (EMD Millipore, Billerica, Mass., USA) centrifugal filter units. Western blot for collagen type I and IV with zymography for *Mmp-2* and *Mmp-9* activity were performed on the conditioned media [19].

Gelatin Zymography

Zymography was performed on the conditioned media from cultured cells at completion of their respective treatments according to the protocol published by our group [10]. Protein (100 μg) was electrophoresed using 10% polyacrylamide gels containing 0.1% gelatin (Bio-Rad, Hercules, Calif., USA). After electrophoresis, the gels were incubated twice for 30 min in 10 volumes of 2.7% Triton X-100 renaturing buffer and once for 30 min in 10 volumes of development buffer [200 mM NaCl, 50 mM HEPES (pH 7.5), 5 mM $CaCl_2$ and 20 μM $ZnCl_2$]. The gels were then incubated at 37 ° C in fresh development buffer overnight and subsequently stained in 40% methanol, 10% acetic acid and 0.5% Coomassie blue R-250 for 30 min, followed by destaining in the same solution without the dye. Bands were semiquantitated by reverse-image scanning densitometry (NIH ImageJ Software, NIH, Bethesda, Md., USA). An area of the gel image that was devoid of signal was assigned to be the background value. Each band was then analyzed for the density above the background. The results were calculated and expressed as relative densitometric units/unit area. Relative active protein was calculated by dividing the densitometric value in the active band by the total densitometric value of the active and proactive bands.

Western Blotting

Protein samples obtained either from AKR-2B cell lysates (30 μg) or concentrated conditioned media (100 μg) were resolved on the SDS-PAGE followed by Western blotting as described previously [20]. The blots were probed with respective primary IgG antibodies and then horseradish peroxidase-conjugated goat anti-species secondary IgG antibodies. The immunoreactive protein bands were visualized with SuperSignal West Pico chemiluminescence (ECL) detection reagents (Rockford, Ill., USA) and exposed to X-OMAT AR films (Eastman Kodak, Rochester, N.Y., USA). The films were scanned on an EPSON scanner and the optical density (OD) of each band was determined using ImageJ software. The OD of bands in the control treatment was designated as 100 after being normalized to the respective loading controls (β-actin for α-SMA and Ponceau-S staining for collagen). All experiments were performed in triplicate.

Reverse Transcriptase Polymerase Chain Reaction

RNA from the cells was isolated as per manufacturer guidelines (Qiagen, Gaithersburg, Md., USA). First-strand complementary DNA (cDNA) was synthesized using the Superscript III First-Strand system (Invitrogen, Carlsbad, Calif., USA) according to the manufacturer's guidelines. cDNAs specific for the analyzed genes, *Vegf-R1, Vegf-A, Mmp-2, Mmp-9, Timp-1 and Timp-2*, were amplified using the primers obtained from SA Biosciences (Valencia, Calif., USA). PCR products were analyzed on 1.5% (w/v) agarose gels containing 0.5 μg/ml ethidium bromide. Bands were semiquantitated by scanning densitometry (ImageJ). An area of the gel image that was devoid of signal was assigned to be the

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background value. Each band representing the gene of interest was then analyzed for the density above the background, and then normalized to ensure that there were no differences in the amount of loading of mRNA to the 18S gene.

Statistical Analysis

Data are expressed as mean ± SEM. Two-way ANOVA followed by Student's t test with post hoc Bonferroni's correction was performed. Significant differences between groups were indicated by $p < 0.05$, $p < 0.01$, $p < 0.001$ or $p < 0.0001$.

Results

Mmp-2 shRNA Suppresses the Expression of the Mmp-2 Gene and MMP-2 Protein in AKR-2B Cells

We determined Mmp-2 shRNA efficacy in fibroblasts by measuring the *Mmp-2* gene and MMP-2 protein expression under experimental conditions. This included hyperglycemia and those cells acclimatized to euglycemia at 24 and 72 h of hypoxia and normoxia. Results from RT-PCR analysis revealed that *Mmp-2* gene expression was significantly decreased in *Mmp-2*-silenced fibroblasts compared to scrambled controls under hyperglycemic conditions at 24 ($p < 0.05$) and 72 h ($p < 0.01$) of hypoxia and normoxia (fig. 2a). Similar results were observed in cells acclimatized to euglycemic conditions at 24 h of hypoxia ($p < 0.05$) and normoxia ($p < 0.001$) and at 72 h ($p < 0.01$) in cells silenced for *Mmp-2* compared to controls (fig. 2a).

Next, we performed zymography to assess the effect of *Mmp-2* gene knockdown on pro-MMP-2 activities in fibroblasts. Pro-MMP-2 activity was significantly decreased in hyperglycemic cells at 24 ($p < 0.05$) and 72 h ($p < 0.05$) of normoxia and hypoxia in *Mmp-2*-silenced fibroblasts compared to controls (fig. 2b). Under euglycemic conditions, *Mmp-2*-silenced fibroblasts had significantly decreased pro-MMP-2 activity, when cultured for 24 h under hypoxia and normoxia ($p < 0.05$) when compared to respective controls. However, a reduction in pro-MMP-2 activity was observed only at 72 h of hypoxia ($p < 0.01$) in MMP-2 knockdown fibroblasts acclimatized to euglycemic conditions compared to their respective controls (fig. 2B).

We next assessed active MMP-2 activity under similar conditions. *Mmp-2*- silenced fibroblasts had significantly reduced active MMP-2 activity in hyperglycemic cells at 24 ($p < 0.05$) and 72 h ($p < 0.01$) of normoxia and hypoxia compared to respective controls (fig. 2b). Similar results were observed in cells acclimatized to euglycemic conditions at 24 h of normoxia ($p < 0.05$) and hypoxia ($p < 0.01$) as well as at 72 h of normoxia ($p < 0.05$) in *Mmp-2*-silenced fibroblasts compared to respective controls (fig. 2b).

Loss of Mmp-2 Reverses the Hyperglycemia-Induced Vegf-A and Vegfr-1 Gene Regulation

We have previously shown that *Vegf-A*-silenced fibroblasts under hypoxic conditions have reduced MMP-2 activity compared to normoxic fibroblasts. *Vegf-A* and its cognate receptor *Vegfr-1* are downstream intermediates of the hypoxic transcriptome and so *Vegf-A* and *Vegfr-1* gene expression was assessed by RT-PCR. Our results demonstrate that under

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hyperglycemic conditions, there was a significant reduction in *Vegf-A* gene expression at 24 h of hypoxia ($p < 0.05$) in *Mmp-2*-silenced fibroblasts when compared to controls (fig. 3a). However, under euglycemic conditions, *Vegf-A* expression was significantly increased at 72 h of hypoxia ($p < 0.05$) in *Mmp-2*-silenced fibroblasts when compared to respective controls (fig. 3a).

Under both hyperglycemic and euglycemic conditions, *Mmp-2*-silenced fibroblasts had significantly decreased *Vegfr-1* gene expression after being cultured for 24 h of normoxia ($p < 0.05$) compared to the controls (fig. 3b). These results conclusively demonstrate that the *Mmp-2*-silenced fibroblasts had a profound impact on the expression of *Vegf-A* and its cognate receptor *Vegfr-1*, irrespective of glycemic variance.

Mmp-2 Modulates the Expression of Genes Associated with Matrix Turnover under Varying Glycemic Conditions with Hypoxia and Normoxia

Mmp-2-silenced fibroblasts exposed to hyperglycemic conditions had a significant decrease in the average expression of the *Mmp-9* gene at 24 h of normoxia ($p < 0.05$) and 72 h of hypoxia ($p < 0.05$) compared to respective controls (fig. 4a). However, under euglycemic conditions, the reduction in *Mmp-9* gene expression was observed only at 24 h of normoxia ($p < 0.05$) in *Mmp-2*-silenced fibroblasts compared to respective controls.

Next, we determined the gene expression pattern of *Timp-1*, an inhibitor of *Mmp-9*. Under hyperglycemic conditions, *Mmp-2*-silenced fibroblasts had significantly reduced Timp-*1* gene expression when cultured for 24 h ($p < 0.05$) of normoxia, but this increased significantly ($p < 0.05$) when the cells were exposed to hypoxia for 24 h compared to respective controls (fig. 4b). Under euglycemic conditions, *Mmp-2*-silenced fibroblasts had significantly increased *Timp-1* expression at 24 ($p < 0.01$) and 72 h ($p < 0.05$) of hypoxia only compared to their respective controls.

Finally, we determined the pattern of gene expression of *Timp-2*, an inhibitor of *Mmp-2*. Under hyperglycemic conditions, *Mmp-2*-silenced fibroblasts significantly reduced the expression of the *Timp-2* gene ($p < 0.01$) when exposed to 24 h of normoxia compared to the respective controls (fig. 4c).

Loss of Mmp-2 Reduces Hypoxia with Hyperglycemia-Mediated Fibroblast-to-Myofibroblast Differentiation

We have previously shown that *Vegf-A*-silenced fibroblasts have a significantly reduced α-SMA expression under hypoxic versus normoxic conditions [4]. We determined α-SMA expression by Western blot analysis in fibroblasts silenced for *Mmp-2* and exposed to hyperglycemic and euglycemic conditions for 24 and 72 h with hypoxia or normoxia. Our results demonstrate that under hyperglycemic or euglycemic conditions, *Mmp-2*-silenced fibroblasts had significantly reduced α-SMA protein when exposed to 24 h of hypoxia or normoxia compared to the respective controls (fig. 5a).

We next determined the amount of actin stress fibers in *Mmp-2*-silenced fibroblasts cultured under hyperglycemic and euglycemic conditions and exposed to varying oxygen levels. Under hyperglycemic conditions, *Mmp-2*-silenced fibroblasts had significantly reduced actin

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stress fiber formation at 24 h of normoxia (fig. 5b; $p < 0.01$) and hypoxia ($p < 0.0001$) and at 72 h (of both normoxia and hypoxia; $p < 0.0001$) when compared to controls. Under euglycemic conditions, there was a significant reduction in the average percentage change in actin stress fiber formation at 24 ($p < 0.001$) and 72 h of hypoxia only ($p < 0.0001$) compared to the controls.

Loss of Mmp-2 Alters Collagen Secretion

Collagen I and IV are substrates for MMP-2. We hypothesized that there would be a reduction in their activity in *Mmp-2*-silenced fibroblasts. We have demonstrated that *Vegf-A* shRNA transduced vessels have decreased expression of the *Mmp-2* gene and MMP-2 protein expression, accompanied by a reduction in collagen I and IV staining as determined by Picrosirius red [19]. We determined collagen I and IV expression by performing Western blot analysis on the conditioned media. We observed that under hyperglycemic conditions, *Mmp-2*-silenced fibroblasts when compared to controls have a significant reduction in the average collagen I expression at 24 h of normoxia (fig. 6a; $p < 0.05$) and hypoxia ($p < 0.01$) as well as at 72 h of hypoxia only ($p < 0.01$). A reduction in collagen I expression was observed at 24 h of normoxia only ($p < 0.05$) under euglycemic conditions.

Next, we quantified the collagen IV expression. Results demonstrate that *Mmp-2*-silenced fibroblasts under hyperglycemic conditions have a significant reduction in the average percentage change in collagen IV expression at 24 (fig. 6b; $p < 0.01$) and 72 h ($p < 0.01$) of hypoxia when compared to controls. Under euglycemic conditions, we observed a significant reduction in percentage change in collagen IV expression at 24 h ($p < 0.01$) of normoxia and hypoxia compared to controls.

Loss of Mmp-2 Decreases Cell Migration under Hyperglycemia

Decreasing *Mmp-2* expression can reduce the migratory potential of cells. We determined the migration of such cells using an in vitro invasion assay as assessed by the Boyden's chamber. Our experiments demonstrated that in *Mmp-2*-silenced fibroblasts under hyperglycemic conditions had a significant reduction in the migratory potential (fig. 7a; $p < 0.001$) when exposed to hypoxia and normoxia compared to controls. Under euglycemic conditions, no significant difference was observed between the controls and cells silenced for *Mmp-2*.

Loss of Mmp-2 Alters Cell Proliferation with Varying Concentrations of Oxygen and Glucose

Proliferation was determined using a thymidine incorporation assay. Our results demonstrated that *Mmp-2*-silenced fibroblasts conditioned to hyperglycemic milieu under hypoxic conditions (fig. 7b; $p < 0.001$) or to euglycemic milieu under both hypoxic ($p < 0.001$) and normoxic stress ($p < 0.0001$) underwent a significant increase in the percentage change in the proliferative potential of cells.

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Discussion

Hemodialysis vascular access failure caused by venous stenosis formation is a significant clinical problem for patients with end-stage renal disease and it is associated with worse clinical outcomes in those patients with diabetes [9]. A majority of patients on hemodialysis often have an underlying history of diabetes [9], and a recent study demonstrated that fistula patency was associated with optimal glycemic control [21]. Developing novel therapies to aid in reducing venous stenosis formation is important for improving patient outcomes.

MMP-2, a constitutive matrix metalloproteinase plays a key role in the invasive potential of cells including fibroblasts in vascular stenosis [4, 22]. Pharmacological inhibition of MMPs has been used as a therapeutic strategy for cancer; however, a major problem is the selectivity issues associated with these inhibitors [23]. Furthermore, an antisense approach has been used to decipher the role of MMP-2 in cancer progression [22]. To the best of our knowledge, the role of MMP-2 in vascular stenosis has not been elucidated in diabetic milieu. This prompted us to investigate the simultaneous role of hyperglycemia and hypoxia on the physiognomy of the adventitial /medial fibroblasts. Previous studies from our laboratory suggest that *Mmp-2* plays major role in hypoxia-induced fibroblast-to-myofibroblast differentiation [6]. In this study, we used an in vitro fibroblast cell culture model that employed both hyperglycemia and hypoxia to partly simulate epithelial-to-mesenchymal differentiation, focusing on fibroblast function and biology.

The turnover of the extracellular matrix facilitates the migration of tumor cells causing tumor metastasis [24] and of adventitial fibroblasts to the lumen of blood vessels, thereby significantly contributing to the onset of vascular stenosis [4]. Copious amounts of collagen I and IV were produced by the fibroblasts acclimatized to euglycemia compared to hyperglycemic fibroblasts in this study. In line with these results, glucose-induced MMP-2 and MMP-9 activity with a decrease in collagen synthesis by fibroblasts and endothelial cells has been described [25-27]. Surprisingly, the *Mmp-2* gene did not have a statutory effect either under normoxic or hypoxic conditions, especially at the 72-hour time point. In line with these results, the loss of *Mmp-2* decreased fibroblast migration under hyperglycemic conditions, while it had no effect on the migratory behavior of fibroblasts acclimatized to the euglycemic environment. Another possibility is that *Mmp-9* and *Mmp-2* activity, which was unaltered, might have increased the hypoxic stress-induced migratory behavior of those fibroblasts acclimatized to the euglycemic environment. A similar trend was observed with other physiologically relevant end points such as actin stress fibers, α-SMA, *Mmp-9* and *Timp-1*. Together, these results indicate that fibroblasts that have been silenced for *Mmp-2* gene expression and acclimatized to euglycemia from hyperglycemic conditions behave differently under hypoxic stress when compared to hyperglycemic cells.

Although hypoxia induces *Vegf-A* expression [28], this effect was observed only in *Mmp-2*-silenced cells acclimatized to euglycemic conditions when subjected to hypoxia for 72 h. In line with these results, high glucose disrupts the binding of HIF-1α to p300, a cofactor for HIF-1α via glycosylation leading to impaired HIF-1α-mediated transactivation of genes including *Vegf-A* [29]. Another possibility is that hyperglycemia can impair the hypoxia-dependent HIF-1α stability of proteasomal degradation [30-33]. It is possible that a similar

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interaction might have occurred, not only in the hyperglycemic cells, but also in the cells acclimatized to the euglycemic conditions.

Taken collectively, our studies indicate that glycemia plays an important role in modulating the behavior of fibroblasts. *Mmp-2* gene silencing affected the physiognomy and behavior of the fibroblasts under hyperglycemic conditions. However, acclimatization to euglycemic conditions demonstrated results contrary to our expectations. These results indicate the statutory effect of the *Mmp-2* silencing did not impact the physiognomy or the physiology of cells acclimatization to euglycemic conditions, particularly at the 72-hour time point. Moreover, the cellular response, such as hypoxia-mediated *Vegf-A* expression and the robust reappearance of the cortical stress fibers in fibroblasts acclimatized to euglycemia, is an indication of the ‘metabolic memory’ of these cells. Such results are indicative of the fact that tight management of the glycemic indices alone will not significantly alter or delay venous stenosis formation associated with hemodialysis vascular access failure. Therefore, these findings have implications for diabetic patients with hemodialysis vascular access.

We investigated fibroblast response after the transition from hyperglycemia to a euglycemic environment. One limitation of our study is the lack of negative control cells that were not previously cultured in a hyperglycemic environment. In addition, advanced glycation end product (AGE) levels in fibroblasts cultured under varying glycemic conditions might strengthen the concept of metabolic memory.

In summary, we conclude that *Mmp-2*-silenced fibroblasts under hyperglycemic conditions during hypoxic stimulus have an impaired response to *Vegf-A/Mmp-9* expression. This results in a decrease in collagen I and IV secretion with a decrease in cell migration. These findings demonstrate a rationale for novel site-specific anti-MMP therapies aimed at maintaining the AVF patency. Moreover, maintenance of glycemic indices could help in significantly improving the performance of the AVF in diabetic patients. Further studies are required to validate our in vitro observations.

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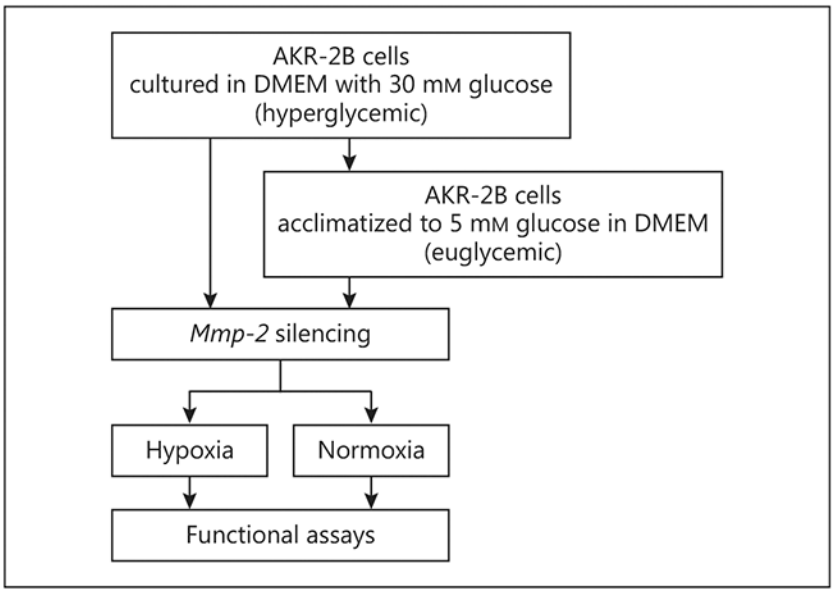

Fig. 1.

Schematic representation of the experimental approach. Murine fibroblast (AKR-2B) cells were initially preconditioned to hyperglycemia (maintained in DMEM with 30 mM glucose). These cells were then acclimatized to euglycemia (subcultured in DMEM with 5 mM glucose). Both hyperglycemic and euglycemic cells were then silenced for the *Mmp-2* gene using shRNA. The cells transduced with LV either silenced for *Mmp-2* (LV) or scrambled shRNA (controls) were further subjected to functional experiments with varying indices of oxygen (normoxia and hypoxia).

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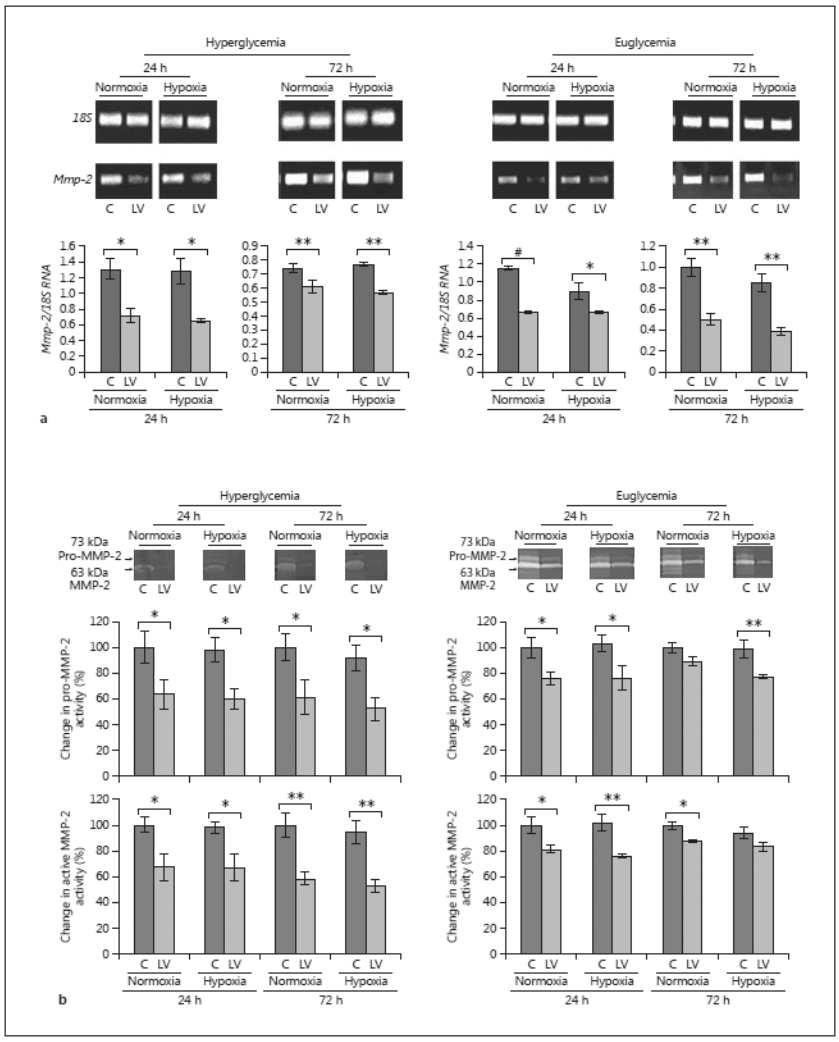

Fig. 2.

Mmp-2 gene and MMP-2 protein expression in cells silenced for *Mmp-2*. AKR-2B cells acclimatized to hyperglycemic and euglycemic conditions were silenced for *Mmp-2* (LV) or scrambled shRNA (controls, C). *Mmp-2* gene expression was assessed by RT-PCR (**a**) and MMP-2 protein and enzyme activity by zymography (**b**) at 24 and 72 h of hypoxia and normoxia. Top: representative gel images. Bottom: densitometric quantification for respective treatment conditions shown in the upper panels. Each bar represents the mean + SEM of 4–6 experiments per group. Two-way ANOVA followed by Student's t test with post hoc Bonferroni's correction was performed. * $p < 0.05$, ** $p < 0.01$ and $^{\#}$ $p < 0.001$, significant difference from control value.

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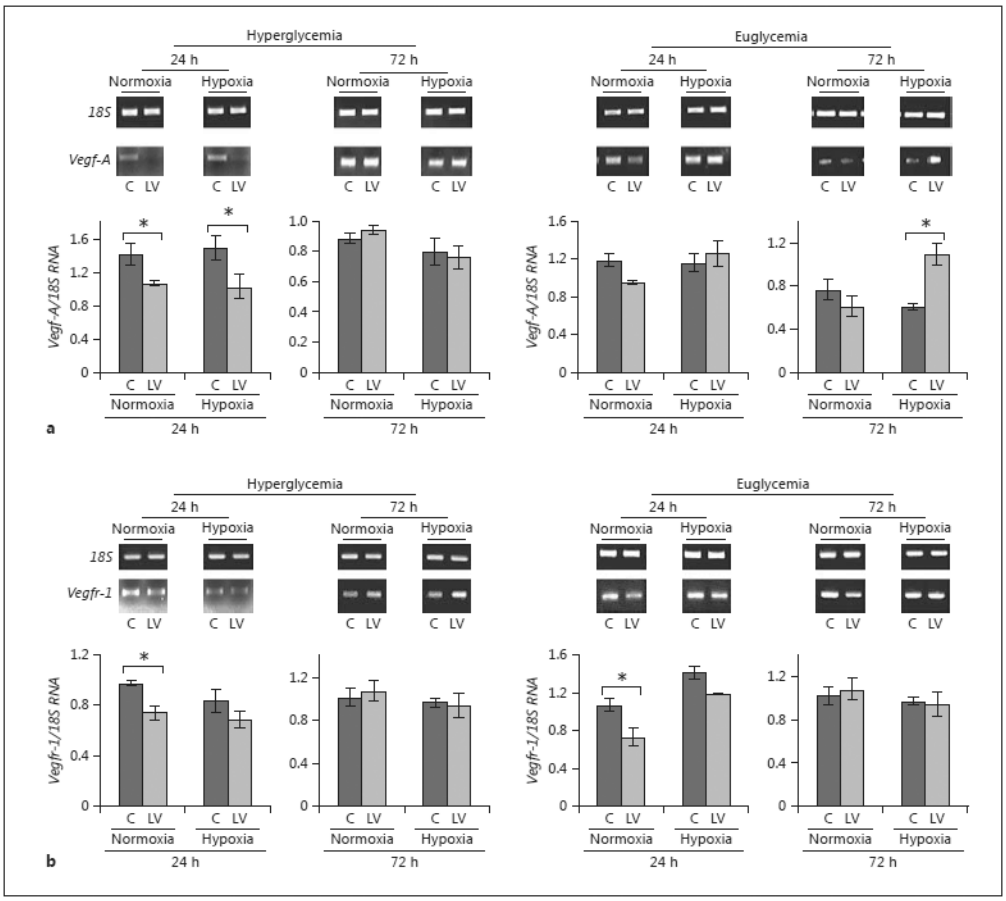

Fig. 3.

Gene expression of *Vegf-A* and *Vegfr-1* in hyperglycemic and euglycemic cells during hypoxia and normoxia. Expression of the *Vegf-A* (**a**) and *Vegfr-1* (**b**) genes was assessed by RT-PCR in AKR-2B cells cultured in hyperglycemic and euglycemic media silenced for *Mmp-2* (LV) or scrambled shRNA (controls, C) subjected to hypoxia or normoxia for 24 and 72 h. Top: representative agarose gel images of gene expression. Bottom: densitometric analysis of gene expression under the respective glycemic conditions. Each bar represents the mean + SEM of 4–6 experiments per group. Two-way ANOVA followed by Student's t test with post hoc Bonferroni's correction was performed. * $p < 0.05$, significant difference from control value.

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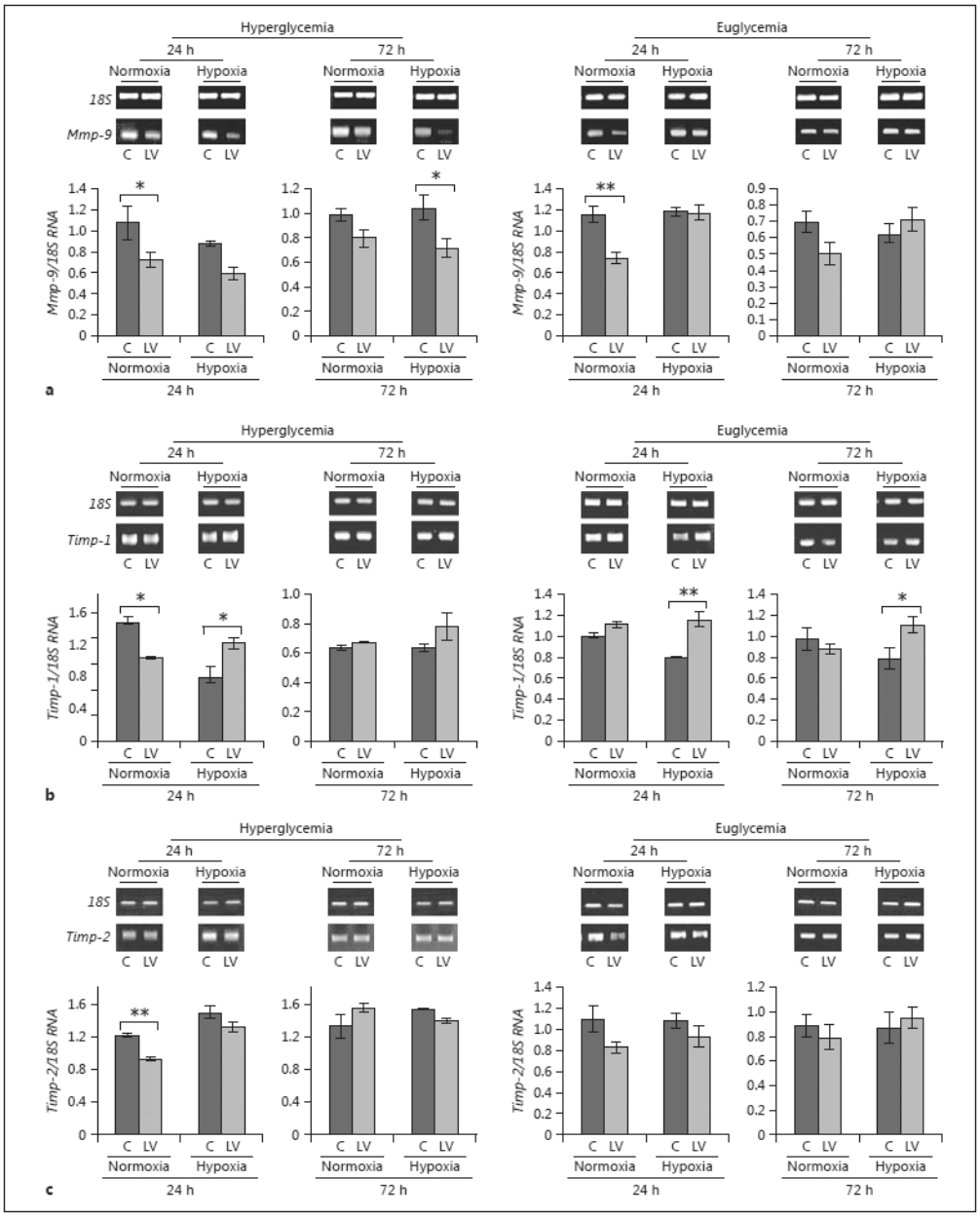

Fig. 4.

Expression of the *Mmp-9* (**a**), *Timp-1* (**b**) and *Timp-2* (**c**) genes was assessed by RT-PCR in AKR-2B cells cultured in hyperglycemic and euglycemic media silenced for *Mmp-2* (LV) or scrambled shRNA (controls, C) subjected to hypoxia or normoxia for 24 and 72 h. Top: representative agarose gel images of gene expression. Bottom: representative densitometric analysis of gene expression under the respective glycemic conditions. Each bar represents the mean + SEM of 4–6 experiments per group. Two-way ANOVA followed by Student's t test with post hoc Bonferroni's correction was performed. * $p < 0.05$ and ** $p < 0.01$, significant difference from control value.

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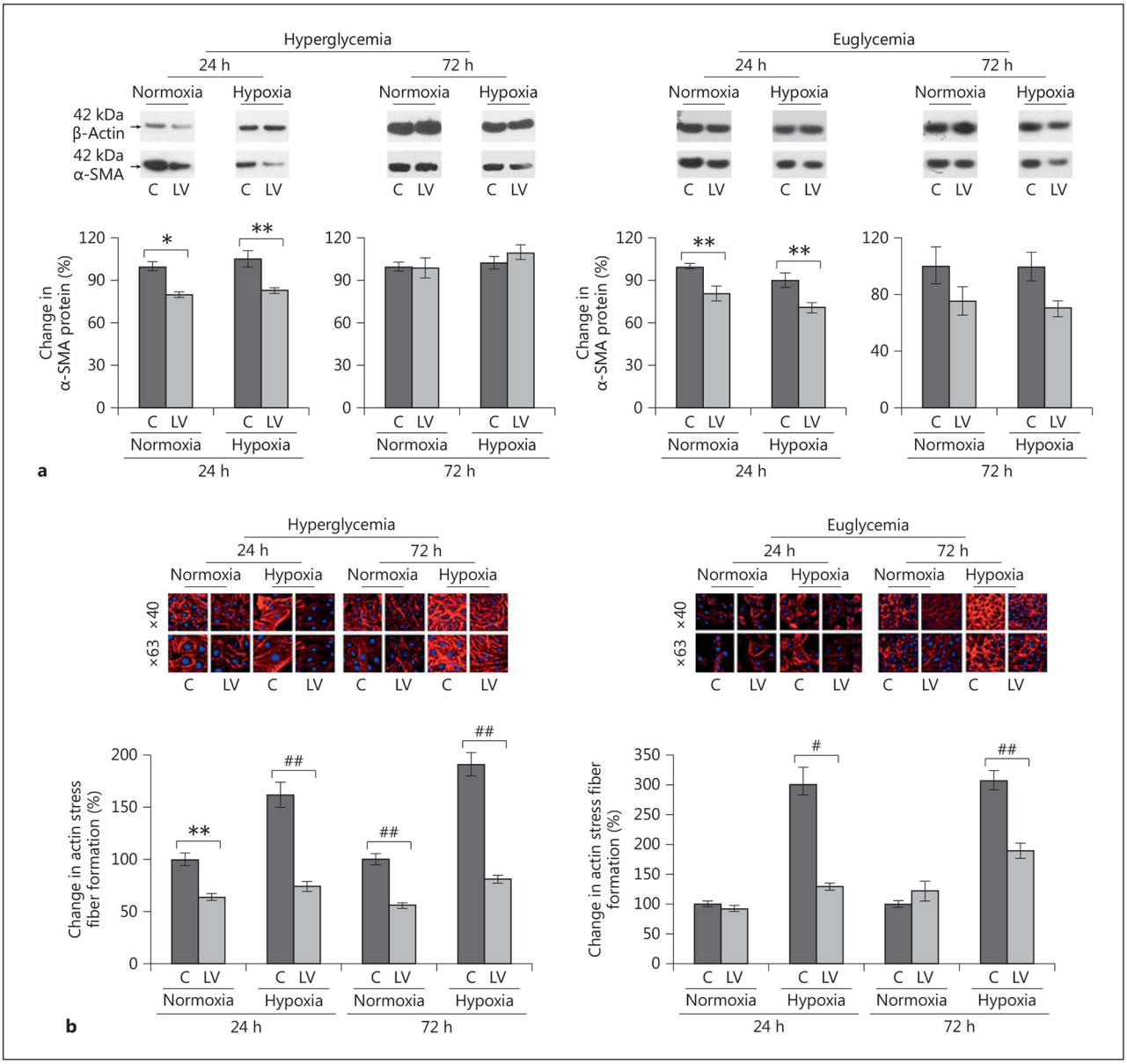

Fig. 5.

Western blots of α-SMA and phalloidin staining to assess stress fiber formation. Conversion of fibroblasts to myofibroblasts was assessed by measuring α-SMA protein using Western blot (**a**) and immunofluorescence staining for phalloidin (**b**) in cells silenced for *Mmp-2* (LV) or scrambled shRNA (controls, C) subjected to hyperglycemic or euglycemic conditions under hypoxia or normoxia for 24 and 72 h. **a** Representative images of α-SMA (top) and densitometric quantification (bottom). **b** Representative images of phalloidin staining (top) with image quantification (bottom) of the respective glycemic conditions. Each bar represents mean + SEM of 4–6 experiments per group. Two-way ANOVA followed by Student's t test with post hoc Bonferroni's correction was performed. * $p < 0.05$, ** $p < 0.01$, $^{\#}$ $p < 0.001$ and $^{\#\#}$ $p < 0.0001$, significant difference from control value.

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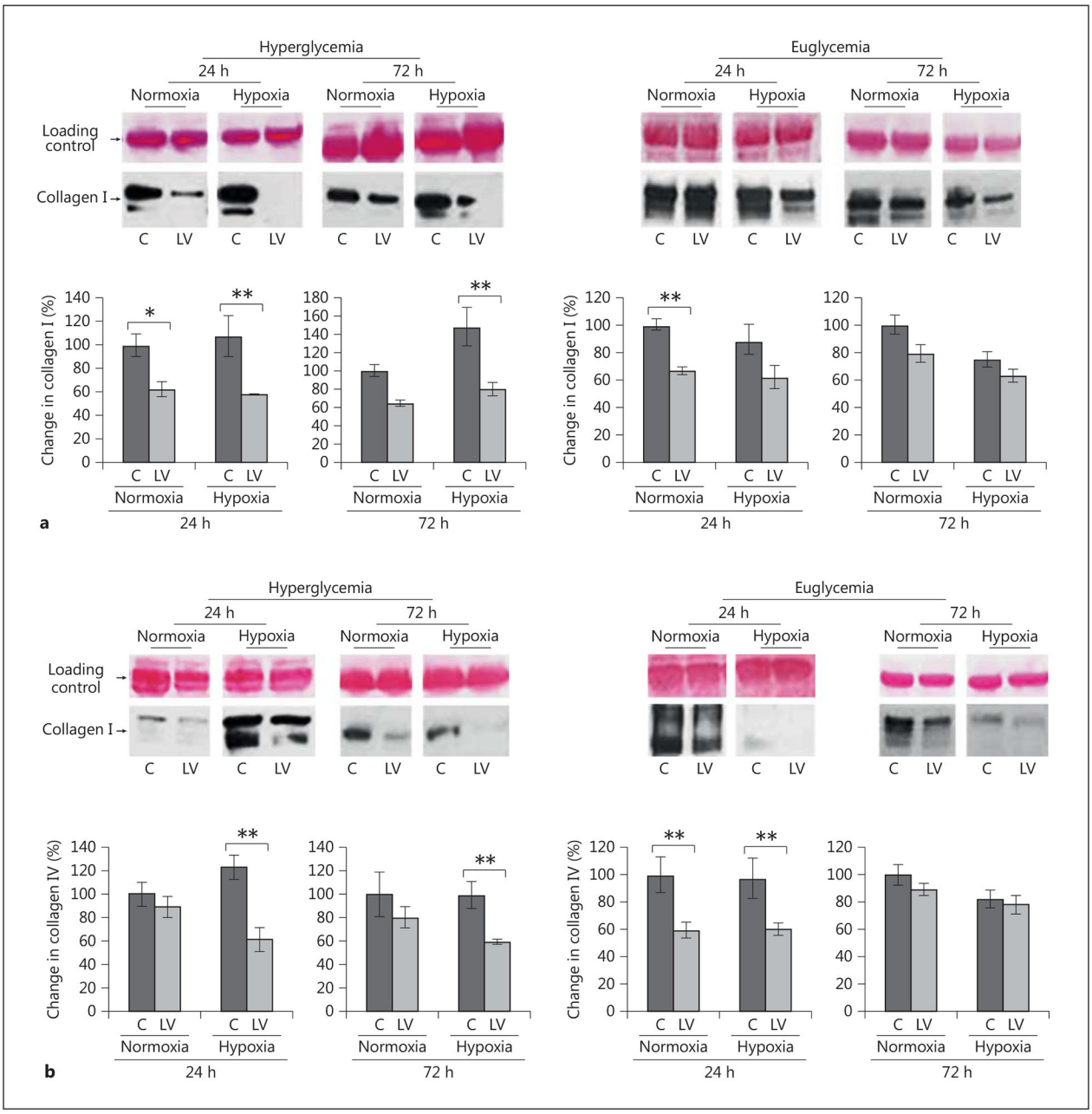

Fig. 6.

Collagen I and IV expression in hyperglycemic and euglycemic cells during hypoxia and normoxia. Expression of collagen I (**a**) and IV (**b**) was assessed using Western blot performed on the conditioned media from cells silenced for *Mmp-2* (LV) or scrambled shRNA (controls, C) and subjected to hyperglycemic or euglycemic conditions during hypoxia or normoxia for 24 and 72 h. Top: representative images of Western blots. Bottom: densitometric analysis. Each bar represents mean + SEM of 4–6 experiments per group. Two-way ANOVA followed by Student's t test with post hoc Bonferroni's correction was performed. * $p < 0.05$ and ** $p < 0.01$, significant difference from control value.

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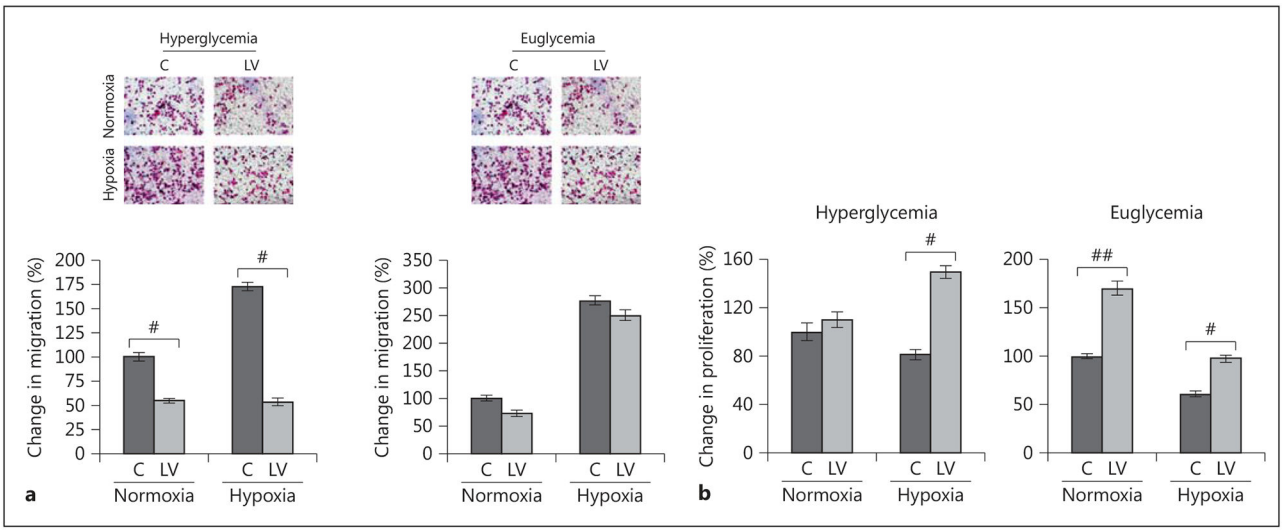

Fig. 7.

Cell migration and proliferation. Boyden chamber cell migration assay (**a**) and thymidine incorporation assay for proliferation (**b**) were assessed on fibroblast cells silenced for *Mmp-2* (LV) or scrambled shRNA (controls, C) subjected to hyperglycemic or euglycemic conditions during hypoxia or normoxia for 24 and 72 h. **a** Representative images for cell migration (upper panel) and the average percentage change in cell migration (lower panel) under varying glycemic conditions. Each bar represents mean percentage change of cell proliferation + SEM of 4–6 experiments Two-way ANOVA followed by Student's t test with post hoc Bonferroni's correction was performed. $^{\#}$ $p < 0.001$ and $^{\#\#}$ $p < 0.001$, significant difference from control value.