Diabetes Causes Bone Marrow Endothelial Barrier Dysfunction by Activation of the RhoA–Rho-Associated Kinase Signaling Pathway

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Objective—Diabetes mellitus causes bone marrow (BM) microangiopathy. This study aimed to investigate the mechanisms responsible for BM endothelial dysfunction in diabetes mellitus.

Methods and Results—The analysis of differentially expressed transcripts in BM endothelial cells (BMECs) from type-1 diabetic and nondiabetic mice showed an effect of diabetes mellitus on signaling pathways controlling cell death, migration, and cytoskeletal rearrangement. Type-1 diabetic-BMECs displayed high reactive oxygen species levels, increased expression and activity of RhoA and its associated protein kinases Rho-associated kinase 1/Rho-associated kinase 2, and reduced Akt phosphorylation/activity. Likewise, diabetes mellitus impaired Akt-related BMEC functions, such as migration, network formation, and angiocrine factor-releasing activity, and increased vascular permeability. Moreover, high glucose disrupted BMEC contacts through Src tyrosine kinase phosphorylation of vascular endothelial cadherin. These alterations were prevented by constitutively active Akt (myristoylated Akt), Rho-associated kinase inhibitor Y-27632, and Src inhibitors. Insulin replacement restored BMEC abundance, as assessed by flow cytometry analysis of the endothelial marker MECA32, and endothelial barrier function in BM of type-1 diabetic mice.

Conclusion—Redox-dependent activation of RhoA/Rho-associated kinase and Src/vascular endothelial cadherin signaling pathways, together with Akt inactivation, contribute to endothelial dysfunction in diabetic BM. Metabolic control is crucial for maintenance of endothelial cell homeostasis and endothelial barrier function in BM of diabetic mice. (Arterioscler Thromb Vasc Biol. 2013;33:555-564.)

Key Words: bone marrow ■ diabetic microangiopathy ■ endothelial dysfunction ■ oxidative stress ■ RhoA
Materials and Methods

Animal Procedures
Experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals and with approval of the British Home Office. Type 1 diabetes mellitus (T1D) was induced in male CD1 mice (Charles River, Margate, Kent, UK) by streptozotocin. Age-matched male CD1 mice injected with the streptozotocin vehicle served as controls. Diabetes mellitus was monitored by measurements of glycaemia at fast and glycosuria.

Insulin Implants
Four weeks after induction of diabetes mellitus, mice were randomized to receive continuous insulin supplementation, through subcutaneous implants (LinBit, LinShin, Toronto, Canada), at the rate of 0.1 unit/implant per day or vehicle. The number of insulin implants was titrated according to the mouse body weight, according to manufacturer’s instructions. Glycaemia was monitored every 4 weeks, whereas glycosuria was assessed at 2 weeks after diabetes mellitus induction and reassessed at the end of the study (Figure I in the online-only Data Supplement).

Cell Cultures
Human BM endothelial cells (hBMECs) were kindly provided by Prof van der Schout (Sanquin University, Amsterdam, The Netherlands) and cultured as described previously. In selected experiments, cells were cultured in normal glucose (5 mmol/L D-glucose) or high glucose (HG; 15 mmol/L and 25 mmol/L D-glucose) for 96 hours before use in expression and functional studies. Equivalent concentrations of L-glucose were used as osmotic control.

BMECs were also isolated from T1D mice (14–18 weeks after diabetes mellitus induction) and age-matched nondiabetic controls, as described previously. Purity was assessed by flow cytometry detection of the endothelial marker MECA32, which was consistently expressed by >90% of the isolated cells.

Infection of BMECs
BMECs were infected separately with 2 different adenoviruses: an adenovirus carrying constitutively active myristoylated Akt and an adenovirus carrying the dominant negative form of RhoA. AdNull (empty vector Ad66) was used as control. BMECs were grown in complete medium in 10-cm petri dishes until 60% to 70% confluent, and then infected overnight with adenoviral vectors at 200 multiplicity of infection for both adenoviruses. The medium was then changed and cells were used for experiments after 2 days.

Illumina Gene Array
An Illumina bead-array interrogating 24,600 murine transcripts was performed on T1D and control BMECs (4 biological replicates each). Microarray expression data are available at the NCBI Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE14035.

Differentially expressed genes were associated with biofunction networks and canonical pathway using Ingenuity Pathway Analysis software core analysis (Ingenuity Systems, Redwood City, CA, www.ingenuity.com). Transcripts with expression changes at false discovery rate (q-value) <0.05, induced or repressed >1.25-fold, were associated with biological functions in Ingenuity Knowledge database, and the association was tested for statistical significance as described below.

Flow Cytometry Staining of BMECs
Freshly harvested BM cells were washed with ice-cold Hank balanced salt solution containing 0.5% bovine serum albumin and 0.02% sodium azide. To recognize endothelial cells (ECs), BM cells were stained with anti-MECA-32 biotin-conjugated antibody (BD Biosciences, Oxford, UK), followed by streptavidin–allophycocyanin–conjugated secondary antibody.

Detection of Oxidative Stress Markers
HBMECs were seeded on 6-well plates, cultured until reaching confluence, and then assessed for reactive oxygen species (ROS) levels by flow cytometry detection of DsRedox (Invitrogen, Carlsbad, CA), a mitochondria-specific dihydroethidium-derivative fluorescent dye, or DCF (Molecular Probes, Paisley, UK), a marker of total oxidative stress. Four separate experiments in triplicates were analyzed and averaged.

In Vivo Permeability Assay
HBMECs and BMECs were seeded onto transwell inserts (pore size: 0.3 µm) coated with 0.5 µg/mL fibronectin, and grown until they reached confluence. Then, the media was replaced in the lower chamber and fresh media containing 70 KDa fluorescein isothiocyanate-labeled dextran was added to the upper chamber. The increase in fluorescence in the lower chamber was evaluated at different time points over 16 hours, with identical exposure settings using FLUOstar Optima fluorimeter (Berthold Technologies Wildbad, Germany). Three independent experiments in triplicate were analyzed and averaged.

In Vitro Permeability Assay
HBMECs and BMECs were seeded onto transwell inserts (pore size: 0.3 µm) coated with 0.5 µg/mL fibronectin, and grown until they reached confluence. Then, the media was replaced in the lower chamber and fresh media containing 70 KDa fluorescein isothiocyanate-labeled dextran was added to the upper chamber. The increase in fluorescence in the lower chamber was evaluated at different time points over 16 hours, with identical exposure settings using FLUOstar Optima fluorimeter (Berthold Technologies Wildbad, Germany). Three independent experiments in triplicate were analyzed and averaged.

Transendothelial Migration of BM-MNCs
Transendothelial migration of BM-MNCs was assessed using transwell cell culture inserts equipped with 3-µm pore size filters (BD Biosciences) using stromal cell–derived factor 1 as the chemotactrant. BM-MNCs from T1D and control mice were labeled with chloromethyl-dyalkylcarbocyanine (Life Technologies, NY), and then added to the top compartment (3×105 cells per well in 300 µL). After 16 hours incubation at 37°C, nonmigrated cells on the upper side of the membrane were removed by scraping. All inserts were fixed for 10 minutes in methanol and mounted on slides counterstained with 4',6-diamidino-2-phenylindole. Three separate experiments in triplicates were analyzed and averaged.

BMEC Migration
Migration of cultured BMECs was studied, as described previously. Briefly, BMECs were seeded on the upper part of 24-transwell plate filters coated with fibronectin. The lower wells contained basal medium supplemented with vascular endothelial growth factor A (100 ng/mL, R&D, Abingdon, UK). After 8 hours incubation, BMECs migrated to the lower part of the filter membrane were counted. Five random fields per each filter were evaluated at ×200 magnification using a fluorescent microscope (Olympus). Four separate experiments in triplicate were performed.

Matrigel Assay
BMECs (3×104 in a total volume of 100 µL Dulbecco’s modified Eagle’s medium) were added on top of 100 µL gelified, growth-enriched Matrigel (BD Biosciences, Oxford, UK) in each well of 8-well chamber slides. After 8 hours at 37°C, gels were washed gently with sterile PBS and fixed with 2% paraformaldehyde, and then mounted with glycerol. Three samples per group were analyzed in triplicate to compute the cumulative tube length of the endothelial network.
Immunohistochemistry and Immunocytochemistry

Paraffin-embedded sections (5 μm thick) of BM from T1D and control mice (n=6 per group) were used for in situ identification of vascular structures (Alexa Fluor 568-conjugated isoclinet B4 [Invitrogen]) expressing phosphorylated VE-cadherin (VE-cad pY731 or pY658 rabbit polyclonal antibodies [Invitrogen]). A goat antirabbit Alexa488 was used as secondary antibody (Invitrogen). All samples were counterstained with 4',6-diamidino-2-phenylindole. Microphotographs were taken with a Leica SP5 confocal imaging system (Leica, Milton Keynes, UK) at ×200 magnification.

To study cytoskeletal rearrangements, BMECs were stained with rhodamine phallolidin (Sigma). Five images per field were captured at ×200 magnification using a fluorescent microscope (Olympus, Southend on Sea, UK).

Quantitative PCR

Total RNA was isolated from murine BMECs (RNeasy, Qiagen, Manchester, UK), and RNA quality confirmed using the RNA Nano LabChip in a bioanalyzer (Agilent, Stockport, UK). RNA was reverse transcribed (Senscript reverse transcriptase, Qiagen, Crawley, UK) and quantitative PCR was performed in a LightCycler480 (Roche). Primers for PCR amplification for housekeeping gene, 18S rRNA, and quantitative PCR was performed in a LightCycler480 (Roche). Primers for PCR amplification for housekeeping gene, 18S rRNA, and quantitative PCR was performed in a LightCycler480 (Roche). Primers for PCR amplification for housekeeping gene, 18S rRNA, and quantitative PCR was performed in a LightCycler480 (Roche). Primers for PCR amplification for housekeeping gene, 18S rRNA, and quantitative PCR was performed in a LightCycler480 (Roche). Primers for PCR amplification for housekeeping gene, 18S rRNA, and quantitative PCR was performed in a LightCycler480 (Roche). Primers for PCR amplification for housekeeping gene, 18S rRNA, and quantitative PCR was performed in a LightCycler480 (Roche).

Western Blot

The analysis of protein expression was performed on lysates from confluent BMECs and BMECs using phosphospecific antibodies against anti-phospho Ser 1177 endothelial nitric oxide synthase (eNOS) (Cell Signaling Technologies, MA), antibodies raised against respective total proteins and a monoclonal antibody for recognition of tubulin. Blots were analyzed with an enhanced chemiluminescence detection system (Amersham Biosciences, Buckinghamshire, UK).

Immunoprecipitation

Membrane samples were obtained, as described previously, in lysis buffer, 20 mMol/L Tris-HCl, 150 mMol/L NaCl, 1 mMol/L EDTA, 1 mMol/L ethylene glycol tetraacetic acid, 1% Triton-X, 2.5 mMol/L sodium pyrophosphate, 1 mMol/L β-glycerophosphate, 1 mMol/L Na,VO₃, 1 μg/mL leupeptin. Cav1 was immunoprecipitated using anti-Caveolin-1 (Cav-1) antibody (Abcam) and protein A (Santa Cruz). After washing of the immune complexes in wash buffer, 20 mMol/L Tris-HCl, 137 mMol/L NaCl, 1% Triton-X, 2 mMol/L EDTA, the complexes were run on a SDS-PAGE gel and blotted for total eNOS (Cell Signaling Technologies). Samples incubated with nonimmune rabbit IgG, instead of anti-Cav-1 antibody, were used as controls.

Data Analysis and Statistical Procedures

Values are presented as mean±SEM. If data failed to pass normality and equal variance tests, a nonparametric analysis was applied and results were expressed as median with 5 to 95 percentile distribution. Multiple groups were compared by parametric 2-way ANOVA, followed by Bonferroni post t test, 1-way ANOVA, followed by Bonferroni Multiple Comparison test, or nonparametric ANOVA on ranks, followed by Tukey pairwise comparison or Dunnnett test for multiple comparisons against a single control group. Comparison of 2 groups was performed by paired or unpaired Student t test. In gene array studies, the right-tailed Fisher exact test was used to evaluate the probability that the association of differentially expressed genes and biological functions or canonical pathways is because of chance. The significance of the association between the data set and a given canonical pathway was also measured as the ratio of the number of differentially expressed genes in a pathway and the total number of genes in the same pathway. A P value <0.05 was considered significant.

Results

To determine the mechanisms underlying BM endotheliopathy, we performed an Illumina gene array on primary BMECs isolated from T1D (18 weeks from diabetes mellitus induction) and age-matched nondiabetic mice. Of 792 transcripts with expression changes at false discovery rate (q value) <0.05, 448 were induced or repressed >1.25-fold. Table II in the online-only Data Supplement shows the list of differentially expressed genes within canonical pathways. Among top-ranked functions, Ingenuity Pathway Analysis showed a highly significant effect of diabetes mellitus on signaling pathways associated with cellular death, assembly, organization, trafficking, and inflammation (Figure 1A).

Functional enrichment analysis identified small GTPases (RhoA and CDC42), actin cytoskeleton dynamics, integrin, leukocyte extravasation, and tight junctions, as the signaling pathways most enriched with differentially expressed genes (Figure 1B). Moreover, within the actin cytoskeleton and leukocyte extravasation/vascular permeability signaling pathways, we found that 14 of 209 and 12 of 183 genes, respectively, were modulated by diabetes mellitus (Figure II in the online-only Data Supplement). Actin-related protein 2/3 (nucleation site for actin filaments polymerization), membrane-organizing extension spike protein (moesin, a cross-linker between the endothelial plasma membrane and actin-based cytoskeleton), and the Rho-associated kinase-2 (ROCK2, an activator of moesin through phosphorylation on Thr558) were all upregulated in diabetic BMECs.

Taken together, these gene array data indicate transcriptional alterations compatible with loosened adhesive intercellular contacts and increased endothelial permeability. Alterd RhoA/ROCK and Akt Activity in Diabetic BM Endothelium

RhoA and ROCK regulate a wide range of cellular functions, including cytoskeletal rearrangement, migration, and proliferation. Using a RhoA-GTP-bound pulldown assay, we found that diabetes mellitus increases Rho activity in BMECs (Figure 2A). It is known that oxidative stress is a potent inducer of RhoA. Here, we confirm our previous finding of increased oxidative stress at the mitochondrial level in T1D-BMECs (Figure IIIA in the online-only Data Supplement). Moreover, we found polyADP-ribose polymerase 1 to be upregulated and transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2) downregulated in T1D-BMECs (Figure IIIB in the online-only Data Supplement). It is known
that oxidative stress induces DNA strain breaks, which in turn activate polyADP-ribose polymerase,\(^1\),\(^2\) whereas Nfr2 exerts antioxidant activity to protect vascular cells from HG-induced damage.\(^3\),\(^4\) In contrast, the expression of NADPH oxidase isoform 2, another important source of ROS, was similar in BMECs from healthy and diabetic mice (Figure IIIC in the online-only Data Supplement). Thus, oxidative stress in BM endothelium is attributable to increased ROS production in mitochondria and reduced antioxidant defense. In accordance, we found that preconditioning T1D-BMECs with the ROS scavenger, N-acetyl-cysteine (NAC), inhibits RhoA activation (Figure 2A). Furthermore, RhoA upregulation was accompanied by increased ROCK1 and ROCK2 mRNA levels, which was again prevented by NAC (Figure 2B).

The influence of activated RhoA on Akt is controversial, with reports indicating that RhoA/ROCK causes induction \(^22\)
or suppression of Akt activity in ECs. We found that Akt activity is remarkably depressed in diabetic BM endothelium. Notably, this deficit was partially reverted by NAC, the ROCK inhibitor Y27632, or by transfecting cells with adenovirus carrying the dominant negative form of RhoA (Figure 2C), thus suggesting that small GTPase activation by oxidative stress is responsible for Akt inhibition.

Akt activation in ECs reportedly induces the release of angiocrine factors that support BM stem cell expansion. Several of these angiocrine substances, such as fibroblast growth factor 2, JAGGED1, and JAGGED2, were downregulated in diabetic BMECs, but restored after antioxidant treatment (Figure 2D and 2E). The ROCK inhibitor Y27632 and RhoA knockdown recovered fibroblast growth factor 2, but not JAGGED1 and JAGGED2 mRNA expression (Figure 2D).

**Rescue of Endothelial Dysfunction by ROCK Inhibition or Akt Activation**

We next investigated whether an altered RhoA–Akt axis has specific consequences for the BMEC function-type. Akt is a potent inducer of eNOS activity, which synthesizes nitric oxide, a key molecule in EC function. In total membrane fractions from T1D BMECs, we observed a decrease in eNOS phosphorylation as well as a reduction in Cav-1 expression. Cav-1 negatively regulates eNOS by directly interacting with it. Immunoprecipitation of Cav-1 confirmed that Cav-1 and eNOS interact both in Ctrl and T1D BMECs (Figure 3A and 3B). Taken together, these data suggest a reduced nitric oxide (NO) availability in diabetic cells.

We next investigated the effect of Akt activation (by transfection of BMECs with a constitutively active form), of RhoA knocking down (by a transfection with a dominant negative form), and of pharmacological ROCK inhibition with the compound Y27632. Successful transduction of cells by adenovirus carrying constitutively active myristoylated Akt and adenovirus carrying the dominant negative form of RhoA was documented by Western blot for Akt and Rho activity assay (Figure IIID and IIIE in the online-only Data Supplement).

In a network formation assay on matrigel, T1D-BMECs showed reduced tube-formation capacity, which was restored by constitutively active Akt, adenovirus carrying the dominant negative form of RhoA, and ROCK inhibition with Y27632 (Figure 3C). Successful rescue of endothelial dysfunction was confirmed by Western blot for eNOS and Cav-1 activity and interaction with Cav-1 (Figure 3D). These data also suggest that nitric oxide availability is partially restored by ROCK inhibition or Akt activation.

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**Figure 3.** Rho-associated kinase (ROCK) inhibition or Akt activation rescues endothelial dysfunction. A, Endothelial nitric oxide synthase (eNOS) activity (ii) along with Caveolin-1 (Cav-1) (iii) expression were measured by western blot (WB) (i) in membrane fractions. B, Diabetes mellitus impairs eNOS activation and interaction with Cav-1, as demonstrated by Western blot for eNOS in Cav-1 immunoprecipitates (IP Cav-1). Nonimmunogenic IgG was used as control. C, Representative microphotographs (scale bars 100 μm; i) and bar graph (ii) showing network-forming activity of control and diabetic bone marrow endothelial cells (BMECs). The deficit of network formation in diabetic BMECs was rescued by ROCK inhibition with Y27632 or transfection with constitutively active Akt or adenovirus carrying the dominant negative form of RhoA. Images were captured 16 hours after BMEC seeding. D, Bar graph showing percentage of migrated BMECs after stimulation with vascular endothelial growth factor A or vehicle (BSA). Diabetic BMECs showed impaired migratory capacity toward vascular endothelial growth factor A that was rescued, in part, by constitutively active Akt, but not by ROCK inhibition or adenovirus carrying the dominant negative form of RhoA infection. Data are expressed as means±SEM. *P<0.05; **P<0.01; ***P<0.001; ****P<0.001 vs Ctrl; #P<0.05; ##P<0.01 vs type-1 diabetic (T1D); P<0.01; and ###P<0.001 vs BSA. Each experiment was performed in triplicate.
negative form of RhoA, or ROCK inhibition (Figure 3C). In addition, T1D-BMECs displayed a reduced migratory response to vascular endothelial growth factor A, with this defect being partially recovered by Akt activation, but not by RhoA/ROCK inhibition (Figure 3D). ROS are known to induce the rearrangement of F-actin stress fibers and cell contraction via RhoA–ROCK activation and phosphorylation of moesin, resulting in increased endothelial permeability. We asked whether this mechanism is activated in T1D-BMECs. Accordingly, we found that diabetes mellitus triggers the formation of F-actin stress fibers in BMECs, which is reduced by ROCK inhibition and to a lesser extent by Akt activation (Figure 4A). Furthermore, moesin mRNA (Figure 4B) and protein phosphorylation levels (Figure 4C) were increased in T1D-BMECs, with the latter effect being blunted by NAC and ROCK inhibitor Y27632.

We next asked whether ROS- and ROCK-dependent activation of BMEC cytoskeleton translates into increased endothelial permeability and barrier dysfunction. Size-selective assessment of paracellular permeability was performed using fluorescently labeled dextran. Figure 4D shows that the T1D-BMEC monolayer is more permeable to dextran compared with BMECs from healthy mice. This increased permeability was prevented by NAC, myristoylated Akt, and RhoA/ROCK inhibition. The presence of endothelial barrier dysfunction was further assessed using a transendothelial migration assay on BM-MNCs. Results confirm our previous findings indicating that spontaneous transendothelial migration of BM-MNCs is increased in the presence of diabetic BMECs compared with control BMECs, whereas directed migration toward stromal cell-derived factor-1 is abolished. Moreover, we newly show that endothelial barrier function is rescued, in part, by ROS scavenging and RhoA/ROCK inhibition (Figure 4E). In contrast, Akt activation did not reduce the increased basal migration of BM-MNCs, but restored responsiveness to stromal cell-derived factor-1.

Figure 4. Rho-associated kinase (ROCK) inhibition and Akt activation rescues diabetes mellitus–induced endothelial barrier dysfunction in bone marrow endothelial cells (BMECs). A, Microphotographs (scale bars 100 µm) showing BMECs stained for F-actin with rhodamine-associated phalloidin. Diabetes mellitus induces actin fiber activation, which is blunted by ROCK inhibitor Y27632, adenovirus carrying constitutively active myristoylated Akt or adenovirus carrying the dominant negative form of RhoA. B and C, Representative blots and graphs showing the effect of diabetes mellitus on mRNA expression (B) and the phosphorylation state of moesin (C) in BMECs. Upregulation of moesin is blunted by N-acetyl-cysteine (NAC) and ROCK inhibition. D, Bar graph showing the effect of diabetes mellitus on endothelial permeability using fluorescein isothiocyanate-labeled 70-kDa dextran. The increase in endothelial permeability is blunted by NAC, ROCK inhibition, constitutively active Akt or the dominant negative form of RhoA. E, Bar graph showing BM-MNC transendothelial migration after exposure to stromal cell–derived factor-1 (directed migration) or PBS (vehicle, spontaneous migration). Spontaneous transmigration was enhanced and directed migration suppressed by diabetes mellitus. These alterations were prevented by treating BMECs with NAC and ROCK inhibitors. Constitutively-active Akt improves directed migration without reducing the exaggerated spontaneous migration of bone marrow mononuclear cells (BM-MNCs). Data are mean±SEM. *P<0.05; **P<0.01; ***P<0.001; ##P<0.01 vs Ctrl; #P<0.05; and ##P<0.01 vs type-1 diabetic (T1D). Each experiment was performed in triplicate.
Altogether, these data indicate that the Rho/ROCK–Akt axis plays a crucial role in the functional alterations of diabetic BMECs.

**HG Increases BMEC Permeability Through VE-Cadherin Phosphorylation**

We next investigated the direct effect of HG on BMEC permeability. To this end, we established an in vitro model consisting of hBMECs cultured in normal (5 mmol/L, normal glucose) or high (15 and 25 mmol/L, HG) D-glucose for 96 hours. ROS levels were augmented by progressive increases of glucose concentration, as assessed by flow cytometry detection of MitoSox and 2′,7′-dichlorofluorescein-2A. The ROS production was brought back to control levels totally by catalase treatment, and partially reduced by superoxide inhibitor and antioxidant diethyldithiocarbamate (Figure IV A in the online-only Data Supplement). Moreover, HG alters hBMEC permeability in a dose-dependent manner, as assessed in an in vitro assay using 70 kDa dextran (Figure IVB in the online-only Data Supplement). The increase in permeability was completely reversed by treating hBMECs with NAC or catalase; however, neither the hydroxyl scavenger MCI-186 nor diethyldithiocarbamate modified the effect of HG on permeability. The inhibition of detoxifying chain at superoxide level suggests that this ROS, and the ones generated as peroxynitrite, can trigger molecular changes leading to increased permeability.

ROS reportedly modifies the activity of several tyrosine kinases. Among them, Src increases vascular permeability through phosphorylation of VE-cadherin, a key component of EC adherens junctions. We found that HG increases the phosphorylation of VE-cadherin at Y731 and Y658, which are binding sites for β-catenin and p120, respectively. Moreover, VE-cadherin phosphorylation was prevented by both NAC treatment and Src inhibition, suggesting a pivotal role of Src kinase in adherens junction disassembly through a redox-sensitive mechanism (Figure VA and VB in the online-only Data Supplement). Of note, the HG-induced increase in permeability was reverted by Src inhibitor SU6656 (Figure IVB in the online-only Data Supplement). Another redox-sensitive kinase controlling adherens junctions is represented by the proline-rich kinase 2 (Pyk2), which has the same targets as Src. In accordance, the active phosphorylated form of Pyk2 was increased in hBMECs under HG. This effect was totally prevented by NAC (Figure VC in the online-only Data Supplement).

In addition, we found that the proapoptotic and proinflammatory redox-sensitive kinases p38 and c-Jun N-terminal kinases are activated in both HG-treated hBMECs and T1D-BMECs. This effect was reversed by NAC and catalase. Finally, the MAPK kinase kinase, MEK1, which control angiogenesis and proliferation in ECs, was found increased in HBMECs treated with HG, but not in diabetic cells (Figure VI in the online-only Data Supplement).

**Redox-Dependent Activation of VE-Cadherin in BMEC Leads to Endothelial Barrier Dysfunction in T1D Mice**

We next asked whether phosphorylation events associated with VE-cadherin activation occur in BMECs from diabetic mice. As for HG-treated hBMECs, phosphorylation of VE-cadherin and Pyk2 was increased in diabetic murine BMECs, but reduced by NAC (Figure 5A–5C). Fluorescence microscopy demonstrated in situ phosphorylation of VE-cadherin in BM vascular cells of T1D mice (Figure 5D and Figure VII in the online-only Data Supplement).

Finally, we assessed the abundance of BMECs by flow cytometry of MECA32-positive cells and BM endothelial barrier function in vivo using a double tracer technique. We found that MECA-32–positive ECs are reduced in BM of T1D mice (Figure 6A and 6B). Moreover, vascular permeability is increased by diabetes mellitus (Figure 6C and 6D), which was confirmed at different times from diabetes mellitus induction (Figure VIII A and VIII B in the online-only Data Supplement). To verify whether the observed changes can be contrasted by metabolic control, we treated diabetic animals with insulin implants. Of note, insulin replacement resulted in maintenance of BMECs abundance and normalization of vascular permeability (Figure 6A–6D). Furthermore, in vitro insulin treatment of BMECs was capable of reducing VE-cadherin phosphorylation at site Y731. Conversely, p-Pyk2 seemed not to be affected by insulin (Figure VIII A and VIII B in the online-only Data Supplement).

**Discussion**

This study provides new mechanistic insights into BM endothelial dysfunction induced by diabetes mellitus. BMECs from T1D mice showed a spectrum of functional alterations, including defects in angiocrine activity, migration, network formation, and permeability. Endothelial dysfunction can be traced back to mitochondrial oxidative stress triggered by high levels of glucose and alteration of the RhoA/ROCK/Akt signaling pathway. Moreover, BMEC availability and endothelial barrier dysfunction were confirmed in vivo and corrected by insulin.

RhoA controls several cellular function, including migration, angiogenesis, and apoptosis. In ECs, this Ras-like protein is committed to the formation of stress fibers through its effector ROCK. In recent years, RhoA has gained attention in the field of diabetes mellitus, being acknowledged as a primary target for oxidative stress or advanced glycation end products, and as an initiator of a series of transcriptional and posttranscriptional events leading to endothelial dysfunction. Here, we newly demonstrate that diabetes mellitus increases RhoA expression and activity, as well as the mRNA levels of ROCK isoforms in diabetic BMECs. ROCK1 activation is involved in permeability changes under inflammatory conditions, whereas ROCK2 contributes to the increase in adhesion molecules via nuclear factor-κB p65. Activation of moesin by ROCK-mediated phosphorylation induces rearrangement of the actin cytoskeleton and cell contraction instrumental to endothelial permeability. Importantly, we found that moesin is transcriptionally upregulated and phosphorylated in BMECs of T1D mice, leading to the activation of stress fibers and increased permeability to MNCs and macromolecules. These effects were prevented by the ROS scavenger and ROCK inhibitor, thus delineating a causal association between oxidative stress, RhoA/ROCK activation, stress fiber contraction, and endothelial barrier dysfunction.

Diabetic endotheliopathy is characterized by an alteration in the phosphorylation state and activity of several kinases.

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**References**

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We have previously reported that diabetic BMECs have higher phosphorylation levels of VE-cadherin (at tyrosine 731, the β-catenin-binding site) and Pyk2 (at tyrosine 402, which is the autophosphorylation site for Pyk2) compared with control BMECs. Here, we newly report that HG-induced oxidative stress causes phosphorylation of VE-cadherin via the redox-sensitive kinases Src and Pyk2, thereby favoring the disassembly of adherens junctions and BM-MNC extravasation. Furthermore, we found that both diabetes mellitus and HG trigger the phosphorylation of apoptosis-related kinases, such as p38 and c-Jun N-terminal kinases, in human and murine cells. The redox-sensitive MAPK kinase kinase, MEK1, which in turn activates extracellular-signal-regulated kinases 1/2 exerts a modulatory control of angiogenesis.

Figure 5. Vascular endothelial cadherin (VE-cadherin) phosphorylation is increased in diabetic bone marrow endothelial cells (BMECs). A–C, Representative Western blots (i) and bar graphs (ii) showing phosphorylation of VE-cadherin pY658 (A) and VE-cadherin p731 (B) and pPyk2 (C) in BMECs. D, Immunostaining for residues pY658 (top) and pY731 (bottom) of VE-cadherin (arrowhead) and isolectin B4 (arrow) in bone marrow (femur) of type-1 diabetic (T1D) and control non-diabetic mice (scale bars: main pictures 80 μm; enlargement 20 μm). Data are mean±SEM. **P<0.01 vs Ctrl; and *P<0.05 vs T1D. Each experiment was performed in triplicate.

Figure 6. Diabetes mellitus increases vascular permeability in bone marrow of type-1 diabetic (T1D) mice. A, Gating strategy for flow cytometry assessment of MECA-32–positive bone marrow endothelial cells (BMECs). B, Insulin replacement restores the abundance of MECA-32–positive ECs in bone marrow (BM) of T1D mice. C, Line graph showing the concentration of fluorescein isothiocyanate-labeled dextran in femoral BM of T1D (dotted line) and control mice (full line), at different time points from dextran injection. D, Bar graph showing restoration of proper vascular permeability in BM of T1D mice given insulin implants (grey bars) in comparison with untreated T1D mice (black bars). Reference value of nondiabetic control group (white) is also shown. Data are mean±SEM. *P<0.05; **P<0.01 and vs Ctrl; and ***P<0.01 vs T1D. Each experiment was performed in triplicate.
found that in vitro exposure of hBMECs to HG increases the phosphorylation of MEK1, however, MEK1 levels were similar in BMECs from diabetic or nondiabetic mice. Thus, this specific pathway seems to be particularly sensitive to acute increases in glucose levels. We also observed a differential effect of various antioxidants on vascular permeability. The alteration of endothelial barrier function diabetes mellitus is more likely to depend on the formation of peroxynitrite, which is an activator of the RhoA/ROCK pathway, whereas redox-sensitive kinases are triggered by an increase in hydrogen peroxide production.

Another hallmark of BM endotheliopathy consists of Akt inactivation. NAC, RhoA dominant negative transfection, and ROCK inhibitor Y27632 were able to rescue Akt activity, suggesting an intertwined relationship between redox-dependent activation of RhoA–ROCK and Akt suppression. In fact, either inhibiting ROCK or enhancing Akt activity rescued diabetes mellitus–induced dysfunctions, including migratory and angiogenic defects, and increased permeability. Akt seems to be crucial for BMECs to manifest a migratory phenotype, as Akt inactivation in diabetes mellitus reduces their migratory and network-forming capacity, whereas Akt reactivation rescues both defects. In accordance with this hypothesis, we observed an impairment in eNOS activity. Therefore, the picture that emerges from a joint analysis of molecular and functional readouts is that of a contracted and leaky BM endothelium, incapable of responding to migratory signals as a consequence of dysfunctional Akt. In addition, it has been recently shown that Akt is essential for BMECs to convey self-renewal and differentiation signals to long-term hematopoietic stem cells through the release of angiocrine factors.3,4 We newly report the decreased expression of some Akt-dependent factors in diabetic BMECs, that is, the Notch ligands JAGGED1 and JAGGED2 and the angiogenic factor fibroblast growth factor 2. Further studies are warranted to gather new evidence that microvascular rarefaction occurs together with hematopoietic tissue remodeling and stem cell depletion in BM of diabetic patients.32 Hence, preserving the fitness of BM microvasculature represents a novel therapeutic target in the management of patients with diabetes mellitus.

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**Disclosures**

None.

**References**


9. Gadan S, Emanueli C, Van Limthout S, Graiani G, Todaro M, Meloni M, Campestrini I, Inverni G, Spillmann F, Ward K, Madeddu P. Benfotiamine identifies candidate mechanistic targets for rectification of the dysfunctional phenotype (Figure IX in the online-only Data Supplement). Importantly, insulin replacement exerts significant protection of BM vasculature. The notion that insulin is a potent inducer of Akt, and an inhibitor of RhoA in vascular cells, confirms the validity of the proposed molecular network.

BM-specific microangiopathy might have relevant clinical consequences. First, microvascular rarefaction endangers BM stem cell viability through reduction of perfusion and suspension of paracrine trophic signaling. Second, plasma extravasation is particularly harmful for a tissue like the marrow that is contained in nonexpandable bone. Third, barrier dysfunction might impinge on the release of stem cells, as illustrated by experiments showing exaggerated spontaneous transendothelial migration and reduced directed migration toward chemotactants. These considerations call for urgent investigation into the status of BM in patients with complicated diabetes mellitus. Here, we show that hBMECs develop typical molecular and functional alterations when exposed to HG. We have also gathered new evidence that microvascular rarefaction occurs together with hematopoietic tissue remodeling and stem cell depletion in BM of diabetic patients.32 Hence, preserving the fitness of BM microvasculature represents a novel therapeutic target in the management of patients with diabetes mellitus.


Diabetes Causes Bone Marrow Endothelial Barrier Dysfunction by Activation of the RhoA–Rho-Associated Kinase Signaling Pathway

Giuseppe Mangialardi, Rajesh Katare, Atsuhiko Ōikawa, Marco Meloni, Carlotta Reni, Costanza Emanueli and Paolo Madeddu

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Supplemental material

Cell cultures
Human BM endothelial cells (hBMECs) were kindly provided by Prof. van der Schout (Sanquin University, Amsterdam, The Netherlands) and cultured, as described previously, in Medium 199 (Gibco BRL), supplemented with 10% (v/v) heat-inactivated human serum (Invitrogen), 10% (v/v) heat-inactivated FCS, 1 ng/mL Basic Fibroblast Growth Factor (bFGF, Boehringer Mannheim, Mannheim, Germany), 5 U/mL heparin (Leo Pharmaceutical Products, Weesp, The Netherlands), 300 μg/mL glutamine (Sigma), 100 U/mL penicillin, 100 μg/mL streptomycin. In selected experiments, cells were cultured in normal glucose (NG) (5 mM D-glucose) or high glucose (HG) (15 mM and 25 mM D-glucose) for 96 hour.

BMECs were also isolated from 14 to 18 weeks T1D mice and age-matched non-diabetic controls as described previously. Briefly, freshly harvested BM cells were immunomagnetically depleted of CD11b-expressing cells to eliminate myeloid/monocyte fraction and cultured on 0.1% gelatin in DMEM containing supplemented with 20% FBS and tetrapeptide Ac-Ser-Asp-Lys-Pro (AcSDKP) to avoid stem cells and fibroblasts contamination. Cells were grown until confluence and then used for experiments. Purity was assessed by flow cytometry detection of the endothelial marker MECA32, which was consistently expressed by > 90% of the isolated cells.

Western Blot
Confluent hBMECs and BMECs were washed twice with ice-cold Ca\textsuperscript{2+}- and Mg\textsuperscript{2+}- containing PBS, and lysed in lysis buffer (25 mM Tris, 150 mM NaCl, 10 mM MgCl\textsubscript{2}, 2 mM EDTA, 0.02% (w/v) SDS, 0.2% (w/v) deoxycholate, 1% Nonidet P-40, 0.5 mM orthovanadate with the addition of fresh protease-inhibitor-mixture tablets (Roche Applied Science, Burgess Hill, UK). After 30min on ice, cell lysates were collected and the supernatant was separated from the insoluble fraction by centrifugation (14,000 g, 10min at 4°C). Supernatants were diluted with boiling 2x SDS-sample buffer containing 4% 2-mercaptoethanol (Bio-Rad, Hemel Hempstead, UK). The samples were analyzed by SDS-PAGE. Proteins were transferred to 0.45-μm nitrocellulose and the blots were blocked with 5% BSA in Tris-buffered saline with Tween-20 (TBS-T) for 1h and subsequently incubated at RT with phospho-specific antibodies against Nox2 (BD Biosciences, Oxford, UK), VE-cadherin-Y731, VE-cadherin-Y658 and Pyk2-Y402 and with a monoclonal antibody against Tubulin, followed by incubation with goat-α-rabbit-IgG-horseradish peroxidase or a goat-antimouse-IgG-horseradish peroxidase for 1h at RT. Between the various incubation steps, the blots were washed 5 times with TBS-T and finally developed with an enhanced chemiluminescence detection system (Amersham Biosciences, Buckinghamshire, UK).

Immunohistochemistry
Paraffin embedded BM sections (5 μm-thick) were kept at 60°C overnight and then deparaffinized in xylene and rehydrated through passages in alcohol (100% to 70%) and distilled water. Heat-induced antigen retrieval was carried out in 10 mM citrate buffer (Ph6) for 15min in a microwave oven. Sections were cooled down and then washed in distillate water/PBS. Samples were incubated with blocking solution of %10 goat serum in PBS Triton X 0.1%. Samples were incubated overnight with Alexa Fluor 568-conjugated isolectin B4 and then with VE-cad pY731 or pY658 rabbit polyclonal antibody for 2h at RT. A goat anti-rabbit Alex488 was used as secondary antibody for 1h at RT. All samples were counterstained with DAPI and then mounted in Fluoromont-G mounting media.
Microphotographs were taken with Leica SP5 confocal imaging system (Leica, Milton Keynes, UK), at 400x magnification.
Rhodamine staining
BMECs were seeded in 8-well chamber slides to reach semi-confluence. Cells were fixed with 2% paraformaldehyde for 15min at RT. After incubation in rhodamine phallodin staining solution (200µM) for 20min at 4°C, cells were washed twice with PBS and then mounted with Fluoromont G mounting media. Representative microphotographs for each condition were taken on a fluorescent microscope (Olympus, Southend on Sea, UK) at 200x magnification.

Rho activity assay
Guanosine triphosphate (GTP)-bound active Rho was assessed by pulldown assays, following the manufacturer’s instructions (Millipore, Billerica, Ma, USA). Briefly, BMECs were cultured until 70-80% confluent. The medium was changed to DMEM without serum, and after an overnight incubation, cell lysates were harvested. Precipitated GTP-bound Rho was run on a 10% SDS-PAGE and subjected to immunoblotting, using anti-Rho monoclonal antibodies. After washing and applying anti-mouse HRP-conjugated antibody, enhanced chemiluminescence reagent (Amersham Biosciences, Bath, UK) was used for visualization.

Trans-endothelial migration
Trans-endothelial migration (TEM) of BM mononuclear cells (BM-MNCs) was assessed using transwell cell culture inserts equipped with 3µm pore size filters (BD Biosciences) using SDF-1 as chemoattractant. Inserts were pre-coated with 0.5 µg/mL fibronectin in 0.1% gelatin. BMECs were seeded on coated transwell filters (8x10⁴/well) and cultured for 24h before the experiment was performed. SDF-1 (100ng/mL) or vehicle was added to the lower compartment of the transwell system, in DMEM containing 0.1% BSA. Freshly isolated BM-MNCs from T1D and control mice were labeled with CM-DiL (Life Technologies, NY, US) following the manufacturer’s instructions, resuspended at 1x10⁶ cell/mL and then added to the top compartment (3x10⁵ cells per well in 300µL). After 16h incubation at 37°C, non-migrated cells on the upper side of the membrane were removed by scraping; all inserts were fixed for 10min in methanol, and mounted on slides counterstained with DAPI. The number of fluorescent BM-MNCs transmigrated to the lower side of the membrane was quantified in duplicates. Five random view-fields at x200 magnification were captured and the number of CM-DiL-positive BM-MNCs was determined. Three separate experiments in triplicate were analyzed and averaged.

In vitro permeability assay
HBMECs and BMECs were seeded onto transwell inserts (0.3 µm, pore size) coated with 0.5 µg/mL fibronectin and grown until they reached confluence. Cells were preconditioned with Src inhibitors, PP2 (10 µM, Sigma, Saint Louise, MO, USA) and SU6656 (2.5 µM, Calbiochem, Darmstadt, Germany) for 30min. Then media were replaced in the lower chamber and fresh media containing 70 kDa FITC-labelled dextran were added in the upper chamber. The increase in fluorescence in the lower chamber was evaluated at different time points over 16h with identical exposure settings using FLUOstar Optima fluorimeter (Berthold Technologies Wildbad, Germany). The intensity of fluorescence was plotted versus time by linear regression in order to obtain the endothelial permeability slope, Pₜₛ. The Pₜₛ value was divided by the area of the porous membrane and the result was the permeability coefficient (Pₑ, cm/s). Results were expressed as fold change of relative control. Three independent experiments in triplicate were analyzed and averaged.
**In vivo permeability assay**
Mice were intra-cardially injected with a 500µl bolus of 70 kDa FITC-labeled dextran followed, after 3, 5 or 7 min by an equal amount of 70 kDa TRITC-labeled dextran (n=3 each time point). Animals were sacrificed 30sec from last injection. Peripheral blood and bones (femura and tibiae) were collected. Peripheral blood was spun down at 1.3x10^4 rpm for 15min in order to obtain plasma. BM was flushed out from bones with sterile saline solution with equal volume for all samples and weighed. Bones were weighted before and after drying. FITC and TRITC fluorescence was measured at FLUOstar Optima fluorimeter with identical exposure settings for all conditions in plasma and flush.

Data were plotted as mg FITC (I_F) in BM interstitium per mg tissue (W) against time. W is given by:

\[ W = (F-P) + (B+D) \]

Where F is the weight of flush, P the weight of saline solution, B the weight of wet bones and D the weight of dry bones.

I_F is given by:

\[ I_F = V.(F_F - F_T.(P_F/P_T)) \]

where V is the volume of flushing, F_F is the concentration of FITC-dextran in the flush-out, F_T is the concentration of TRITC-dextran in the flush-out., P_F and P_T are the concentrations of FITC and TRITC dextran in plasma, respectively. Three independent experiments were carried out for each time point and condition.
**Supplemental Figure I**: Experimental plan for insulin treatment of T1D mice. Four weeks after induction of diabetes, mice received subcutaneous implants for continuous insulin supplementation at the rate of 0.1 unit/implant/d or vehicle. Glycemia was monitored every 4 weeks, while glycosuria was assessed at 2 weeks after diabetes induction and reassessed at the end of the study (A). Glycemia values at different time points in control group (white bars), T1D group (black bars), and T1D group receiving insulin supplementation (grey bars) (B). Data are expressed as mean ± SEM. *** P<0.001 vs. ctrl, ### P<0.001 vs. T1D.

**Supplemental Figure II**: IPA analysis highlights the remarkable effect of diabetes on key components of the signaling network controlling vascular permeability and leukocyte extravasation. Molecules are represented as nodes, and the biological relationship between two nodes is represented by an edge line. All edges are supported by at least 1 reference from the literature, a textbook, or canonical information stored in Ingenuity Pathways Knowledge database. The color indicates up (red) or down (green) regulation. Nodes are displayed using various shapes that represent the functional class of the gene product.

**Supplemental Figure III**: (A) Microphotograph (i) (scale bars 100 µm) and bar graphs (ii) showing BMECs stained with MitoTracker® Red CM-H2ROS, a marker of mitochondrial ROS. (B) DNA damage repair enzyme PARP-1 is upregulated whereas antioxidant transcription factor NRF2 is downregulated in T1D-BMECs. Expression was assessed by qPCR. (C) WB analysis of Nox2 expression in BMECs. Data (mean±SEM) indicate fold changes vs. control non-diabetic BMECs. Each experiment was performed in triplicate. (D) Transfection of T1D-BMECs with Ad.myrAkt was verified by western blot of active Akt. Data (mean±SEM) indicate fold changes relative to control. (E) Rho pulldown assay after transfection of T1D-BMECs with an adenovirus carrying RhoA N19 dominant negative. *P<0.05 and **P<0.01 vs. Ctrl, ### P<0.001 vs. T1D.

**Supplemental Figure IV**: HBMECs were cultured under increasing glucose concentrations from 5mM (white bars) to 15mM (grey bars), and 25mM (black bars). (A) Representative FACS images (i) and bar graph (ii) showing ROS levels in hBMECs assessed using flow cytometry detection of mitochondrial superoxide by MitoSox and intracellular peroxide and superoxide free radicals by DCF-2DA. The latter was measured after treatment with catalase (cat.) (100 U/ml), edavarone (MCI-86)(100 µL), and diethyldithiocarbamate (DDC)(100 µM). (B) In vitro permeability of hBMECs exposed to increasing concentrations of glucose in the presence of antioxidants DDC (100 µM), catalase (100 U/ml), MCI-186 (10 µM), and NAC or Src inhibitors (PP2, SU6656) or vehicle. Data (mean±SEM) indicate fold changes relative to normal glucose (NG). *P<0.05, **P<0.01 and ***P<0.001 vs. NG; #P< 0.05 vs. HG15; §P <0.05, §§§P<0.001 vs. HG25. Each experiment was performed in quadruplicate.

**Supplemental Figure V**: HBMECs were cultured under increasing glucose concentrations from 5mM (white bars) to 15mM (grey bars), and 25mM (black bars). Western blot was performed for VEcadherin p731 (A), and VEcadherin pY658 (B), and pPyk2 (C). Data are expresses as mean ± SEM. ǂP<0.05, ǂǂP<0.01 and ǂǂǂP<0.001 vs. vehicle of the same group, *P< 0.05 and ***P<0.001 vs. 5 mM; ǂP< 0.05 vs. 15 mM. Each experiment was performed in triplicate.
Supplemental Figure VI: (A) Expression of phosphorylated form of redox sensitive kinase p38, JNK, and mapk kinase kinase MEK1 was assessed by western blot. (B) p-p38 (i) and p-JNK (ii) were increased in T1D-BMECs (upper panels) and HBMEC treated with HG (lower panels). Antioxidants NAC (250 μM) and catalase (100 U/ml) reverted the effect of HG and diabetes. p-MEK1 (iii) was also increased in HBMEC exposed to HG but not in T1D-BMECs. **P< 0.01 vs. ctrl; #P<0.05 vs. T1D; §P<0.05 vs. NG; ¥P<0.05 vs. HG25.

Supplemental Figure VII: Representative confocal single channel and merged microphotographs (scale bars, 80 µm) of BM sections stained with isoelectin B4 (Red) and VEcadherin pY658 or VEcadherin pY731 (green) counterstained with DAPI to identify nuclei (blue). Diabetes increases VEcadherin phosphorylation in BMECs.

Supplemental Figure VIII: Bar graph showing the plateau concentration of FITC-labeled dextran in BM from femura (A) and tibiae (B) of T1D mice at 14 (white bars) and 18 weeks (black bars) from diabetes induction as compared with age-matched non-diabetic mice. Effect of insulin (100 nM) treatment in BMECs on VE-cadherin pY731 (C), VE-cadherin pY658 (D), and pPyk2 (E). Data are expressed as mean ± SEM. **P<0.01 vs. ctrl, #<0.05 vs. T1D. Each experiment was performed from triplicate to quadruplicate.

Supplemental Figure IX: Proposed mechanistic network responsible for endothelial dysfunction in diabetic bone marrow and therapeutic targets (green).
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Table II: Summary of microarray analysis comparing the expressional profile of BMECs from T1D and non-diabetic mice. List of single genes modulated in the enriched canonical pathways according to the IPA analysis.

a. Cdc42 signaling

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b. Actin Cytoskeleton signaling

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### c. Integrin signaling

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### a. Leukocyte extravasation signaling

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### b. RhoA signaling

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<th>FDR (q-value)</th>
<th>Location</th>
<th>Type(s)</th>
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### c. Tight Junctions signaling

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Supplemental Figure I

A

Inj. STZ  Insulin implant  Insulin implant

0 2 4 6 8 10 12 14 16

Time (weeks)

Serum glucose check  Serum glucose check  Serum glucose check  Serum glucose check

Urine glucose check  Urine glucose check  Urine glucose check

B

Serum Glucose (mg/dL)

0 100 200 300

4 8 12 16

Weeks

*** *** ### ### ###

*** *** ### ###

*** *** ### ###

*** *** ### ###

*** *** ### ###