Online supplemental material

Material

Roswell Park Memorial Institute (RPMI) 1640 medium and Dulbecco's Modified Eagle's Medium (DMEM) were purchased from Thermo Fisher Scientific (Karlsruhe, Germany). Endothelial Cell Basal Medium was purchased from PromoCell (Heidelberg, Germany). FITC-labeled dextran 150,000, glycerine gelatine, Hoechst 33342, CY-09, neutral red solution, penicillin, rhodamine 6G, STZ and Tween20 were purchased from Sigma-Aldrich (Taufkirchen, Germany). Bovine serum albumin (BSA) was from Santa Cruz Biotechnology (Heidelberg, Germany). Linsitinib was purchased from Selleckchem (München, Germany). Cell lysis reagent QIAzol was from Qiagen (Hilden, Germany). The qScriber cDNA Synthesis Kit and ORA SEE qPCR Green ROX L Mix were from HighQu (Kraichtal, Germany). Collagenase NB 4G was purchased from SERVA GmbH (Heidelberg, Germany). Collagenase NB 8 Broad Range was purchased from Nordmark Biochemicals (Uetersen, Germany). HepatoQuick® and Annexin-V-Fluos Staining Kit were purchased from Roche (Basel, Switzerland). Matrigel was purchased from Corning (Wiesbaden, Germany). Polyvinylidene difluoride (PVDF) membrane was purchased from Bio-Rad (Feldkirchen, Germany). Accutase was purchased from BioLegend (Koblenz, Germany). Propidium iodide was purchased from BD Biosciences (San Jose, CA, USA). Calcein was purchased from Molecular Probes (Eugene, OR, USA). Hematoxylin was purchased from Morphisto (Offenbach am Main, Germany).

Antibodies

The anti-CD31 antibody (DIA310) was purchased from Dianova (Hamburg, Germany). The anti-GAPDH (sc-25778), the anti-MafA (sc-390491) and the anti-PDX-1 (sc-25403) antibodies were purchased from Santa Cruz Biotechnology (Heidelberg, Germany). The anti-insulin (Ab7842), anti-somatostatin (Ab30788), anti-glucagon (Ab92587), anti-MPO (ab9535), anti-CD3 (ab16669) and anti-CD68 (ab125212) antibodies were purchased from Abcam (Cambridge, UK). The anti-TXNIP antibody (D5F3E) and the anti-NLRP3 antibody (D4D8T) were purchased from Cell Signaling (Danvers, USA). The peroxidase-labeled anti-rabbit

antibody (NIF 824) and the peroxidase-labeled anti-mouse antibody (NIF 825) were purchased from GE Healthcare (Freiburg, Germany).

Ca^{2+} measurements

Min6 cells were seeded on coverslips and incubated for 24 h with vehicle or 10 µM of CY-09. Moreover, WT and *Nlrp3*$^{-/-}$ islets were seeded on coverslips. Then, the cells or islets were loaded with 1 µM Fura-2 AM for 40 min in Krebs Ringer buffer (KRB) without glucose. After incubation, the cells were washed with PBS twice and imaged using excitation 340/380 nm and emission 505 nm (Axio Observer 7; Zeiss, Oberkochen, Germany). The measurements were performed every 2 s over the entire observation period of 25 min. The protocol was divided in three steps: first, the cells or islets were cultivated in KRB without glucose followed by addition of high glucose (20 mM). Finally, 30 mM K^+ was added to depolarize the cells or islets. Delta glucose was calculated by averaging the Fura-2 ratio at min 18, subtracted by average of Fura-2 ratio before glucose addition in min 5.

Western Blot analysis

Hypoxic MIN6 cells exposed to CY-09 (10 µM) or vehicle (DMSO) for 24 h or hypoxic WT and *Nlrp3*$^{-/-}$ islets were harvested and whole cell extracts, cytoplasmic and nuclear extracts were generated as described previously in detail [1]. Protein extracts were then separated through a 12.5% SDS polyacrylamide gel and transferred onto a PVDF membrane. The membrane was incubated in 5% dry milk in phosphate-buffered saline (PBS) (0.1% Tween20) for 1 h and exposed to anti-PDX-1, anti-MafA, anti-TXNIP, anti-NLRP3, anti-GAPDH and anti-nucleolin antibodies, which were diluted (1:500) in PBS (0.1% Tween20) containing 1% dry milk. After incubation of the membrane with a peroxidase-coupled secondary antibody (anti-rabbit 1:2,000 or anti-mouse 1:2,000) for 1 h, the protein expression was visualized by the incubation of the membrane with enhanced chemoluminescence (ECL) Western blotting substrate (GE Healthcare) in a Chemocam device (Intas). The intensity of the measured signals was quantified using ImageJ software and normalized by the corresponding housekeeping protein.

Quantitative real time-polymerase chain reaction (qRT-PCR)

Total RNA from hypoxic MIN6 cells exposed to CY-09 (10 µM) or vehicle (DMSO) for 24 h or hypoxic *Nlrp3*$^{-/-}$ and WT islets were isolated using QIAzol lysis reagent (Qiagen). The corresponding cDNA was synthesized from 1 µg of total RNA by QuantiNova Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. ORA qPCR Green ROX L Mix (highQu) was used for qRT-PCR. The data analysis was performed by the MiniOpticon Real-Time PCR System (Bio-Rad). Murine GAPDH served as internal control for mRNA detection.

Forward and reverse primers were used in a concentration of 700 nM solved in RNase/DNase-free H_2O. Primer sequences for qPCR were coded as follows: Mouse Ins-1 forward 5′-AACAACTGGAGCTGGGAGGAAG-3′, reverse 5'-GGTGCAGCACTGATCCACAATG-3′; Mouse Ins-2 forward 5′-GCAGCACCTTTGTGGTTCC-3′, reverse 5'-CTTGTGGGTCCTCCACTTC-3′; Mouse GAPDH forward 5′-CGGTGCTGAGTATGTC-3′, reverse 5′-TTTGGCTCCACCCTTC-3′. Mouse VEGF-A forward 5′-GCTGTACCTCCACCATGCCAAG-3′, reverse 5′- CGCACTCCAGGGCTTCATCG -3′.

Intravital fluorescence microscopy

Dorsal skinfold chamber-equipped mice were anesthetized. Subsequently, they received a retrobulbary intravenous injection of 0.05 mL FITC-labeled dextran (5%) for plasma staining and 0.05 mL rhodamine 6G (2%) for the visualization of microvessel fenestration [2] on day 0 as well as 3, 6, 10 and 14 after islet transplantation. Thereafter, the dorsal skinfold chamber was positioned under a fluorescence microscope (Zeiss) with a 100 W mercury lamp attached to a blue (excitation wavelength: 450-490 nm/emission wavelength: >515 nm) and a green (530-560 nm/>585 nm) filter block. The microscopic data were recorded for off-line evaluation.

Microscopic images were analyzed off-line by the computer-assisted image analysis system CapImage (Zeintl, Heidelberg, Germany). The revascularized area represents the islet size in mm², which exhibits blood-perfused microvessels, and the rhodamine 6G-positive area (calculated by the ratio of the rhodamine 6G-positive area and the revascularized area on that

day in %) of islets were assessed as previously described [3, 4]. In addition, we measured the diameter (µm), centerline RBC velocity (µm/s) and volumetric blood flow (pL/s) of 4-8 individual microvessels within the grafts [3, 4]. Moreover, the take rate (%), i.e. the number of engrafted islets in relation to the number of transplanted islets per group on day 14, was determined.

Immunohistochemistry

For the preparation of histological sections, dorsal skinfold chamber-equipped mice were anesthetized and euthanized by a cervical dislocation. The dorsal skinfold chamber as well as pancreatic tissue were excised and fixed for 24 h in 4% formalin. In addition, isolated islets were incubated for 45 min at 37°C in 100 µL HepatoQuick®, 50 µL human citrate plasma and 10 µL 10% $CaCl_2$ solution. The resulting clot was also fixed for 24 h in 4% formalin. The formalin-fixed specimens were embedded in paraffin and 3-µm-thick sections were cut.

The sections were stained with antibodies against insulin (1:300), glucagon (1:300), somatostatin (1:300), CD31 (1:300), PDX-1 (1:300), MPO (1:300), CD68 (1:300) and CD3 (1:300) and visualized by their corresponding secondary antibodies. Cell nuclei were stained with Hoechst 33342 for fluorescence microscopy and with hematoxylin for bright field microscopy. The sections were analyzed by means of fluorescence microscopy (BX60F fluorescence microscope (Olympus)). The quantification of positively stained cells was done by FIJI software (NIH) and is given in % of all islet cells.

Insulin enzyme-linked immunosorbent assay (ELISA) and intraperitoneal glucose tolerance test (IPGTT)

The amount of secreted insulin was measured, as described previously in detail [5]. Briefly, 10 isolated islets were washed with KRB (115 mM NaCl, 4.7 mM KCl, 1.28 mM $CaCl_2$, 1.2 mM $MgSO_4$, 0.1% BSA) and incubated for 1 h at 37 °C and 5% CO_2. The supernatants were discarded and the islets were incubated for 30 min in KRB containing 16.5 mM glucose. The supernatants were collected and the amount of secreted insulin was determined by using an insulin ELISA kit according to the manufacturer's protocol.

The IPGTT was performed on day 28 after islet transplantation under the kidney capsule of diabetic mice. After 16 h of fasting, the mice were i.p. injected with a 10% glucose solution. The blood glucose levels were determined 0, 15, 30, 45, 60, 120, and 180 min after glucose injection from the tail vein and analyzed by a portable blood glucose monitoring system (GL50; Breuer). Additional mice were anesthetized 15 min after glucose injection and the blood samples were collected from the vena cava. The blood plasma was separated by centrifugation and stored at -80°C. The plasma insulin levels were analyzed by means of an insulin ELISA (Invitrogen, USA).

Isolation of MVF and spheroid sprouting assay

Mice were anesthetized and euthanized by a cervical dislocation. Subsequently, MVF were isolated by mechanic and enzymatic digestion (collagenase NB 4G) of epididymal fat pads of mice, as described previously in detail [6]. After isolation, MVF were cultivated in DMEM (10% (v/v) FCS, 100 U/mL penicillin and 0.1 mg/mL streptomycin) at 37 °C and 5% CO_2. MVF spheroids were generated by means of the liquid overlay technique in a 96-well plate covered with 1% agarose. Subsequently, 750 MVF were seeded per well and cultivated for 5 days to allow the formation of one spheroid at 37°C under a humidified 95% to 5% (v/v) mixture of air and CO_2. After 5 days, the spheroids were harvested. The angiogenic activity of MVF spheroids was determined by means of a sprouting assay, as previously described in detail [7].

References:

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