

MATERIAL/METHODS

Animals

To investigate the role of *Txnrd2* specifically in the endothelium, we crossed *Txnrd2*^{wt/lox} mice¹ with transgenic mice expressing the tamoxifen-inducible Cre recombinase CreERT under the control of the endothelial *Cdh5* promoter (a kind gift of Dr. Ralf Adams, MPI Münster)². Progeny was further crossed with *Txnrd2*^{lox/lox}, *Txnrd2*^{wt/lox}, and *Txnrd2*^{wt/wt} mice to result in offspring expressing *Txnrd2*^{fl/fl-Tg[Cdh5-CreERT]}, referred to as *Txnrd2*^{IECKO} upon tamoxifen exposure, and various control littermates, referred to as *Txnrd2*^{control} (Figure 1A). Primer pairs for genotyping are listed in the supplemental table 1. In order to delete *Txnrd2* in the endothelium of mice transgenic for *Cdh5-CreERT2*, Tamoxifen (T5648, Sigma-Aldrich, Deisenhofen, Germany) was administered intraperitoneally for five consecutive days to a total concentration of 10mg in medium-chain triglyceride-rich oil. At the time of Tamoxifen administration all mice were at least 8 weeks old. To control for tamoxifen-specific effects, control mice received the same treatment regimen. *Txnrd2* deletion was allowed to proceed for at least four weeks before experiments were performed. All mice were held under standard conditions with chow (ssniff, Soest, Germany) and water available *ad libitum* on a 12 hour light/dark cycle. Animal experiments were performed in agreement with the German Animal Welfare Law and were approved by the Institutional Committee on Animal Experimentation and the Government of Upper Bavaria.

Isolation of ECs from mouse tissue and confirmation of *Txnrd2* deletion by immunoblotting

In order to confirm the successful deletion of *Txnrd2* in the endothelium, kidneys were excised from *Txnrd2*^{IECKO} and *Txnrd2*^{control} mice, homogenized and digested with Collagenase A (2 mg/ml; #10103586001, Roche Diagnostics, Mannheim, Germany). ECs were labelled with a rat anti-mouse CD31 antibody (1:100; BM 4086, Acris Antibodies GmbH, Herford, Germany) and goat anti-rat IgG Micro Beads (20µl/10⁷ cells; No.130-048-501, Miltenyi Biotec, Bergisch Gladbach, Germany), and isolated using MACS LS columns (No. 130-042-401, Miltenyi Biotec) according to the manufacturer's recommendation. Isolated kidney ECs were subsequently lysed with RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% Sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8) supplemented with a phosphatase/protease inhibitor cocktail (#5872, New England Biolabs, Frankfurt am Main, Germany), and *Txnrd2* detected using a custom-made, monoclonal rat anti-mouse *Txnrd2* IgG by immunoblotting³. To validate that the deletion of *Txnrd2* is limited to ECs and does not affect the hematopoietic system, we analyzed bone marrow tissue as well as CD45-positive cells derived thereof for *Txnrd2* expression by PCR analysis. For isolation of CD45-expressing cells, the tissue was homogenized and digested as described above. Subsequently, cells were labelled with CD45 MicroBeads (Miltenyi Biotec, No. 130-052-301), and isolated using MACs L columns (Miltenyi Biotec) according to the manufacturer's instructions. Extraction of total RNA and reverse transcription into cDNA was performed as described previously⁴. RT-PCR was carried out using the primer pairs listed in the suppl. Table 2.

Femoral Artery Ligation

Unilateral ligation of the femoral artery was performed to induce peripheral angiogenesis (calf muscle) and arteriogenesis (adductor muscle) as described⁵. The contralateral hind-limb was sham-operated and served as an in-subject control.

Assessment of perfusion recovery

Hind-limb perfusion was measured by laser Doppler imaging (LDI) using the Moor LDI 5061 and Moor Software Version 3.01 (Moor Instruments, Remagen, Germany) as previously described⁶. The occluded (occ) to sham ratio of blood flow was calculated for each mouse and recovery observed for a total duration of seven or 21 days after which mice were sacrificed.

Quantification of angiogenesis and arteriogenesis and histology

Subsequent to the final LDI measurement, the descending aorta was catheterized in order to perfuse the hind-limbs with adenosine buffer (5 mg bovine serum albumin, 1 ml adenosine/ml PBS), and, if the tissue was to be embedded in paraffin, with a 4% paraformaldehyde solution. Adductor and calf muscles were excised and embedded in either paraffin or Tissue Tek (Sakura, Staufen, Germany) for cryo preservation, respectively.

Cryo-conserved calf muscles were sectioned with a cryostat (Cryotome HM 560, Microm, Walldorf, Germany) in 10 μm thick slices from the anterior, posterior and intermediate region of the muscle and fixed with ice cold acetone. Capillaries were detected by fluorescence microscopy (AxioPhot, Zeiss, Munich, Germany) after staining the sections with a rat anti-mouse CD31 antibody (Acris Antibodies) and a Cy3-conjugated secondary antibody (Dianova, Hamburg, Germany). Three distinct sites per slide were photographed (AxioCam, Zeiss) and vessel density assessed as CD31-positive area/total area using ImageJ (National Institutes of Health, USA).

Paraffin-embedded adductor muscles were cut into 5 μm thick slices with a microtome (Biocut 2030, Reichert-Jung, Leica Microsystems, Wetzlar, Germany). Slices were stained with haematoxylin and eosin (H&E) and collateral vessels at the medial region of the muscle, i.e. at the narrowest area of the vessels, were photographed at 400x magnification with a bright field microscope (BX41, Olympus, Hamburg, Germany) featuring a camera (Camedia C5050, Olympus). Inner and outer vessel circumference were measured with ImageJ and values employed to calculate wall area, wall thickness and lumen using standard circle formulas.

In order to characterize the observed luminal deposits within the adductor muscle as well as within renal glomeruli of Txnr2^{IECKO} mice, we stained paraffin-embedded sections either with Periodic acid–Schiff staining (PAS) and/or with antibodies against CD31, CD45, fibrin, ICAM, MCP1 and VCAM (listed in the supplemental table 3). After antigen retrieval and blocking of endogenous peroxidase activity, sections of paraformaldehyde-fixed and paraffin-embedded tissues were incubated with a rat anti-mouse CD45 antibody (1:50, #550539, BD Pharmingen, Heidelberg, Germany) and/or with a mouse anti-human fibrin II beta antibody (1:200, NYBT2G1, Accurate Chemical and Scientific Corporation, Westbury, NY, USA) and a mouse on mouse kit (Vector Laboratories, Burlingame, CA, USA). Using appropriate biotinylated secondary antibodies, binding was visualized with either 3,3'-diaminobenzidine (DAB) or 3-amino-9-ethylcarbazole (AEC) substrate (Vector Laboratories).

Isolation of mouse arteries and determination of flow-mediated relaxation

Three weeks following FAL, Txnr2^{IECKO} and Txnr2^{control} mice were euthanized by cervical dislocation, and femoral arteries dissected and cannulated as described previously⁷. The ends of both pipettes were connected to a silicone tube and a height-adjustable reservoir containing MOPS buffer (3 mM 3-morpholinopropanesulfonic acid, 145 mM NaCl, 4.7 mM KCl, 3 mM CaCl₂, 1.2 mM NaH₂PO₄, 2 mM Pyruvate, 0.02 mM EDTA, 5 mM Glucose, pH 7.4) via a three-way valve. Initially, the reservoir was set to a height representing a transmural pressure in the vessel of 60 mmHg.

Smooth muscle viability was confirmed prior to each experiment by constricting the vessel to at least 30 % with 300 nM of the thromboxane mimetic U46619 (Tocris, R&D Systems GmbH, Wiesbaden-Nordenstadt, Germany). When the constriction was stable, 10 μM acetylcholine (ACh, Sigma-Aldrich), was applied to obtain at least 90% of the initial diameter (endothelial viability test). 10 μM acetylcholine were chosen based on previous concentration response curves (10 nM-3 μM , n=5 vessels per group, data not shown) which indicated that (1) both groups did not differ in their response to acetylcholine and (2) 10 μM corresponded to the maximal pharmacologic effect. If the vessels did not meet these pre-specified criteria they were discarded. Smooth-muscle reactivity to nitric oxide was unaltered in KO vs WT vessels as verified by SNAP ((S)-Nitroso-N-acetylpenicillamine, Tocris, R&D Systems GmbH) concentration response curves (1-100 μM , data not shown).

With both three-way valves open, femoral arteries were pre-constricted to a level comparable to the *in vivo* situation by administration of 300 nM U46619 (Tocris). To assess flow-mediated dilation, the position of the inlet pressure reservoir was increased stepwise by 2.5 mmHg whereas the outlet reservoir was lowered by 2.5 mmHg. This led to an increase in the pressure difference of 5 mmHg per step while keeping the mean pressure in the artery constant⁸. We assessed pressure differences of 5, 10, 15, 20, 25 and 30 mmHg. At each pressure difference step, the resulting flow velocity was calculated by dividing the fluid volume that had travelled through the artery during by the duration each pressure step was maintained. The diameter changes were recorded via videomicroscopy and the dilation calculated as described elsewhere⁷.

After flow-mediated dilation, the arteries were incubated with 100 μ M N ω -Nitro-L-arginine methyl ester (L-NAME, Sigma-Aldrich) for 30 minutes at 37 °C and 60 mmHg with no flow. The flow-mediated dilation protocol was then repeated. At the end, the pressure difference was set back to zero again. After the vessel had re-constricted, 10 μ M acetylcholine was applied. The resulting dilation was compared to the endothelial viability test.

In a second set of experiments femoral arteries were pre-constricted with 300 nM U46619 and concentration response curves were constructed ranging from 10 nM to 10 μ M ACh before and after 30 min incubation with 100 μ M L-NAME and 1250 U/ml Catalase (Merck Millipore, Darmstadt, Germany). Catalase was present throughout the experiment.

Intravital microscopy on the mouse cremaster muscle

The analysis of the inflammatory properties of the vessel wall of Txnrd2^{control} and Txnrd2^{iECKO} mice was studied in the cremaster muscle. Four hours before commencing the surgical preparation, recombinant murine interleukin-1 (IL-1, R&D Systems, Abingdon, United Kingdom; 50 ng per animal, intrascrotal) was injected in male mice in order to induce inflammation and leukocyte recruitment to the cremaster muscle. The subsequent surgical preparation was performed as originally described by Baez⁹ with minor modifications¹⁰. Briefly, male mice were anaesthetized with an i.p. administration of ketamine/xylazine (100 mg/kg ketamine, 10 mg/kg xylazine). The right cremaster muscle was excised by a ventral incision of the scrotum, opened ventrally and spread over the pedestal of a custom-made microscopy stage. Leukocyte rolling, adhesion and transmigration were analyzed in a minimum of five unbranched sections of postcapillary venules (diameter between 21 and 36 μ m) per mouse during a 15 minute period by intravital microscopy (AxioTech-Vario 100 Microscope, Zeiss). Digital movie files were evaluated off-line using ImageJ software.

Isolation of embryonic endothelial progenitor cells (eEPCs)

Due to the low proliferation rates of adult ECs, we opted to investigate the effects of Txnrd2 depletion *in vitro* in eEPCs. Txnrd2^{+/+} (wildtype) and Txnrd2^{-/-} (knockout) eEPCs were isolated at embryonic day E7.75, as described¹¹. Briefly, pregnant females were sacrificed after mating of hemizygous Txnrd2 mice¹, and the embryonic body trunk was dissected and digested in Trypsin/EDTA at 37°C for 15-20 minutes. Subsequently, dissociated cells were cultured on a feeding layer of γ -irradiated, non-proliferating murine fibroblasts in high glucose DMEM (supplemented with 15% FCS, 1% L-Glutamine, 1% Penicillin-Spreptomycin, 1% non-essential amino acids 100x, 2% HEPES, and 0.03% β -Mercaptoethanol (50 mM)) at 37°C, 5% CO₂ and 5% O₂ until cobblestone-like cell colonies emerged. Upon reaching confluence, cells were grown on 10 cm dishes covered with γ -irradiated, non-proliferating murine fibroblasts for two passages after which they could be cultured in gelatin-coated (0.1%) dishes in the absence of a feeding layer. Correct gene expression was confirmed by RT-PCR with the primer pairs listed in supplemental table 2. Due to the reducing activity of the thiol β -Mercaptoethanol, the culture media was replaced on four consecutive days with β -Mercaptoethanol-free DMEM prior to any experiment. For reconstitution experiments, mouse wildtype Txnrd2 was cloned into a bicistronic lentiviral vector as described previously¹² and stably expressed in Txnrd2^{-/-} eEPCs. Expression of an empty vector served as control.

Tube formation assay

To verify whether the observed restriction of post-ischemic angiogenesis can also be observed *in vitro*, we tested eEPCs in a tube formation assay. BD Matrigel® (Becton Dickinson) served as extracellular matrix and the assay was performed as described elsewhere¹³. In brief, wildtype and Txnrd2 deficient cells (30.000 cells per each well of a 24-well culture plate) were grown for 24h on Matrigel. Subsequently, pictures were taken from each well using a microscope (Olympus IX50, 10 x objective) and a digital camera (Canon IXUS 55). The number of branching points was determined by manual counting.

Flow cytometry

eEPCs were cultivated as above. 10^6 cells were loaded with 5 μM of the ROS-sensor CellROX™ (Invitrogen, Life Technologies, Karlsruhe, Germany) and incubated for 30 minutes according to the manufacturer's recommendations. After three successive washes, cells were harvested and ROS measured using a Gallios 2/8 flow cytometer (Becton Coulter, Krefeld, Germany) at an excitation wave length of 644 nm and emission collected at 665 nm. Values for fluorescence were corrected for autofluorescence using unloaded controls. For the assessment of the mitochondrial membrane potential ($\Delta\Psi\text{m}$), 10^6 eEPCs were incubated with 2 μM of the MitoProbe™ JC-1 Assay Kit (Invitrogen) for 30 minutes according to the manufacturer's guidelines. The $\Delta\Psi\text{m}$ was detected by flow cytometry with an excitation wavelength of 488 nm and emission at 590 nm (J-aggregates) and 529 nm (J-monomers). Prior incubation with the potential disruptor carbonyl cyanide 3-chlorophenylhydrazone (CCCP) served as positive control.

Immunoblotting

Cells were lysed with RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% Sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8), supplemented with a phosphatase/protease inhibitor cocktail (#5872, New England Biolabs) and immunoblotting was performed as described before¹⁴. All antibodies used are listed in the supplemental Table 4. If necessary, blots were stripped (15 g glycine, 1 g SDS, 10 mL Tween 20 per liter of H₂O, pH 2.2) and reprobed. Finally, equal loading was determined by probing for either β -actin or Tubulin. Semi-quantitative analysis was achieved using ImageJ software.

Statistical Analysis

Statistical analysis was performed using SigmaStat software v11 (Jandel GmbH, Erkrath, Germany) and results expressed as mean \pm SEM unless stated otherwise. Normally distributed values were compared with a student's t-test, one-way ANOVA or two-way repeated measures ANOVA followed by Holm-Sidak multiple comparison. Non-normally distributed data was compared with the Mann-Whitney Rank Sum Test or a Kruskal-Wallis one-way ANOVA on Ranks followed by Dunn's multiple comparison. Flow-dependent dilation values were fitted to a 3-parameter Gompertz function and displayed with their 95% confidence intervals. Statistical significance of the individual regression curves was calculated as described elsewhere¹⁵. Acetylcholine concentration response curves were fitted to a 3-parameter Hill equation. Differences were computed by two way ANOVA with subsequent Tukey test. P values < 0.05 were considered significant. One asterisk (*) in a figure denotes P values < 0.05, two asterisks (**) denote P values < 0.01, and three asterisks (***) denote P values < 0.001.

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