Supplementary material

Coronary Serum Obtained After Myocardial Infarction Induces Angiogenesis and Microvascular Obstruction Repair. Role of Hypoxia-inducible Factor-1A

Experimental Protocol

As described previously,[1,2] during the surgery protocol, animals were mechanically ventilated employing a 50% oxygen gas mixture, and heart rate, rhythm, and ST-segment changes were continuously monitored. In short, intramuscular ketamine (8 mg/kg) and medetomidine (0.1 mg/kg) for sedation and continuous intravenous 10 mg/kg/h infusion of 2% propofol for anesthesia induction were administrated. Furthermore, to diminish life-threatening arrhythmias, the pigs were pretreated with intravenous amiodarone (300 mg) and lidocaine (30 mg). A 7-Fr sheath was introduced into the right femoral artery to monitor blood pressure and to access the proximal left anterior descending coronary artery. A 7-Fr Amplatz Left 0.75 catheter was used to selectively engage the proximal left anterior descending and a standard hydrophilic angioplasty wire was advanced and placed in the distal left anterior descending. A 2.5 mm x 15 mm over-the-wire angioplasty balloon was inflated at 6-atm in the midleft anterior descending distal to the first diagonal branch. Coronary artery occlusion was confirmed by contrast injection and by electrocardiographic ST-segment elevation.

Blood samples were collected from a transjugular 7-Fr multipurpose catheter placed in the coronary sinus (coronary veins) before balloon inflation, after 5 minutes and 90 minutes of ischemia (immediately before reperfusion) as well as 1 minute, 1 week and 1 month after reperfusion. Coronary serum obtained

from the coronary veins was obtained by centrifuging blood samples at 2500 rpm for 15 minutes and stored immediately at -80°C until further analyses were performed.

To determine the presence and extent of microvascular obstruction in the area at risk, 20 mL of 4% thioflavin-S solution (Sigma Aldrich, MO, USA) was infused through the lumen of the inflated over-the-wire balloon immediately before sacrifice. In the no-reperfusion group (n = 5) thioflavin-S was administered at the end of the 90-minute ischemia period. In the 1-minute, 1-week and 1-month after reperfusion groups (n = 5 each), prior to the sacrifice of the animal, the angioplasty balloon was reinflated at the same location used to induce the infarction (midleft anterior descending) and the colorant was injected through the lumen of the inflated balloon. Thereafter, the heart was arrested with potassium chloride and excised.

Macroscopic Study of Myocardial Samples

Once the heart was excised, the left ventricle was sectioned into 5-mm thick short-axis slices. First, to determine the area at risk and the extent of microvascular obstruction, each slide was viewed under ultraviolet light and photographed. Light blue represents the thioflavin-S myocardial uptake after infusion into the area at risk, while dark blue indicates lack of perfusion (Figure 1A, upper panel). Microvascular obstruction was defined as dark blue zones in the core of the area at risk and was expressed as the percentage of the area at risk.

Second, to determine the extent of the infarcted area, slices were incubated in 2% 2,3,5-triphenyltetrazolium chloride (Sigma Aldrich, MO, USA) solution at 37ºC for 20 minutes. Afterward, they were viewed under room light and photographed (Figure 1A, lower panel). The infarct area was defined as the triphenyltetrazolium-negative myocardial region and was expressed as the percentage of the left ventricle volume.

After digitizing the images, manual offline quantification of all short-axis slices was performed in a dedicated laboratory by a trained observer unaware of the experimental protocol applied. All numerical data analysis was performed using the software package MATLAB 8.4 (The MathWorks Inc, Natick, MA, USA). The predefined slice thickness (5 mm) and the presence of a ruler beside heart slices in all images were required to calculate left ventricular myocardial volumes.

In all short-axis slices, the infarcted and remote areas within the left ventricle were defined. The infarcted area was regarded as the myocardium located in the area at risk that did not stain with triphenyltetrazolium (thioflavin-S+ and triphenyltetrazolium-). The remote area was considered as the region outside the area at risk (thioflavin-S- and triphenyltetrazolium+).

Microscopic Analysis and Immunohistochemistry of Myocardial Samples

Tissue samples from infarcted and remote areas were fixed in 4% paraformaldehyde acid, embedded in paraffin, and stained with hematoxylin-eosin (Sigma Aldrich, MO, USA) for histological analysis (Figure 2A, upper panel). To further characterize swine tissue, fibrosis was detected by staining myocardial samples with 0.1% Direct Red 80 (Sigma Aldrich, MO, USA) in saturated picric acid[2] and photographed at 20 x magnification in an optic microscope Leica DMD 108 using a polarized filter that permitted the visualization of collagen (Leica Microsystems, Wetzlar, Germany) (Figure 2A, middle panel).

For immunohistochemistry, representative sections (5 μm) of paraffin tissues from all 5 experimental groups were selected. After peroxidase inactivation (H_2O_2 0.3%) and blockade with horse serum, sections were incubated overnight (4ºC) with the following primary antibodies; a rabbit antiporcine CD31 (dilution 1:100, ab28364, Abcam, Cambridge, UK) (Figure 2A, lower panel) was used to identify microvessel density and a mouse antiporcine *HIF-1A* (dilution 1:100, ab16066, Abcam, Cambridge, UK) (Figure 4B) was applied to assess *HIF-1A* expression. Amplification of the primary antibody signal was carried out by a 45-

minute incubation with a biotin-conjugated goat antirabbit secondary antibody (1:1000 dilution, Dako, Glostrup, Denmark) or biotin-conjugated goat antimouse secondary antibody (1:500 dilution, Dako, Glostrup, Denmark).[3,4] Photographs at 20 x magnification were taken in independent field. Microvessel formation was determined by counting $CD31^+$ microvessels in high power field and expressed as $CD31^+$ vessels per mm^2 using a phase contrast microscope (Axio Observer A1, Carl Zeiss, NY, USA). Scoring was performed blinded on coded slides.

Quantification of Hypoxia-inducible Factor-1A in Serum and in Myocardial Tissue

HIF-1A levels were determined in the 5 experimental groups in coronary serum obtained from coronary sinus blood samples using a commercial sandwich enzyme-like immunoassay, following the manufacturer's instructions (Elabscience Biotechnology Co, Bethesda, MD, USA). Absorbance was measured at 450 nm and the data were processed by GraphPad Prism software. Results are expressed as pg/mL serum.

HIF-1A mRNA expression was also assessed in tissue samples from the infarcted and remote areas isolated from the 5 experimental groups. To extract RNA, RNeasy Plus Mini Kit (QIAGEN GmbH, Hilden, Germany) was employed following the manufacturer's instructions. *HIF-1A* gene expression was determined by real-time polymerase chain reaction using a 7900HT Fast Real-Time Polymerase Chain Reaction System (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA). The values of the threshold cycle (Ct) were calculated and normalized to the housekeeping gene 18S ribosomal RNA. We used specific primers predesigned by Applied Biosystems (Thermo Fisher Scientific, Waltham, MA, USA) for analysis of porcine *HIF-1A* (Ss03390447_m1) and the endogenous ribosomal 18S (4319413E).

Porcine Coronary Artery Endothelial Cells Culture

Porcine coronary artery endothelial cells (Cell Applications, Inc, San Diego, CA) were cultured in porcine endothelial growth medium (Cell Applications, Inc, San Diego, CA). The cells were routinely grown in a humidified atmosphere (21% O_2) at 37°C and 5% carbon dioxide (CO_2). All experiments were performed in endothelial cells at passage 3.

Coronary Endothelial Cell Differentiation Assay. Functional Analysis

Growth factor reduced matrigel (100 μl, BD Biosciences, Madrid, Spain) was pipetted into 96 culture wells and polymerized for 30 minutes at 37°C, as described previously.[3,4] Porcine coronary artery endothelial cells were seeded on matrigel 30 x 10^3 cells/well and incubated for 6 hours with DMEM medium containing 10% coronary serum drawn from coronary veins of controls and at different times of ischemia and reperfusion: after 5-minute and 90-minute ofischemia (immediately before reperfusion) as well as 1 minute, 1 week and 1 month after reperfusion.

Thereafter we aimed to investigate whether *HIF-1A* blockade attenuates the angiogenic response induced by porcine serum on the coronary endothelial cell differentiation assay. For this purpose, some samples were incubated in the presence of a mouse monoclonal antiporcine *HIF-1A* antibody (10 μg/mL) (ThermoFisher Scientific, Waltham, MA, USA) or irrelevant isotype and concentration-matched IgG (R&D System). Phase contrast micrographs were recorded (Axio Observer A1, Carl Zeiss microscope, Germany) and the mean number of tubes in 5 low-power (× 100) random fields were quantified and used as a surrogate of the angiogenic effect induced on the coronary endothelial cell differentiation assay by the coronary sinus serum obtained from experiments (Figure 3A). An investigator blinded to treatments performed scoring of tubulogenesis.

Statistical Analysis

Continuous variables are expressed as the mean ± standard deviation. One-way ANOVA analysis was used for comparisons and statistical significance was considered for 2-tailed *P*-values < .05. SPSS 19.0 (SPSS, Inc, Chicago, IL, USA) was used throughout.

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