Supplementary Material

Surgical Preparation and Electrocardiography

Animals were anaesthetized with subcutaneous urethane (1.2 g/kg birth weight, Sigma, St. Louis, Missouri, United States). The body temperature was maintained at 37°C with an infrared light. The trachea was exposed via a midline cervical incision and intubated. One piece of PE-10 tubing was put in place in the jugular vein. A mouse’s trachea was intubated for artificial ventilation (Small Animal Ventilator Model 683, Harvard Apparatus, Holliston, Michigan, United States) at 50 breaths/min with a tidal volume of 8 mL/kg and a positive end-expiratory pressure of 5 cm H2O. Right front leg, left front leg and left hind leg limb leads were attached. The electrocardiography waveforms were recorded with an iWorx 214 data recorder (iWorx Systems, Dover, New Hampshire, United States).

Induction of Myocardial Ischaemia/Reperfusion

Under anesthesia, midline sternotomy was performed followed by peri-cardiotomy. The left anterior descending artery close to its origin, approximately 3 mm away from the left coronary ostium, was ligated with 7-O Prolene (Ethicon Inc, Somerville, New Jersey, United States) and a slipknot was tied to establish reversible coronary artery occlusion as described previously. After 1 hour of ischaemia, the slipknot was released, and the heart was reperfused for 4 hours. After this experiment, the anaesthetized animals were sacrificed with intravenous KCl.

Heart Micro-Circulation Determination

To examine the in vivo response of coronary arterial constriction to vasoactive agents and myocardial ischaemia/reperfusion, a full-field laser perfusion imager (MoorFLPI, Moor Instruments Ltd., Devon, UK) was used to continuously quantitate the cardiac micro-circulatory blood flow intensity. In brief, the imager used laser speckle contrast imaging, which would exploit the random speckle pattern generated when tissue was illuminated by laser light. The random speckle pattern changed when blood cells moved within the region of interest (ROI). When there was a high level of movement (fast flow), the changing pattern became more blurred, and the contrast in that region reduced accordingly. The contrast image was processed to obtain a 16-colour coded image that correlated with blood flow in the heart. Blue was defined as low flow and red as high flow. The micro-circulatory blood flow intensity of each ROI was recorded as flux with perfusion unit, which was related to the product of average speed and concentration of moving red blood cells in the heart sample volume. The negative control value was set at 0 perfusion unit (blue colour) and the positive value was at 1,000 perfusion unit (red colour). The perfusion units were real-time analysed by the MoorFLPI software version 3.0.

Coronary Effluent Thromboxane B$_2$, Nitric Oxide and Superoxide Anion Measurements

We determined the coronary effluent thromboxane B$_2$ (TXB$_2$), nitric oxide (NO) and superoxide anion (O$_2^-$) in response to myocardial ischaemia/reperfusion ex vivo. After injecting heparin (1,000 IU/kg intraperitoneally) and urethane, the hearts from four groups of mice (n = 6 each group) were rapidly excised through a midline sternotomy, and the aorta was catheterized. Retrograde perfusion was performed at 37°C from a reservoir 900 mm above the aortic cannula and perfused with non-recirculating Krebs–Henseleit solution. The perfusate was saturated with a gas mixture of 95% O$_2$ and 5% CO$_2$. To explore the myocardial ischaemia/reperfusion effects on TXA$_2$, NO and O$_2^-$ release, stable thromboxane A$_2$ metabolite, TXB$_2$, NO and O$_2^-$ concentrations in the coronary effluent were measured before and after myocardial ischaemia/reperfusion. Hearts subjected to myocardial ischaemia/reperfusion underwent 30 minutes of baseline perfusion, 60 minutes of global ischaemia and 60 minutes of reperfusion. TXB$_2$ concentrations in the coronary effluent of perfused mice hearts were measured using a commercially available enzyme immunoassay (Thromboxane B$_2$ ELISA kit 519031, Cayman Chemical, Ann Arbor, Michigan, United States). Effluent samples were collected and stored at −70°C until TXB$_2$ was measured. Some effluent samples were used to detect NO by a modified luminal-amplified chemiluminescent method and the O$_2^-$ amount was assessed by a lucigenin-amplified chemiluminescent method.

Platelet Adhesiveness to the Mesenteric Artery In Vivo

Whole blood was collected from the dorsal vein and heart of donor B6 mice by puncture in 1.5-mL polypropylene tubes containing 300 μL of heparin (30 U/mL). Platelet-rich plasma was obtained by centrifugation for 5 minutes at 1,200 rpm and 4°C. Plasma and buffy coat containing some red blood cells was transferred to fresh polypropylene tubes and re-centrifuged at 1,200 rpm. The platelet-rich plasma was transferred to fresh tubes containing 2 μL of prostacyclin (PGI$_2$) (2 μg/mL) and incubated for 5 minutes at 37°C. Then, the pellets were re-suspended in Tyrode-Hepes buffer (137 mM NaCl, 0.3 mM Na$_2$HPO$_4$, 2 mM KCl, 12 mM NaHCO$_3$, 5 mM Hepes, 5 mM glucose and 0.35% bovine serum albumin) containing 2 μL PGI$_2$ and were centrifuged for 5 minutes at 2,800 rpm and 4°C. After centrifugation, the pellets were incubated for 5 minutes at 37°C. To remove the PGI$_2$, two washing steps were conducted, and platelets were fluorescently labelled with 2.5 μg/mL calcein AM (catalogue no. 1674595; Life Technologies, Waltham, Massachusetts, United States) for 10 minutes at room temperature and then stored at −20°C. The fluorescently labelled platelets (PFs) were injected into test mice through the PE-10-catheterized jugular vein. Aspirin (Sigma Aldrich, Milan, Italy) was dissolved in water (2 mg/mL) and administered by oral gavage at the dose of 10 mg/kg/mouse (corresponding to the dose of 75 mg for humans, using the body surface area normalization method) 60 minutes before PFs injection. Two hundred seconds after injecting PFs, we applied 10 μL FeCl$_3$ particles to the mesenteric artery (which were ~200–300 μm in diameter) to induce an acute artery thrombosis with detection by fluorescent dissecting microscopy (Leica DMLFA; Wetzar, Hessen, Germany).
Infarct Size Calculation

After myocardial injury, 1 mL of methylene blue was injected via the jugular vein catheter, and the heart was harvested 2 minutes later. The heart was sliced from the base to the apex at 3-mm intervals. The right ventricle was removed from each slice, and the remaining left ventricle in each slice was weighed. Slices were incubated for 20 minutes at 37°C in 1% triphenyltetrazolium chloride (Sigma) to distinguish the infarct (pale) from the viable (red) myocardial area. Heart slices were photographed with a digital camera (Nikon, Tokyo, Japan).

Statistical Analysis

All data are expressed as the mean ± standard error of the mean. Differences within groups were evaluated by a paired t-test. A one-way analysis of variance was used to compare differences among groups. Inter-group comparisons were made with Duncan’s multiple-range test. Differences were regarded as significant at \( p < 0.05 \). Graphs were prepared using Sigma Plot 12.0. All statistical analyses were performed using the SPSS software system (SPSS, Chicago, Illinois, United States).

References