

Ivabradine induces cardiac protection by preventing cardiogenic shock-induced extracellular matrix degradation

SUPPLEMENTARY DATA

Supplementary figure 1

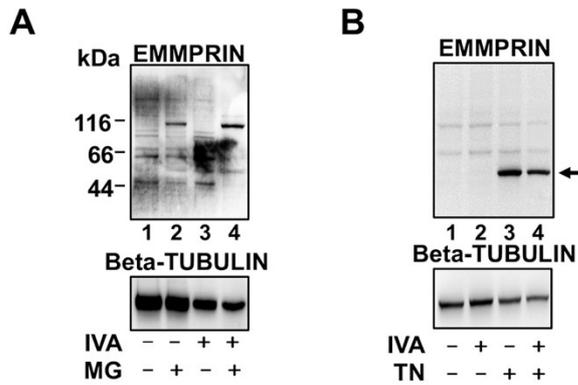


Figure 1. Expression of EMMPRIN in HL1B cells under hypoxic conditions. A: immunoblotting of EMMPRIN in HL1B cardiac cells in the presence of the proteasome inhibitor MG-132 showing no difference in the high-molecular-weight form of EMMPRIN in response to MG-132. B: immunoblotting of EMMPRIN in HL1B cardiac cells in the presence of 5 mg/mL of the N-glycosylation inhibitor tunicamycin showing no difference in the low-molecular-weight 20-kDa native nonglycosylated EMMPRIN form in response to ivabradine or placebo (arrow).

Supplementary data: METHODS

Reagents

Hematoxylin & eosin, Masson's trichrome staining reagents, TTC, tunicamycin D, actinomycin D, and fetal bovine serum were from Sigma (Spain). Horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibody and liquid 3,3'-diaminobenzidine (DAB) substrate were from Dako (United States). Anti- β -tubulin (ab6046), anti-EMMPRIN (ab64616), and anti-caveolin-3 (ab30750) for immunoprecipitation and anti-HCN4 (ab66501) and anti-caveolin-3 (ab87770) for western blot were from Abcam (United Kingdom). Anti-MMP9 (sc10737), anti-EMMPRIN (sc53064), and anti-HCN4 (sc58622) for immunofluorescence, immunochemistry, and western blotting were from Santa Cruz (United States). Conjugated secondary antibodies were from Thermo Fisher (United States). HRP-conjugated anti-rabbit and anti-mouse secondary antibodies were from Sigma-Aldrich. The chemiluminescence ECL detection kit was from GE (GE Healthcare Life Sciences, Spain). Centrifugation concentrators were from Sartorius (Fischer Scientific, Spain). Ivabradine was from Servier (Spain).

Evans blue/TTC staining

Myocardial infarction extension was evaluated by perfusion with 5% Evans blue (in serum) and TTC staining. Briefly, by day 7, a catheter was inflated at the same position as in day 0 to avoid Evans blue perfusion downstream to the area at risk and a pigtail catheter was inserted from the femoral artery up to the left ventricle for Evans blue perfusion into the systemic circulation. One minute after perfusion, the animals were sacrificed by injection of a potassium chloride solution, and the hearts were then isolated, washed 3 times with saline buffer, frozen for 2 hours at -20°C , and chopped into 0.5-cm slices from base to apex (8 slices/heart). The slices were incubated with 1% TTC dye dissolved in saline buffer for 20 minutes at 37°C and then washed with 10% paraformaldehyde. Images were acquired with ImageJ software to differentiate healthy areas (dark blue), the area at risk (red), and the pale necrotic area (white). The area of necrosis was calculated as a percentage of the area at risk.

Cardiac ultrasound

Pig hearts were visualized by echocardiography using a Vivid Q ultrasound system from GE Healthcare (United States) equipped with a 1.9 to 4.0-MHz scan head. In anesthetized animals, short- and long-axis images of the heart were recorded in B- and M-mode. From these recordings, the following parameters were determined using the on-site cardiac software package: endsystolic and diastolic interventricular septum thickness, endsystolic and diastolic left ventricular internal diameter, endsystolic and diastolic left ventricular posterior wall thickness, left ventricular ejection fraction, left ventricular shortening fraction, heart rate, cardiac output, and stroke volume.

Cells

Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 50 mg/mL penicillin, and 50 mg/mL streptomycin (Invitrogen, United States) and incubated at 37°C in a humidified atmosphere of 5% carbon dioxide and 95% oxygen. Cells were cultured under hypoxic conditions in hypoxia incubator chambers in a humidified atmosphere containing 1% oxygen, 5% carbon dioxide, and 94% nitrogen. Reoxygenation was performed by growing cells in fresh medium in the above humidified atmosphere.

RNA isolation and quantitative real-time polymerase chain reaction

Total RNA was extracted with TRIzol as previously described (reference 8 of the main text, and see below). First-strand cDNA was synthesized from 1 µg of total RNA in a 20-µl reaction mixture using the High-Capacity cDNA Achieve kit according to the manufacturer's instructions (Thermo Fisher). The following primers were used for quantitative real-time polymerase chain reaction:

EMMPRIN-Forward: 5'-GGC ACC ATC GTA ACC TCT GT-3'

EMMPRIN-Reverse: 5'-CAC TGG CGT GTT CCG ATT TC-3'

β-actin-Forward: 5'-CTT AGT TGC GTT ACA CCC TTT CT-3'

β -actin-Reverse: 5'-CTG TCA CCT TCA CCG TTC CAG TT-3'.

Three replicates were performed for each experimental condition, and differences were assessed with a 2-tailed *t* test. Results were normalized using the housekeeping gene β -actin and the $\Delta\Delta$ cycle threshold method and are expressed in arbitrary units.

REFERENCES

References cited in this supplementary data, with the citation number of the main text reference list.

8. Yu Y, Hu Z, Li B, Wang Z, Chen S. Ivabradine improved left ventricular function and pressure overload-induced cardiomyocyte apoptosis in a transverse aortic constriction mouse model. *Mol Cell Biochem.* 2019;450:25–34.