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Isodunnianol alleviates doxorubicin-induced myocardial injury by activating protective autophagy

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Supporting information

Cell viability assay

The H9C2 Cells were seeded at 10^4 cells/well into 96-well plates and incubated overnight, then treated with different concentrations of DOX for desired time. The medium of each well was replaced by 200 μL fresh medium and 20 μL freshly prepared MTT (5 mg/mL in PBS) and the plate was incubated in the dark at 37 ℃ with shaking for 15min. The culture medium was then removed and 200 μL DMSO

was added to each well to dissolve formazan crystals, and absorbance was read at 570 nm using a microplate reader. All assays were performed in triplicate.

Western blot analysis

The protein extracts from H9C2 cells or cardiac muscle tissue were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane. Membranes were blocked with TBST containing 5% fat free milk for 1h and incubated with the different primary antibodies as indicated overnight at 4 °C. The membranes were then incubated with horseradish peroxidase conjugated secondary antibodies for 1h and visualized by the enhanced chemiluminescence system. The quantitative analyses were conducted by ImageJ software.

Flow cytometry

Flow cytometry was used to determine the apoptosis rate among cells. Apoptotic cells rate were detected by the combined application of Annexin V-FITC and PI. Cells were washed twice and adjusted to a concentration of 1×10^6 cells/mL with cold Hanks buffer. Then, the cells were incubated with Annexin V-FITC and PI for 15 min at room temperature in the dark. Finally, the apoptotic cell rates were determined using flow cytometry and data were analyzed by FlowJo software. Each experiment was performed at least in triplicate.

GFP-LC3 transfection and immunofluorescence staining

H9C2 cells were transfected with a peGFP-LC3 plasmid. Transfected cells were treated with DOX, isodunnianol, EBSS culture or their combination. Transfected

cultures were observed using fluorescence microscopy (Axio Observer A1, Zeiss, Germany) and cells containing GFP-LC3 puncta were defined as Autophagy-positive cells. At least 100 cells per condition were counted and the percentage of autophagy-positive cells was reported.

Transmission electron microscopy

H9C2 cells treated with 2.0 μM isodunnianol for 24 h were collected and washed with 4℃ PBS, followed by fixing with 4% glutaraldehyde in 0.1 M sodium cacodylate for 2 h. Next, the samples were post-fixed with 1% OsO4 for 1.5 h, washed, dehydrated and embedded in Epon-Araldite resin. Ultrathin sections (80 nm) cut in a Reichert ultramicrotome were stained with 3% aqueous uranyl acetate for 1 h, and counterstained with 0.3% lead citrate. Samples were examined by transmission electron microscopy (HT7700, Hitachi, Japan).