Molecular BioSystems

Supplementary information

Title:

Hydrogen sulfide donor micelles protect cardiomyocytes from ischemic cell death

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1. Materials and Methods

PAM-PADT polymer synthesis

The PAM-PADT block polymer was prepared as shown in Supplementary Scheme 1 by reacting block copolymer (**1**)[1] with (**2**)[2] using dicyclohexylcarbodiimide/*N*-hydroxysuccinimide coupling chemistry following the procedure previously reported.[3]

Micelle formation of PAM-PADT

A DMF solution of PAM-PADT (50 mg/mL) was added drop wise to endotoxin-free water (1:9 v/v DMF/H_2O) under vigorous stirring at room temperature. After stirring for 30 min, the solution was transferred to a Slide-A-Lyzer G2 (MWCO 2 kDa) and dialyzed against milliQ water. The micelle solution was then sterile filtered in the cell culture hood.

Dynamic light scattering (DLS)

The hydrodynamic diameter of the micelles was obtained on an Otsuka ELSZ machine. The mean diameter (Z-average) and polydispersity index (PDI=μ_2/Γ^2) were calculated by the cumulant method.

Primary culture of neonatal rat ventricular myocytes

All animal experiments were performed in compliance with the relevant laws and institutional guidelines, and were approved by the Animal Care and Use Committee of Mukogawa Women's University. Primary cultures of ventricular cardiac myocytes from 1- to 2-day-old Wistar rats (Japan SLC, Hamamatsu, Japan) were prepared according to a previously described procedure[4-8]. This procedure yielded cell preparations containing 90 - 95% myocytes, as assessed by microscopic observation of cell beating. The myocytes were seeded in 96-, 48-, or 24-well microplates at a density ~80% confluence and were cultured for 3 days in Dulbecco's modified Eagle's medium (DMEM)/F-12 (Invitrogen, Carlsbad, CA, USA) containing 5% defined newborn calf serum (Invitrogen), pyruvic acid (3 μM) (Nacalai Tesque, Kyoto, Japan), insulin (5 μg/mL), transferrin (5 μg/mL), selenium (5 ng/mL) (Roche Diagnostics, Mannheim, Germany), and 5-bromo-2-deoxyuridine (100 μM) (Nacalai Tesque) to prevent the proliferation of nonmyocytes. The culture medium was replaced daily.

Ischemia model

To mimic hypoxic conditions for *in vitro* research, the culture medium was replaced to fresh culture medium, and the cells were exposed to an anaerobic environment prepared using an Anaeropack system (Mitsubishi Gas Chemical Company, Inc., Tokyo, Japan) at 37 °C for 24-48 hr. During this period, the concentration of O_2 in the medium was 0.02% and that of CO_2 was 5%. NaHS, ADT-OH, and ADT micelles were added to medium at the same time as ischemic stimulation. As a control, cells were incubated under normoxic conditions at 37 °C in culture medium, which was equilibrated with a 5% CO_2-95% air atmosphere.

Cell viability assay

Primary cultured rat cardiomyocytes were grown in a 96-well microplate at 80% confluency. After ischemic experiments, cell beating was observed by using a microscopy system (Nikon, Tokyo, Japan) and cell viability was evaluated by using a CellTiter 96® Non-Radioactive Cell Proliferation Assay based on the cellular conversion of a tetrazolium salts into a formazan product according to the manufacturer's instructions (Promega, Madison, WI, USA). The medium was replaced with fresh medium containing a tetrazolium compound and an electron coupling reagent, and then incubated for 2 hr at 37 °C. The quantity of formazan products was measured by the absorbance at 490 nm using a 96-well microplate reader (Bio-Rad Laboratories, San Jose, CA, USA). The experiments for cell viability were performed in triplicate and repeated at least 3 times. The number of normoxia control cells was 1.4×10^4 cells and the 490 nm absorbance reading is directly proportional to the number of the cells.

Evaluation of ischemia-induced apoptosis

Apoptosis was monitored by quantification of apoptotic nuclei (fragmentation and condensation of nuclei), which were blocked by a cell-permeable and irreversible pan-caspase *inhibitor*, zVAD-fmk (Merck Millipore, Darmstadt, Germany)[4,5]. Primary cultured rat cardiomyocytes were grown in a 12-well microplate at 80% confluency (1.6×10^5 cells). After ischemic experiments, to visualize apoptotic nuclei, the cells were rinsed twice in PBS and stained with Hoechst 33342 (5 μM) (Sigma-Aldrich) for 15 min at room temperature. Fluorescence images were captured using a fluorescent microscopy system (Nikon, Tokyo, Japan). More than 100 nuclei were counted for each sample and repeated at least 3 different primary culture preparations. The percentage of apoptotic nuclei was calculated as the ratio of fragmentation and condensation of nuclei to the total amount of nuclei. Further evaluation of apoptosis was performed with staining by a FITC-labelled Annexin V (x 1/20; Molecular Probes,

Inc., Eugene, OR, USA) to detect phosphatidylserine residues on the surface of apoptotic cells. The cells were washed with PBS and the fluorescence intensities of the cells were analyzed using the Tali Image-Based Cytometer (Invitrogen).

H_2S determination by fluorescent dye (WSP-1 assay)

Primary cultured rat cardiomyocytes at 80% confluency (12-well microplate at 80% confluency (1.6×10^5 cells)) were lysed with CelLytic™ M (Sigma-Aldrich, St. Louis, MO, USA) and centrifuged at 15,000 rpm for 15 min at 4°C. Measurement of H_2S release in cell lysate and serum were performed as described previously[9]. Cell lysate (1.8×10^5 cells) /phosphate-buffered saline (PBS, pH 7.4) or newborn calf serum (10%) /PBS (pH 7.4) containing 10 μM WSP-1 and 8.9 μM cetyl trimethyl ammonium bromide (Sigma-Aldrich) were prepared. The mixture solutions were added NaHS (Wako Pure Chemical Industries, Osaka, Japan), ADT-OH (Cayman Chemical Company, Ann Arbor, MI, USA), and ADT micelles at final concentration of 50 μM as H_2S donor. Cell lysate or serum without donor was applied to control sample. Immediately after adding, fluorescence intensity (λex = 465 nm, λem = 515 nm) was measured over time by using a spectrofluorometer (JASCO Corporation, Tokyo, Japan).

H_2S determination by fluorescent dye (HSip-1 DA assay)

Primary cultured rat cardiomyocytes (2×10^5 cells) were plated onto a 35-mm glass dish (Iwaki, Tokyo, Japan) and incubated for 3 days in Dulbecco's modified Eagle's medium (DMEM)/F-12 (Invitrogen, Carlsbad, CA, USA) containing 5% defined newborn calf serum (Invitrogen) at 37°C under 5% CO_2. After complete adhesion, the cells were washed with cell culture medium and treated with each H_2S donor sample (100 μL/well) in the presence of HSip-1[10] (30 μM) 3 hr at 37°C under 5% CO_2. The cells were then washed with fresh cell culture medium and analysed using a FV1200 confocal laser scanning microscope (Olympus, Tokyo, Japan) equipped with a 20× objective without cell fixation.

Statistical analysis

All results are expressed as the mean ± standard error (SE). All statistical analyses were performed using GraphPad Prism software (ver. 5.00; GraphPad, San Diego, CA, USA). According to the design of the experiment, statistical significance was determined by using either one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test or two-way ANOVA

followed by Bonferroni's post-hoc test. Differences were considered significant when the calculated p value was <0.05.

References

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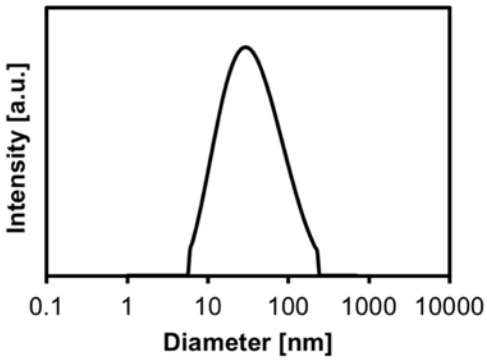
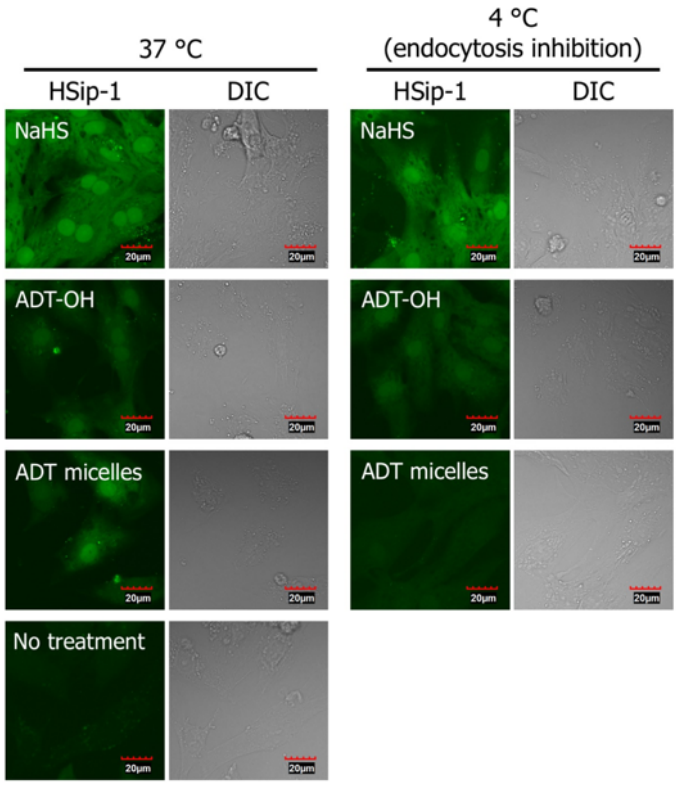
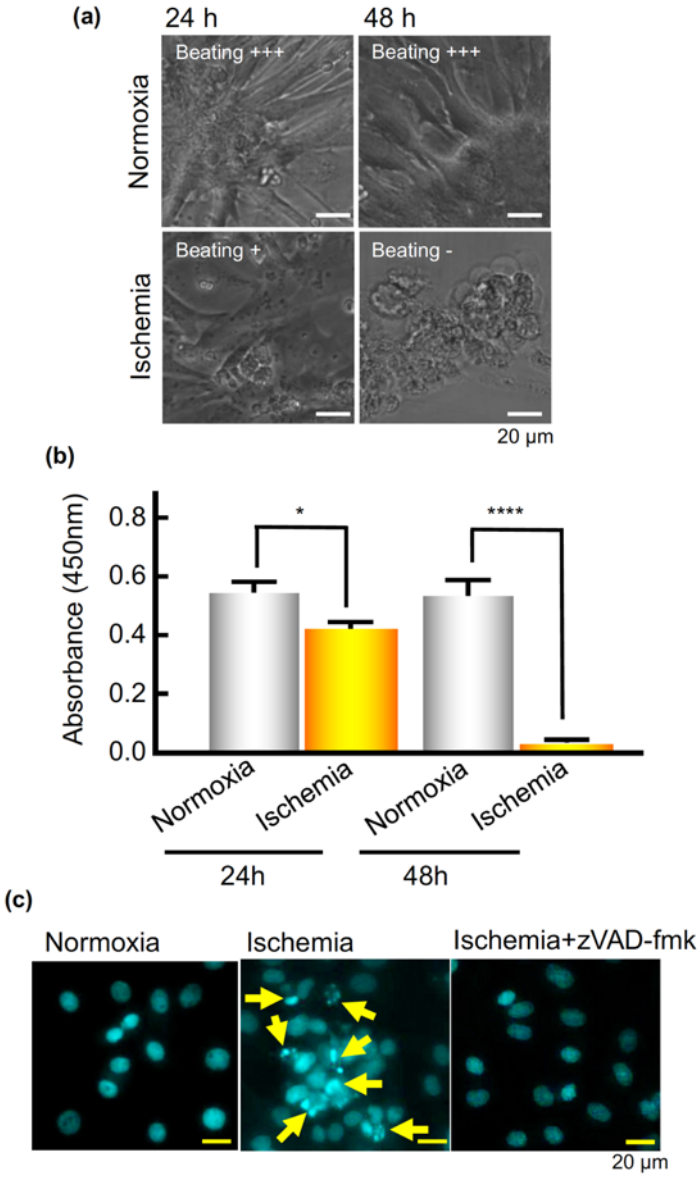
2. Supplementary Figures



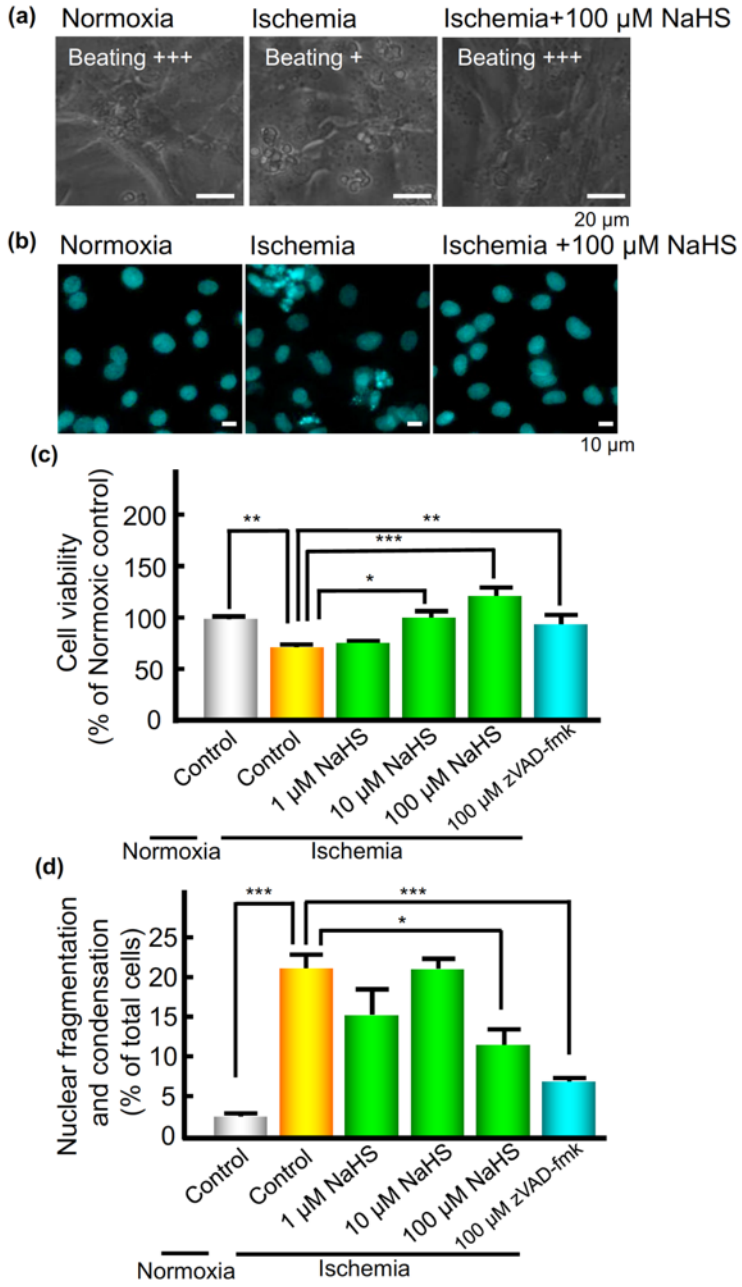
Supplementary Figure 1. **Size distribution of ADT micelles analyzed by DLS.**



Supplementary Figure 2. **H_2S release from NaHS, ADT-OH and PAM-PADT micelles.** Intracellular detection of H_2S in rat cardiomyocytes using HSip-1 DA. Cells were incubated with the different H_2S donors (each 100 μM) at 37 ^{o}C or 4 ^{o}C (endocytosis inhibition) for 1 h in cell culture medium before acquiring the fluorescent images.



Supplementary Figure 3. **Prevention of ischemic cell death of rat cardiomyocytes by treatment of NaHS.** (**a**) Microscopy observations of the rat cardiomyocytes under ischemia condition for 24 or 48 hr at 37 °C. Scale bar: 20 μm. Cell beating assessments are shown in each cell image. (**b**) MTT assay of the cardiomyocytes under ischemia condition for 24 or 48 hr at 37 °C. The data are expressed as the average (± SE) of six experiments. *$p < 0.05$, ****$p < 0.0001$. (**c**) Fluorescence microscopy observation of the rat cardiomyocytes with or without treatment of caspase inhibitor, zVAD-fmk (100 μM) under ischemia condition in cell culture medium for 48 hr at 37 °C, prior to nuclear staining with Hoechst33342. Scale bar: 20 μm.



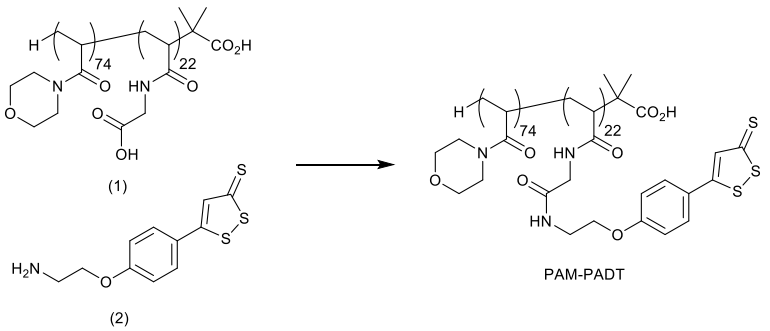
Supplementary Figure 4. **Cell protection effects of NaHS treatment on ischemic cardiomyocytes.** (**a**) Microscopy observations of the rat cardiomyocytes with or without treatment of NaHS (100 μM) under ischemia condition in cell culture medium for 48 hr at 37 °C. Scale bar: 20 μm. Cell beating assessments are shown in each cell image. (**b**) Fluorescence microscopy observation of the rat cardiomyocytes with or without treatment of NaHS (100 μM) under ischemia condition in cell culture medium for 48 hr at 37 °C, prior to nuclear staining with Hoechst33342. Scale bar: 10 μm. (**c**) Cell viability of the cardiomyocytes with or without treatment of NaHS (100 μM) or zVAD-fmk (100 μM) under ischemia condition in cell culture

medium for 48 hr at 37 °C analyzed by MTT assay. The data are expressed as the average (± SE) of four-seven experiments. (**d**) Nuclear fragmentation and condensation of the cardiomyocytes with or without treatment of NaHS (100 μM) or zVAD-fmk (100 μM) under ischemia condition in cell culture medium for 48 hr at 37 °C. The data are expressed as the average (± SE) of three-eleven experiments. **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

Apoptotic cell death (% of Total cells)	
Normoxia	2.4 ± 0.5 ***
Ischemia	20.8 ± 1.8
Ischemi+zVAD-fmk	6.4 ± 0.5 ***

Mean±S.E. n=10-11, ***p<0.001 vs ischemia.
zVAD-fmk; Caspase inhibitor (100 μM)

Supplementary Table 1. **Apoptotic cell death of rat cardiomyocytes under ischemia condition.**



Supplementary Scheme 1. **Synthetic scheme for the PAMPADT block copolymer.**