# **Supplementary Information**

# Two-photon fluorescence imaging reveals a Golgi apparatus superoxide anion-mediated hepatic ischaemia-reperfusion signalling pathway

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#### SUPPLEMENTAL METHODS

## General materials and methods

All chemicals used for synthesis were purchased from Adamas Reagent, Ltd. (China) or Energy Chemical Ltd. (China), and analytical grade solvents were used without further purification. MTT and annexin V-FITC were purchased from Beyotime (China). The commercial organelle dyes, including Golgi-Track Red, Mito-Track Deep Red, Lyso-Track Deep Red and ER-Track Red was obtained from Invitrogen. The human TNF-α ELISA kit was purchased from Shanghai Enzyme-linked Biotechnology Co., Ltd. The method used to prepare reactive oxygen species (ROS) was adapted from previous reports.<sup>1, 2</sup>  $H_2O_2$ , tert-butylhydroperoxide (TBHP), and hypochlorite (NaOCl) were delivered from 30%, 70%, and 10% aqueous solutions respectively. The hydroxyl radical (•OH) was generated through the reaction of 1 mM Fe2+ with 200 µM H2O2. Nitric oxide (NO) was used as a stock solution prepared from sodium nitroprusside. Singlet oxygen ( $^{1}O_{2}$ ) was prepared using the ClO<sup>-</sup>/H<sub>2</sub>O<sub>2</sub> system. Peroxynitrite (ONOO<sup>-</sup>) was used from a 10 mM stock solution in 0.3 M NaOH. Superoxide ( $O_2^{-}$ ) was delivered from KO<sub>2</sub> in a DMSO solution. <sup>1</sup>HNMR spectra were examined at 400 MHz using Bruker NMR spectrometers. The mass spectra were obtained using the Bruker maXis ultra-high resolution-TOF MS system. All onephoton fluorescence measurements were performed at room temperature on an F-4600 fluorescence spectrometer. The two-photon excited fluorescence spectra were measured using a Zeiss LSM 880 NLO with lambda-mode. In vivo fluorescence images were acquired on a Zeiss LSM 880 NLO microscope with an objective lens (20X water objective, N/A 1.0) The Ti:sapphire laser was used to excite the specimen at 800 nm, and the laser power was 80 mW. The co-localized images were recorded on a Leica TCS SP8 microscope with a 63x oil-immersion objective (N/A 1.3).

#### Synthesis

**Synthesis of the CCA probe.** Under nitrogen protection, caffeic acid (10.0 mM) was dissolved in dimethyl formamide (20 mL), followed by the addition of 1.380 mL of triethylamine. Following the addition of dicyclohexylcarbodiimide (DCC) and dimethylaminopyridine (DMAP), the mixture were stirred for 15 minutes. Finally, L-cysteine (12 mM) was added and the mixture was stirred for 24 hours. After the reaction was complete, the reaction product was cooled to form a precipitate by-product, and the supernatant was repeatedly extracted with water and ethyl acetate. The aqueous phase was removed under reduced pressure, affording the crude product, which was purified by thin layer chromatography using ethyl acetate/methanol (v/v 5:1) to afford the yellow solid as the CCA probe. HRMS data, m/z calculated for [C<sub>12</sub>H<sub>13</sub>NO<sub>5</sub>S<sup>-</sup>], 282.0430 found 282.0435. <sup>1</sup>HNMR (400 MHz, DMSO):  $\delta$  8.04 (d, 1H), 7.24 (s, 1H), 7.20 (s, 1H), 6.97 (d, 1H), 6.84 (d, 1H), 6.73 (s, 1H), 6.54 (s, 1H), 6.50 (s, 1H), 4.59 (m, 1H), 3.00 (m, 1H), 2.90 (m, 1H), 1.55 (m, 1H). <sup>13</sup>CNMR (100 MHz, DMSO)  $\delta$ 173.26, 165.65, 147.96 146.01, 139.89, 126.88, 120.95, 118.95, 116.26, 114.59, 49.07, 25.36.

# Cytotoxicity assay

We used the MTT assay to determine cell viability<sup>3</sup>. First, we seeded the hepatic cells (10<sup>6</sup> cells mL<sup>-1</sup>) into replicate 96-well microtiter plates in a total volume of 100  $\mu$ L well<sup>-1</sup>. After 12 h of culture, various concentrations of the probe (1×10<sup>-3</sup>, 1×10<sup>-4</sup>, 1×10<sup>-5</sup>, 1×10<sup>-6</sup> and 1×10<sup>-7</sup> M) were added to the cells and incubated for 24 h. Subsequently, 20  $\mu$ L of a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

bromide (MTT) solution (5 mg mL<sup>-1</sup>, DMEM) were added to each well. The MTT solution was removed after 4 h, and 150  $\mu$ L of DMSO were added to each well. Finally, we measured the absorbance at 490 nm using a TRITURUS microplate reader.

## Cell culture and mouse maintenance

Human hepatic cells (HL7702) were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in high-glucose DMEM supplemented with 10% foetal bovine serum, 1% penicillin and 1% streptomycin at 37°C (w/v) in a 5% CO<sub>2</sub>95% air MCO-15AC incubator (SANYO, Tokyo, Japan). IR hepatic cells were cultured in DMEM without glucose containing a deoxygenated reagent 0.5 mmol/L sodium dithionite for 0.5 h<sup>4</sup>. Afterwards, cells were incubated in DMEM containing glucose in a 5% CO<sub>2</sub> and 95% O<sub>2</sub> atmosphere for 0.5 h. Cells were disrupted with an ultra-vibration metre and cell debris was removed by high speed centrifugation (3000 rpm for 20 minutes at 4°C).

Eight- to ten-week-old wild-type BalB/C mice (females) were used. In the deep tissue imaging experiment, Zeiss 880 NLO microscopy was employed with *z*-stack mode and a water objective (20X). Firstly, mice were anaesthetized with 4% chloral hydrate (3 mL/kg) via an intraperitoneal injection, and a laparotomy was performed to expose the liver. Secondly, CCA (10  $\mu$ M) was injected into the upper epidermis for imaging. The IR procedure was induced in mice using a previously reported method and as briefly described below<sup>5</sup>. Hepatic ischaemia was induced by clamping the artery to the left lobe of the liver. Thirty minutes later, the ischaemic liver was reperfused by opening the vascular clamp. Then, CCA (10  $\mu$ M) was injected into the left lobe of the liver. As a control, the liver of the mice was exposed and CCA (10  $\mu$ M) was injected in the left lobe of the liver. The animal experiments were approved by the Shandong Normal University authorities.

#### Cellular extracts

Live hepatic cells were detached, replated on 10-cm glass-bottom dishes, and cultured for 7 days before extracts were prepared. After trypsinization, the hepatocytes were centrifuged at 1500 rpm for 5 minutes at room temperature. We washed cells three times with PBS by centrifugation and collected cells in 2 mL of secondary water. The mixture was disrupted by a sonicator. Supernatants collected by centrifuging the disrupted cells (4 °C, 12000 rpm, 20 min) were retained as cell extracts.

#### **Fluorescence imaging experiments**

Living cells were detached, replated onto 15-mm glass-bottom dishes, and cultured for 24 h before imaging. After an incubation with the CCA probe for 20 min, the cell culture media were removed and cells were washed with 1.0 mL of PBS three times. Fluorescence images of CCA were obtained with an excitation wavelength of 405 nm and blue channel emission wavelengths of 430-530 nm. Fluorescence images of Golgi Red were obtained at an excitation wavelength of 580 nm and red channel emission wavelengths of 600-750 nm. Analyses were performed using Image-Pro Plus software. For data analysis, the average fluorescence intensity per image under each experimental condition was obtained by selecting regions of interest. Each experiment was repeated at least three separate times with identical results.

#### Flow cytometry

Cells were plated on six-well plates in 2 mL of high-glucose DMEM and incubated for 36-72 hours prior to the day of experiment and maintained at 37 °C in a 5% CO<sub>2</sub> atmosphere until they reached 80-90% confluence. Prior to flow cytometry, the treated cells in each well were washed with PBS and trypsinized with 500  $\Box$ L of 0.05% Trypsin for 1 min. Trypsin was discarded and 1 mL of high-glucose DMEM was added to each well and cells were centrifuged at 1000 rpm for 5 min. Media were discarded and cells were resuspended in 1.5 mL of PBS and again centrifuged at 1000 rpm for 5 min. Next, each pellet was incubated with 195 µL of Annexin V-FITC binding buffer, 5 µL of Annexin V-FITC and 10 µL of propidium iodide (PI) at room temperature in the dark for 20 min; cells were subjected to flow cytometry. Fluorescence signals in the FITC channel and PI channel were measured for 5000 cells on an ImageStream<sup>X</sup> Mark II flow cytometer (Merck Millipore, Seattle, WA). Data were analysed using IDEAS software version 6.2.

## Western blot analysis

All the pre-treated cells were lysed in cell lysis buffer containing the protease inhibitor PMSF. The cell lysates were centrifuged at 15,000 rpm for 10 min at 4°C. After protein concentration was determined using a bicinchoninic acid (BCA) protein assay kit. The proteins were electro transferred to a nitrocellulose membrane in transfer buffer containing 48 mM Tris-HCl, 39 mM glycine, 0.037% SDS and 20% methanol at 4 °C for 1 h. Nonspecific binding to the membrane was blocked for 1 hour at room temperature with 5% nonfat milk in TBS buffer (20 mM Tris-HCl, 150 mM NaCl and 0.1% tween 20). The membranes were incubated for 16 hours at 4 °C with various primary antibodies (Abcam) in TBS buffer containing 5% milk at the dilutions specified by the manufacturers. Binding of primary antibodies was followed by incubation for 1 hour at room temperature with the secondary horseradish peroxidase-conjugated IgG in 1% nonfat milk. The signals were visualized with the enhanced chemiluminescence system (ECL, Amersham).

#### Measurement of two-photon cross section

The two-photon cross sections ( $\delta$ ) were determined using the femtosecond (fs) fluorescence measurement technique<sup>6</sup>. CCA and O<sub>2</sub><sup>--</sup> were dissolved in cell extract buffer at a concentration of 1.0 × 10<sup>-4</sup> M and then the two-photon-induced fluorescence intensity was measured at 800 nm using fluorescein (1.0×10<sup>-4</sup> M, pH 11) as the reference, whose two-photon property has been well characterized in the literature. The intensities of the two-photon-induced fluorescence spectra of the reference and sample emitted at the same excitation wavelength were determined. The value of  $\delta$  was calculated using Eq (1).

$$\delta_s = \delta_r \frac{\Phi_r}{\Phi_s} \frac{C_r}{C_s} \frac{n_r}{n_s} \frac{F_s}{F_r} \quad (1)$$

The subscripts s and r refer to the sample and the reference material, respectively.  $\delta$  is the TPA cross sectional value, C is the concentration of the solution, n is there refractive index of the solution, F is two-photon excited fluorescence integral intensity and  $\Phi$  is the fluorescence quantum yield.

## SUPPLEMENTAL SCHEME AND FIGURES



**Fig. S1** Two-photon fluorescence spectra of 10  $\mu$ M CCA (black line) alone and after the addition of 5  $\mu$ M O<sub>2</sub><sup>-</sup> (red line) in cell extract buffer (pH 7.4) at  $\lambda_{ex} = 800$  nm.



**Fig. S2** Fluorescence responses of a 10  $\mu$ M CCA & 5  $\mu$ M O<sub>2</sub><sup>--</sup> mixture to various reactive oxygen species, reactive nitrogen species and metal ions (1-17: Control, 20  $\mu$ M GSH, 100  $\mu$ M TBHP, 10 mM H<sub>2</sub>O<sub>2</sub>, 1  $\mu$ M <sup>1</sup>O<sub>2</sub>, 1  $\mu$ M •OH, 2  $\mu$ M ONOO<sup>-</sup>, 50  $\mu$ M NO, 100  $\mu$ M NaClO, 10 mM Na<sup>+</sup>, 10 mM K<sup>+</sup>, 500  $\mu$ M Ca<sup>2+</sup>, 500  $\mu$ M Zn<sup>2+</sup>, 500  $\mu$ M Fe<sup>2+</sup>, 500  $\mu$ M Fe<sup>3+</sup>, 20  $\mu$ M Cu<sup>2+</sup>, and 20  $\mu$ M Cu<sup>+</sup>) in cell extract buffer (pH 7.4) at  $\lambda_{ex} = 370$  nm and  $\lambda_{em} = 495$  nm.



**Fig. S3** Fluorescence spectra of 10  $\mu$ M CCA (black line) alone and after the addition of 5  $\mu$ M O<sub>2</sub><sup>-</sup> (red line) in the presence of a solution with various pH values at  $\lambda_{ex} = 370$  nm and  $\lambda_{em} = 495$  nm.



**Fig. S4** Time course of changes in the fluorescence of 10  $\mu$ M CCA (black line) alone and after the addition of 5  $\mu$ M O<sub>2</sub><sup>--</sup> (red line) in cell extract buffer (pH 7.4) at  $\lambda_{ex} = 370$  nm and  $\lambda_{em} = 495$  nm.



Fig. S5 Viability of HL-7702 cells in the presence of CCA, as measured using MTT assay.



**Fig. S6** Fluorescence imaging of  $O_2^{-}$  levels in HL-7702 cells. (A1) HL-7702 cells were incubated with 10  $\mu$ M CCA for 30 min. (A2) IR HL-7702 cells were treated with 10  $\mu$ M CCA for 30 min. (A3) IR HL-7702 cells were treated with 10  $\mu$ M CCA for 30 min. (A3) IR HL-7702 cells were treated with 10  $\mu$ M CCA for 30 min and then simulated with 100  $\mu$ g/mL silymarin for 30 min. (A4)-(A6) Bright field images. (B) The average fluorescence intensity output of A. Images were acquired at an excitation wavelength of 405 nm and emission wavelengths in the blue channel of 430-530 nm.



Fig. S7 The level of TNF- $\alpha$  in HL-7702 cells were measured by using a human TNF- $\alpha$  Elisa kit.

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