Human cardiac organoid model reveals antibacterial triclocarban promotes myocardial hypertrophy by interfering with endothelial cell metabolism

Materials and Methods

**Cell lines.** The human umbilical vein endothelial cells (HUVECs, catalogue No. CP-H082) were obtained from Procell (Wuhan, China). The human cardiac microvascular endothelial cells (HCMECs, CTCC-013-HUM) were obtained from Meisen CTCC (Jinhua, China). The human myocardial cell line AC16 (catalogue No. YC-C134) was purchased from UBIGENE Biosciences (Guangzhou, China). The human induced pluripotent stem cells (hiPSCs) [1] were a kind gift from Dr. Yang Li (Peking University, Beijing, China). For co-culture experiments, the human aortic endothelial cells (HAECs, catalogue No. iCell-0015a) were obtained from Cellverse (Shanghai, China).

**Cell culture.** hiPSCs were cultivated in mTeSR1 medium (Stemcell Technologies, Canada) on Matrigel (BD science, USA) coated culture dishes (Corning, USA). The medium was replaced every day until the cells reached a confluence of 70%. The cells were digested and passaged with accutase (Sigma-Aldrich, Germany). HUVECs and HAECs were cultured in ECM medium (ScienCell, San Diego, USA). The cells were digested and passaged with 0.05% trypsin (Gibco, Grand Island, USA). Cells at passages 4 to 6 were used for experiments. AC16 was cultured in DMEM (Gibco) medium containing 10% FBS (Gibco) and 1% Penicillin-streptomycin (Gibco). Cell cultures were maintained at 37℃ in a humidified atmosphere containing 5% CO2. TCC was used at 1,2 or 5 μmol/L, which have been recognized as environmentally relevant doses. [2, 3] AC16 cells underwent serum starvation for 24h before treatment.

For co-culture experiments, (a) Endothelial cells was first treated with different doses of TCC for 24h. At the same time, AC16 was changed to serum-free medium for starvation. Then the medium supernatant of endothelial cells was transferred to the plates of AC16 cells for another 24h before final harvest and detection. (b) Endothelial cells and AC16 cells were seeded onto the the upper compartment of the insert and the well of the plate, separately. AC16 with a confluence of 70-80% was starved for 24h, then the inserts seeded with endothelial cells were positioned into the well. TCC was added to the upper chamber. 24h later the cells in the wells were harvested. (c) Endothelial cells and AC16 was mixed at a ratio of 1:3 into the same dish in DMEM (Gibco) medium containing 10%FBS (Gibco). Cells were harvested after 48h of TCC addition.

**Construction of cardiac organoids (COs).** hiPSCs were harvested at approximately 70% confluency. Cardiac organoids were generated as previously described [4]. Briefly, 7500 cells were seeded in a volume of 200 mL into ultra-low-attachment 96-well plates (Corning, USA) containing mTeSR1 with 10 μmol/L ROCK inhibitors (Selleck) and centrifugation for 5 minutes at 300 g. Next day, formed aggregates were induced with basic medium CDM3[5] containing 6 μmol/L CHIR99021 (Selleck, USA), 30 ng/mL FGF2 (MCE, USA), 5 mmol/L LY294002 (Selleck), 50 ng/mL Activin A (Novoprotein), 10 ng/mL BMP4 (MCE) and 1 μg/mL of insulin (MCE). After 36h-40h, medium was changed with CDM3 containing 10 ng/mL BMP4, 8 ng/ml FGF2, 1 μmol/L IWR-1-endo (Glpbio, Montclair, USA) and 0.5 mmol/L retinoic acid (Selleck). The medium was changed every day, 4 days later, the medium was changed to CDM3 containing 10 ng/mL BMP4, FGF2 (8 ng/mL) and insulin (10 μg/mL) with another 2 days and the medium changed every day. Obtained cardiac organoids were cultured in CDM3 with 10 μg/mL insulin for maintaining until use.

**Immunofluorescence analysis and cardiomyocyte area measurement of cardiac organoids.** Cardiac organoids were treated with TCC and dissociated into single cells after 48h. Briefly, several cardiac organoids were collected and washed in PBS. Trypsin (0.25%) was used and incubated at 37℃ for 20 min. At the end of dissociation, gently pipetted organoids up and down until complete dissociation to single cells. Dissociated cells were seeded onto Matrigel-coated glass coverslips for confocal in CDM3+insulin with 10% Knock Out Serum Replacement (GIBCO) and 10 mmol/L ROCKi. 24h after seeding, cells were kept in CDM3+insulin without ROCKi until the experiments began.

Coverslips were then fixed in 4% paraformaldehyde, stained with the cardiomyocyte-specific marker cardiac Troponin T (cTnT / TNNT2, Thermo), labeled with Hoechst to detect nuclei, and mounted on glass slides. The antibody information is shown in Table S1. Representative images were acquired for each sample condition using an inverted microscope (Keyence BZ-X810). For each cell, area and perimeter were quantified using ImageJ software. Data was presented as a violin plot showing all the points.

**Lactate dehydrogenase assay.** After the cardiac organoids were treated by different doses of TCC for 24 hours, the supernatant was collected and the LDH cytotoxicity assay kit (Takara, MK401, Japan) was used to quantify the release of lactate dehydrogenase (LDH) according to the manufacturer's instructions. LDH activity was determined with a multifunctional enzyme marker (BMG LABTECH, Germany).

**Heart rate monitoring of cardiac organoids.** Videos of spontaneously beating organoids were recorded 2 minutes before and after different doses of TCC treatment. Every record was done within 10 minutes in order to reduce variation in temperature mediated changes in beating. Recording of the videos was performed with a Keyence Microscope BZ-X810 (Japan). Threshold edge-detecting in ImageJ software was used to calculate the beating frequency.

**Measurement of ADMA.** The amounts of ADMA, were measured in cell medium using the Human asymmetric dimethylarginine (ADMA) ELISA kit (MM-1437H1, Jiangsu Meimian industrial, China) according to the manufacturer’s instructions.

**Measurement of endothelial NO production.** DAF-FM DA (488 nm excitation, 520 nm emission, catalogue No. 40769ES50, Yesen, Shanghai, China) was used to measure NO production in HUVECs. HUVECs were exposed to different concentrations of TCC for 24h and then treated with 10 mmol/L DAF-FM DA at 37°C for 30 min. After washing with PBS for 3 times, cells were detected using Keyence Microscope BZ-X810 (Japan) and flow cytometry (Beckman, USA).

**Detection of ROS production in HUVECs.** ROS production in HUVECs was assessed using the fluorescent dye DCFH-DA (catalogue No. CA1410, Solaibio, Beijing, China). HUVECs were exposed to different concentrations of TCC for 24h and then stimulated and loaded with 1 mmol/L of DCFH-DA in serum-free ECM for 30 min at 37℃. After being washed 3 times with PBS, the cells were digested with 0.05% trypsin, resuspended with 300 mL serum-free ECM and measured by the Keyence Microscope BZ-X810 (Japan) and flow cytometry (Beckman, USA).

**Flow cytometry.** The cell composition in cardiac organoids was analyzed by flow cytometry. 0.25% Trypsin was used and incubated at 37℃ for 20 min to dissociate cardiac organoids to single cells. The cell suspension was filtered with a 100 μmol/L filter and fixed with 4% formaldehyde (10 min) and then permeabilized with 0.1% PBS-Triton X-100 for 15 min. The cells were then incubated in PBS with 10% normal goat serum. Cardiac Troponin T Polyclonal Antibody (TNNT2, PA5-82599, Invitrogen) and VE-Cadherin (CDH5, 2500, CST) primary antibody were used and incubated for 30 min. The secondary antibody used was Donkey Anti-Mouse IgG H&L (Alexa Fluor® 488) (ab150105) at 1/2000 dilution for 30 min.

**Transcriptome analysis.** Total RNA of cardiac organoids was extracted after 2 μmol/L TCC treatment for 24h. The sequencing was done by BGI Technology Co., LTD. (Shenzhen, China), and paired-end (PE) sequencing was conducted on the BGI-DIPSEQ platform. Differentially expressed gene (DEG) analysis was performed by using the edgeR package. Genes with a parameter of false discovery rate (FDR)≤ 0.05 and absolute fold change≥ 2 were filtered as differentially expressed genes (DEGs).

**Sample preparation and targeted mass spectrometry quantification for amino acids.** For amino acids (AA) extraction, endothelial cells or cardiac organoids were collected, washed with PBS and then centrifuged. About 50 mg of precipitated cells or tissue was added to 2 mL of the extract buffer. The extract buffer was composed of 80% methanol and 20% water, containing [13C, 15N]-stable-isotope-labeled cell free AA mixture (Product No: 767964, Sigma-Aldrich, USA) as internal standards. Amino Acid Standard H (Prod No. WAT088122, Waters, USA) was used as standard substance for retention time confirmation and standard curve. Next, the homogenized sample within extract buffer was centrifuged at 14,000 g for 10 min (4–8 ℃). The supernatant was transferred to a new tube and dried under-speed in reduced pressure without heating. Later, the dried samples were re-dissolved with 100 μL methanol and centrifuged at 12,000 g for 10 min at 4°C. Then 80 μL of the supernatant was transferred to an MS vial with a plastic insert.

LC/MS quantification of amino acids was according to the manufacturer’s instructions. Briefly, targeted metabonomic for amino acid was performed on Intrada Amino Acid column 100mm x 3mm, 3 μmol/L (Imtakt Corp, JAPAN) at a flow rate of 0.5 mL/min with an API 6500Q-TRAP mass spectrometer (AB SCIEX, Framingham, MA). A discontinuous gradient was generated to resolve the analytes by mixing solvent A (100 mmol/L Ammonium formate in water) with solvent B (0.1% folic acid in acetonitrile) at different ratios starting from 80% B linearly to 70% B over 4.5 min, then decreased to 40% B in 0.5 min, then decreased to 0% B in 5 min, and hold for 3 min, finally back to 80% B in 0.2 min. Analytes were monitored using electrospray ionization in positive-ion mode with multiple reaction monitoring (MRM) mode. The precursor and characteristic product ion transitions are listed as follows. Relative quantification of analysts were based on (area(analyte)/area(internal standard))/weight(cells/organoids) and normalized to the control group, and the precursor and characteristic product ion transitions of interested analytes are described in the Supplemental Materials section.

**Real-time polymerase chain reaction (RT-PCR).** Total RNA from Human heart organoids and cells exposed to TCC were extracted by TRIzol Reagent (Invitrogen). cDNA was synthesized and amplified using First-strand cDNA Synthesis Mix Kit with gDNA Eraser (Lablead) in an ABI Q3 Real-Time PCR Detection System (Applied Biosystems) using the TransStart® Top Green qPCR SuperMix (TransGen Biotech). The RT-PCR conditions were set to 94°C for 30 s for initial denaturation, 40 cycles of denaturation at 94°C for 30 s, at 60°C hold for 30 s for annealing, and then elongation at 72°C for 30 s. The specificity of the product was determined by analyzing of the melting curve.Allreactions were normalized to the expression of *β­actin*.The fold change of target gene mRNA (sequences of primers were listed in Table S2) expression was calculated according to the 2-ΔΔCTmethod.

**Western blot analysis.** According to the manufacturer’s protocol, the cells were lysed in RIPA buffer (Solarbio LIFE SCIENCES) containing 1 mmol/L protease inhibitors or phosphatase inhibitors on ice. Primary antibodies for SLC7A11 (Abmart), GPX4 (Abmart), eNOS (CST), p-eNOS (CST), Akt (Abmart), p-Akt (Abmart), PI3K (Abmart), p-PI3K (Abmart), GAPDH (Proteintech) and β-actin (Proteintech) were used. The signals of blots were performed with chemiluminescence (ECL) HRP Substrate (Millipore) and visualized with the Image Analyzer (Invitrogen iBright 1500).

**Determination of S-nitrosylation.** HUVECs were treated with 2 μmol/L TCC for 0h, 1h, and 3h respectively. Post-translational modification of total protein S-nitrocysteine was detected by PierceTM S-Nitrosylation Western Blot kit (Thermo Scientific) according to the manufacturer’s instructions.

**Statistical Data Analysis.** Data were presented as standard deviation (SD). Differences were analysed by two-tailed unpaired Student’s t-test for experiments with two groups and ordinary one-way ANOVA test for multiple comparisons as appropriate in experiments including ≥ 3 groups. A value of P < 0.05 was considered statistically significant. All experiments were performed with at least three biological replicates. Statistical analyses and graphical illustrations were processed with GraphPad Prism 9.3 software.

**NMR-based metabolomics.** The collected cells were subjected to freeze-thaw for three times and extracted with pre-colded methanol/water (2/1, V/V) solution for 3 times using TissueLyser (Qiagen, Germany). After centrifugation for 10 min (16099 g and 4°C), the supernatants were combined and lyophilized in vacuo to remove methanol. The aqueous extracts from cells were dissolved in 600 μL phosphate buffer (0.1 M, K2HPO4/NaH2PO4 = 4/1, pH ≈ 7.4) containing 80% D2O and 0.001% TSP-d4 as a reference (δ 0.00). After vortex and centrifugation for 10 min (16099 g and 4 °C), 550 μL of supernatants was pipetted into NMR tubes (5 mm) for later NMR analysis. 1H NMR spectra of cell extracts were acquired at 298K on a Bruker Avance III 600 MHz spectrometer equipped with a Bruker inverse detection cryogenic probe (Bruker, Biospin, Germany). A standard one-dimensional NMR spectrum NOESY pulse sequence (recycle delay-90°-t1-90°-tm-90°-acquisition) was used for water-suppressed. Recycle delay was set to 2 s, t1 of 3 μs and the mixing time, tm, of 80 ms. 90° pulse length was adjusted to about 10 μs and 128 transients into 32 k data points were collected with a 20 ppm width of spectral for each spectrum. For NMR signals identification, 2D NMR spectra including 1H-1H COSY and TOCSY, 1H-13C HSQC and HMBC were recorded for selective samples.

**NMR data processing and multivariate data analysis.** The phase and baseline distortions of all the 1H NMR spectra were manually corrected using Topspin 3.6 (Bruker Biospin, Germany). After removing water signals (δ 4.7-δ 5.0 ppm), the spectral region (δ 0.5-δ 9.6 ppm) was automatically integrated into equal width regions of 0.002 ppm (1.2 Hz) using MestRenova software (version 9.0.1). Each integral region was normalized to the sum of all integrals for each spectrum to compensate for the concentration differences before statistical data analysis. Multivariate data analysis was conducted using SIMCA-P+ 13.0 software package (Umetrics, Sweden). Principal component analysis (PCA) and orthogonal projection to latent structures with discriminant analysis (OPLS-DA) were performed on the NMR spectral data with unit variance scaling. The OPLS-DA models were further assessed by a 7-fold cross validation method CV-ANOVA (p < 0.05). Loading plots were employed to identify those significantly changed metabolites using Matlab script (V7.8, MA).

Table S1 Summary of antibodies

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Antibody | Usage | Application | Dilution | Manufacture |
| Rabbit VE-cad | Primary | Immunofluorescence staining, Flow cytometry | 1:200  1:500 | Cell Signaling Technology, USA |
| Rabbit SLC7A11 | Primary | Western blot | 1:1000 | Abmart, CHN |
| Rabbit GPX4 Antibody | Primary | Western blot | 1:1000 | Abmart, CHN |
| Rabbit eNOS | Primary | Western blot | 1:1000 | Cell Signaling Technology, USA |
| Rabbit Phospho-eNOS | Primary | Western blot | 1:1000 | Cell Signaling Technology, USA |
| Rabbit PI3 Kinase p85 alpha Antibody | Primary | Western blot | 1:1000 | Abmart, CHN |
| Rabbit Phospho-PI3-kinase p85- alpha/ gamma(Tyr467/199) Antibody | Primary | Western blot | 1:1000 | Abmart, CHN |
| Rabbit AKT1/2/3 Antibody | Primary | Western blot | 1:1000 | Abmart, CHN |
| Rabbit Phospho-Akt (Ser473) Antibody | Primary | Western blot | 1:1000 | Abmart, CHN |
| Mouse Actin antibody | Primary | Western blot | 1:50000 | Proteintech, USA |
| Mouse GAPDH antibody | Primary | Western blot | 1:50000 | Proteintech, USA |
| Rabbit TNNT2 | Primary | Flow cytometry, Immunofluorescence staining | 1:500  1:2500 | Invitrogen, USA |
| Alexa Fluor® 555 Donkey anti-rabbit IgG | Secondary | Immunofluorescence staining/Flow cytometry | 1:500 | Abcam, USA |
| Alexa Fluor® 488 Donkey anti-Rabbit IgG | Secondary | Immunofluorescence staining/Flow cytometry | 1:500 | Abcam, USA |

Table S2 Primers used for quantitative real-time PCR

|  |  |  |
| --- | --- | --- |
|  | Forward primer (5’ -3’) | Reverse primer (5’-3’) |
| *SLC7A11* (Human) | TTTTCTGAGCGGCTACTGGG | CATGGAGCCAAAGCAGGAGA |
| *NOS3* (Human) | TGATGGCGAAGCGAGTGAAG | ACTCATCCATACACAGGACCC |
| *ARG2* (Human) | TGTGATAGGAGCCCCGTTCT | AGTCTTTTAGGTGGCAGCCC |
| *NOS1* (Human) | GTGAAGGAGCGGGTCAGTAA | CTTCAGGGCCCCTCAGAATG |
| *NOS2* (Human) | TCCAAATCTTGCCTGGGGTC | AGAAGCTCATCTGGAGGGGT |
| *SLC3A2* (Human) | ATGGAGCTACAGCCTCCTGA | CGCGCTGAGACCCTGG |
| *GPX4* (Human) | GAAGATCCAACCCAAGGGCA | GACGGTGTCCAAACTTGGTG |
| *β-actin* (Human) | AGGATTCCTATGTGGGCGAC | ATAGCACAGCCTGGATAGCAA |
| *DDAH1* (Human) | CCAACAAAGGGCACGTCTTG | AGTTCAGACATGCTCACGGG |
| *DDAH2* (Human) | CTGGATGGCACTGACGTTCT | TCAGGGAGGCATATGGGTGA |
| *TEK* (Human) | GGTCAAGCAACCCAGCCTTTTC | CAGGTCATTCCAGCAGAGCCAA |
| *TIE* (Human) | ATGGCTGCTCTTGTGGATCTGG | CGGTCACAAGTGCCACCATTCT |
| *WT1* (Human) | CGAGAGCGATAACCACACAACG | GTCTCAGATGCCGACCGTACAA |
| *TCF21* (Human) | GCAGATCCTGGCTAACGACA | GTAAAGTGTTCTCGCGGGGT |
| *PECAM*（Human） | AAGTGGAGTCCAGCCGCATATC | ATGGAGCAGGACAGGTTCAGTC |
| *ESAM* (Human) | CGCTGTCCAATACCAGTGGGAT | CCTTGCAGACATAGACTCCAGC |
| *DLK* (Human) | CCCCAAAATGGATTCTGCGAGG | GGTTCTCCACAGAGTCCGTGAA |
| *DDR2* (Human) | GCTATATGCCGCTATCCTCTGG | ACTCTGACCACTGACTGGAAG |
| *NKX2.5* (Human) | AAGTGTGCGTCTGCCTTTCCCG | TTGTCCGCCTCTGTCTTCTCCA |
| *PDGFRA* (Human) | TGGCAGTACCCCATGTCTGAA | CCAAGACCGTCACAAAAAGGC |
| *EOMES* (Human) | TGGCAGTACCCCATGTCTGAA | CTCCTGTCTCATCCAGTGGGAA |
| *MEF2C* (Human) | TCCACCAGGCAGCAAGAATACG | GGAGTTGCTACGGAAACCACTG |
| *ISL1* (Human) | GCAGAGTGACATAGATCAGCCTG | GCCTCAATAGGACTGGCTACCA |
| *TBX18*(Human) | CACAACCGTCACTGCCTATCAG | CCGTAGTGATGGTCGCCAGAAT |
| *NPPA* (Human) | CAACGCAGACCTGATGGATTT | AGCCCCCGCTTCTTCATTC |
| *NPPB* (Human) | TGGAAACGTCCGGGTTACAG | CTGATCCGGTCCATCTTCCT |
| *TNFα* (Human) | CCCAGGCAGTCAGATCATCTTC | GTGAGGAGGACATGGGTGGAG |
| *IL-1β* (Human) | CGTCAGTTGTTGTGGCCATGGA | TTCTGCCAGTGCCTCTTTGCTG |
| *IL-6* (Human) | AGACAGCCACTCACCTCTTCAG | GAGCGTGCAGTTCAGTGATCGTA |
| *SLC7A5* (Human) | GGGAAGGGTGATGTGTCCAA | AGAGGCCGCTGTATAATGCC |

Table S3 1H NMR signal assignment of metabolites obtained from cardiac organoids

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Code | Metabolites | Moieties | δ1H (multiplicity) | δ13C |
| 1 | Alanine (Ala) | CH3 | 1.48(d) | 19 |
|  |  | CH | 3.78(q) | 53.4 |
|  |  | COOH |  | 178.6 |
| 2 | |  | | --- | | Dimethylamine (DMA) | | (CH3)2 | 2.76(s) | 37.2 |
| 3 | Myo-Inositol | C(4)H，C(6)H | 3.537(dd) | 74.6 |
|  |  | C(1)H | 3.628(t) | 75.7 |
|  |  | C(2)H | 4.06(t) | 75.6 |
|  |  | C(3)H | 3.28(t) | 77.7 |
| 4 | Arginine | αCH | 3.77(m) | 57.5 |
|  |  | βCH2 | 1.93(m) | 30.5 |
|  |  | γCH2 | 1.73(m) | 30.5 |
|  |  | δCH2 | 3.25(t) | 44.6 |
|  |  | COOH |  | 177.4 |
|  |  | C=N |  | 159.6 |
| 5 | Glutamate (Glu) | αCH | 3.78(t) | 57.7 |
|  |  | βCH2 | 2.06(m) | 29.9 |
|  |  | γCH2 | 2.35(m) | 36.8 |
|  |  | COOH |  | 178.0 |
|  |  | COOH |  | 184.2 |
| 6 | α-glucose (α-G) | CH | 5.24(d) | 95.4 |
|  |  | 2-CH | 3.54(dd) | 74.9 |
|  |  | 3-CH | 3.73(dd) | 76.2 |
|  |  | 4-CH | 3.42(dd) | 72.7 |
|  |  | 5-CH | 3.83(dd) | 74.4 |
|  |  | 6-CH2 | 3.83(dd) | 63.7 |
| 7 | Lactate | αCH | 1.44 | 23.0 |
|  |  | βCH3 | 1.22 | 23.0 |
|  |  | COOH | 4.12 |  |



**Figure S1 Establishment and characterization of self-organizing cardiac organoids**.

**(a-d)** Temporal log2-transferred gene expression of different cardiac cell lineages markers from day 0 to day 8.5 (each time point contains 10 cardiac organoids).

**(e-f)** Temporal log2-transferred gene expression of first and second heart field (FHF and SHF) markers from day 0 to day 8.5 (each time point contains 10 cardiac organoids).

**(g)** The effect of TCC on LDH leakage in cardiac organoids in culture medium, n=6 per group.

**(h)** The effect of TCC exposure on heart rate of cardiac organoids. n=8 per group.

**(i-k)** mRNA expression of inflammatory response-related markers. n=3 per group.

Data are presented as mean ± SD, by one-way ANOVA.



**Figure S2 Transcriptomic change after TCC exposure indicates reprogrammed amino acid metabolism**.

**(a)** Principal component analysis (PCA) of the transcriptome results.

**(b)** KEGG annotation suggests changes in amino acid metabolism-related pathways. **(c)** RT-PCR analysis of mRNA levels of the amino acids metabolism-related genes in cardiac organoids including *SLC7A11, SLC3A2, SLC7A5* and *ARG2.*

**(d)** WB analysis of SLC7A11 expression in cardiac organoids after 48h TCC treatment.

**(e)** Quantification of SLC7A11 expression levels relative to GAPDH shown in (d).

Data are represented as mean ± SD by one-way ANOVA test.



**Figure S3 1H-NMR metabolic profile changes after 24h TCC treatment of self-organizing cardiac organoids.** (Left) OPLS-DA scores. The black dots represent the 0 μmol/L TCC group, and the red dots represent the TCC treatment groups. (Right) Color-coded coefficient loading plots for models derived from NMR data of aqueous extracts of cardiac organoids exposed to TCC at different doses for 24 hours. TSP-d4 is selected as the reference, and the horizontal coordinate represents the relative chemical shift value (δ) in ppm. The closer the color of the peak is to the warm color, the greater the contribution to the distinction between the two groups. An upward peak indicates a higher concentration in the TCC group compared with 0 μmol/L TCC. The spectral region between 4.7 and 9.6 ppm (middle) has been magnified by a factor of 10 compared to the spectrum on the right. However, the differential metabolite intensity within this range is relatively low.



**Figure S4 Changes of genes and metabolites related to amino acid metabolism in cardiac organoids and endothelial cells**.

**(a)** Quantitative detection of metabolite ADMA in cardiac organoids.

**(b)** Relative levels of 19 amino acids by LC-MS after 24 hours of TCC exposure with different doses in cardiac organoids.

**(c)** RT-PCR analysis of mRNA levels of the amino acids metabolism-related genes in HUVECs*.*

**(d-e)** WB analysis of protein expression of SLC7A11 in TCC-treated HUVECs. Protein quantitative results are shown as SLC7A11/β-actin. Multiple comparison tests were conducted by ordinary one-way ANOVA. Data are represented as mean ± SD.

**(f)** RT-PCR analysis of mRNA expression levels of nitric oxide synthase and dimethylarginine dimethylamino hydrolase in HUVECs after TCC exposure, including *eNOS*, *nNOS, DDAH1, DDAH2.*

**(g)** RT-PCR analysis of inflammatory factor expression in HUVECs after TCC exposure.

**(h-j)** The intensity of the band for protein expression of the PI3K/AKT-eNOS pathway in TCC-stimulated HUVECs was calculated.

**(k)** mRNA levels of *GPX4* in HUVECs exposed by TCC for 24 hours.

Data are represented as mean ± SD by one-way ANOVA test.



**Figure S5. TCC did not directly alter cardiomyocyte amino acid metabolism, possibly causing elevated *BNP* through endothelial-cardiomyocyte interaction**

**(a-i)** RT-PCR analyses ofmRNA levels of amino acid transporter in AC16 cells exposed to TCC for 24 h.

**(j)** Quantitative detection of ADMA in AC16 exposed to TCC for 24 h.

**(k)** mRNA levels of *BNP* in AC16 cells exposed to TCC for 24 h.

**(l)** LDH cytotoxicity assay of AC16 cells exposed to TCC for 24 h.

**(m)** CCK-8 cell viability assay of AC16 cells exposed to TCC for 24 h.

**(n)** mRNA levels of *BNP* in AC16 cells treated with medium from TCC-treated-human aortic endothelial cells (HAEC) for 24 h.

**(o)** mRNA levels of *BNP* in AC16 cells treated with medium from TCC-treated-HUVECs for 24 h.

**(p)** mRNA levels of *BNP* in AC16 cardiomyocytes co-cultured with HUVECs under TCC exposure for 48 h.

**(q)** mRNA levels of *BNP* in AC16 cardiomyocytes co-cultured with HUVECs by transwell under TCC exposure for 24 hours.

**(r)** mRNA levels of *BNP* in AC16 cardiomyocytes co-cultured with HAECs by transwell under TCC exposure for 24 hours.

Data are represented as mean ± SD by one-way ANOVA test.



**Figure S6. Arginine metabolite ADMA promotes a hypertrophic phenotype in cardiomyocytes and cardiac organoids.**

**(a)** mRNA levels of *BNP* in AC16 cells treated with ADMA for 24 hours.

**(b)** Representative images of AC16 cells stained with TNNT2 after treatment with 100 μmol/L ADMA. (scale bar = 50 μm).

**(c)** Surface area of AC16 myocardial cells treated with 100 μmol/L ADMA. *n*=100 cells per group.

**(d)** Perimeter of AC16 myocardial cells treated with 100 μmol/L ADMA. *n*=100 cells per group.

**(e)** mRNA levels of *BNP* in iPSC-CMs treated with 100 μmol/L ADMA for 24 hours.

**(f)** Representative images of iPSC-CMs stained with Actinin and TNNT2 after treatment with 100 μmol/L ADMA. (scale bar = 20 μm).

**(g)** Surface area of iPSC-CMs treated with 100 μmol/L ADMA. *n*=237 cells for control and 179 cells for ADMA group.

**(h)** Perimeter of iPSC-CMs treated with 100 μmol/L ADMA. *n*=237 cells for control group and 179 cells for ADMA group.

**(i)** mRNA levels of *BNP* in cardiac organoids treated with 100 μmol/L ADMA for 24 hours.

Data are represented as mean ± SD by one-way ANOVA test.



**Figure. S7. TCC caused similar changes in human cardiac microvascular endothelial cells (HCMEC).**

**(a)** Quantification of ROS fluorescence staining with DCFH-DA in HCMECs.

**(b)** Representative image of ROS fluorescence staining with DCFH-DA fluorescent probe on HCMECs treated with TCC (scale bar = 50 μm).

**(c)** Flow cytometry quantitative analysis of NO content stained with DAF-FM DA in HCMECs.

**(d)** mRNA levels of iNOS in HCMECs exposed to TCC for 24 hours.

**(e)** ADMA abundance in HCMECs after TCC treatment for 24hours.

**(f)** Immunoblots of nitrosylated cysteines (Cys-SNO) and GAPDH in HCMECs samples after 0, 1 and 3 hours of TCC exposure.

**(g)** Densitometric analysis of total protein Cys-SNO bands in each group.









**Figure S8**. Full-length images of the blots presented in the main figures and supplementary figures.

**Supplementary movie 1**

Cardiac organoids began to beat rhythmically at around 5.5 days.

Reference

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