Science Bulletin xxx (xxxx) xxx

Contents lists available at ScienceDirect

Science Bulletin

journal homepage: www.elsevier.com/locate/scib

Short Communication

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

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ARTICLE INFO

Article history:
Available online xxxx

Environmental pollution is an important but often overlooked risk factor for cardiovascular diseases such as heart failure. Triclocarban (3,4,4′-trichlorocarbanilide, TCC) is a broad-spectrum antibacterial agent commonly used in personal care products such as antibacterial soaps, detergents, toothpaste, and cosmetics [1]. Unfortunately, due to its widespread use, TCC has become one of the top ten most common water pollutants globally, and it is present in the atmosphere, soil, and aquatic sediments [2]. Especially during the post-COVID-19 pandemic era, antibacterial agents such as triclosan (TCS) and TCC have reached alarming levels in the environment [3]. In 2014, the highest concentration of TCS in the surface water of Wuhan East Lake was 6.5 ng/L [4]. While in the winter of 2020, after the outbreak of COVID-19, it reached 466 ng/L [5]. Due to its high lipophilicity and slow environmental degradation rate, TCC can accumulate in the environment and be absorbed by biological organisms [1]. Recently, a study confirmed the association of exposure to antimicrobial agents TCC with coronary heart disease in humans [6]. An animal study in mice also suggests TCC toxicity to the heart [7]. However, the exact impact of TCC environmental exposure on human cardiovascular health and the underlying mechanisms remain unknown.

To better simulate the pathophysiological complexity of the human heart and reduce the reliance on animal experiments, we used human cardiac organoids (hCOs) from human induced pluripotent stem cells (hiPSCs) as a research model to explore the effects and mechanisms of TCC exposure at environmentally relevant doses (1, 2, 5 μmol/L) [8,9]. Throughout the differentiation process (Fig. 1a), hCOs significantly increased in size (Fig. 1c,d), formed chamber-like structures intrinsically (Fig. 1b), and began to beat rhythmically at around 5.5 d (Supplementary Movie 1 online). Based on gene expression data, hCOs showed a higher level of complexity in cardiac cell lineage, including populations of endocardium, epicardium, endothelium and cardiac fibroblasts (Fig. S1a–d online). Moreover, we found cardiac development transcription factor expression of first and second heart fields specification (FHF and SHF) in hCOs (Fig. S1e,f online) was similar to that observed in fetal heart tissue in previous studies [10]. TNNT2 and CDH5 were used for myocardial cells and endothelial cells in cryo-sectioning and 3D immunofluorescence staining, respectively. We found cardiomyocytes are the main cell type, which existed throughout the hCOs differentiation (Fig. 1e). In 3D staining, we

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https://doi.org/10.1016/j.scib.2024.11.037

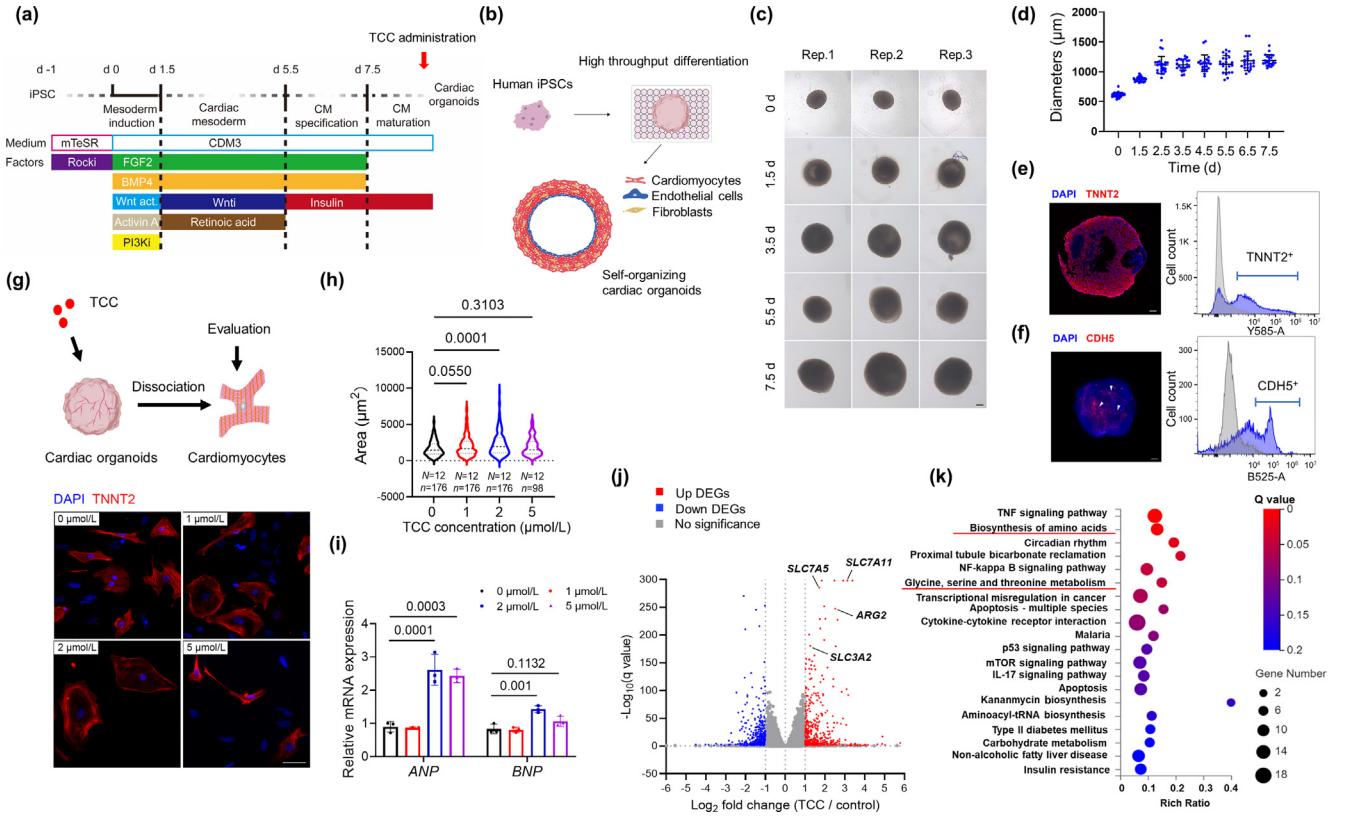

Fig. 1. Establishment and characterization of self-organizing cardiac organoids and transcriptomic change after TCC exposure. (a) Cardiac organoids differentiation scheme. TCC was added for exposure treatment after maturation; (b) Schematic diagram of hiPSCs induced self-organizing cardiac organoids construction; (c) Morphological presentation of organoids on different days with rhythmic autonomous beating, bar = 200 μm; (d) Diameters of cardiac organoids on different days. n = 30 on day 0, and those died during culturing were discarded; (e) Immunofluorescence and flow cytometry images of the cardiac-specific marker gene TNNT2 in myocardial cells on day 10, bar = 200 μm; (f) Positive staining of endothelial-specific marker CDH5 (white arrows) and flow cytometric analysis of the percentage of endothelial cells in the hCOs on day 10. The data were shown in mean ± standard deviation (SD). Scale bar = 200 μm. (g) Top: Schematic diagram of myocardial cell culture after digestion and dissociation of cardiac organoids treated with TCC; bottom: Myocardial cell-specific marker TNNT2 (red) immunofluorescence microscopy imaging. Scale bar = 100 μm; (h) Surface area of myocardial cells; The symbol N represents the number of organoids in each group. The symbol n represents a single myocardial cell. (i) mRNA expression of ANP and BNP, n = 3; Data are presented as mean ± SD, by one-way ANOVA; (j) The volcano plot of differentially expressed genes (DEGs) suggests that the expression change of amino acid transporter-related genes are most significant; (k) KEGG annotation suggests changes in amino acids metabolism-related pathways. ANP: natriuretic peptide A; BMP: bone morphogenetic protein; BNP: natriuretic peptide B; CDH5: VE-Cadherin; CDM: chemical defined medium; CM: cardiomyocyte; FGF: fibroblast growth factor; KEGG: Kyoto Encyclopedia of Genes and Genomes; PI3Ki: phosphoinositide 3-kinase inhibitor; Rocki: Rho-associated kinase inhibitor; TCC: 3,4,4′-trichlorocarbanilide; TNNT2: Troponin T; (h)iPSC: (human) induced pluripotent stem cells. Wnt act.: activator of Wnt pathway (CHIR99021). Wnti: Wnt inhibitor; The cartoon schematic diagrams in (b) and (g) were drawn by the authors using PowerPoint and Adobe illustrator.

observed CDH5$^+$ endothelial-like cells inside the hCOs (Fig. 1f). These data reveal that hCOs closely model human fetal cardiac development and produce main cardiac cell lineages, which are suitable for cardiac disease models.

To evaluate the cardiotoxicity of TCC, we exposed human cardiac organoids to 0, 1, 2, and 5 μmol/L of TCC for 24 h, and detected lactate dehydrogenase (LDH) activity in the supernatant of cardiac organoids culture medium. Compared with the control group, no significant changes were observed at 1 and 2 μmol/L doses (Fig. S1g online), while the LDH activity in the medium containing 5 μmol/L TCC increased significantly, suggesting cell membrane damage. Furthermore, the spontaneous beating was significantly slowed and the heart rate decreased in the 5 μmol/L TCC exposure group, rather than in lower doses (Fig. S1h online). Then, cardiac organoids were digested and dissociated into single cells for adherent culture. TNNT2 labeling of myocardial cells revealed that exposure to 1 and 2 μmol/L TCC caused wider microfilament spacing, blurry fibers, and irregular cytoskeleton. While in the 5 μmol/L group, myocardial cells displayed a change in cellular morphology, with cell membrane damage (Fig. 1g). Examination of myocardial cell surface area revealed that exposure to TCC at 1 and 2 μmol/L resulted in a dose-dependent enlargement of cardiomyocytes. Conversely, no notable change in surface area was observed at 5 μmol/L TCC, which may be attributed to toxic effects resulting in partial death of myocardial cells and cell membrane damage (Fig. 1g,h). Then, we analyzed the mRNA expression levels of heart failure indicators natriuretic peptide A (*ANP*) and natriuretic peptide B (*BNP*), and found that exposure to TCC at 2 and 5 μmol/L significantly upregulated their expression (Fig. 1i). The mRNA expression of inflammatory factors *TNF-α*, *IL-1β*, and *IL-6* also significantly increased in the 5 μmol/L TCC group (Fig. S1i–k online). Overall, exposure to 1 and 2 μmol/L TCC caused myocardial hypertrophy in cardiac organoids, but had no significant effect on heart rate and myocardial toxicity, while exposure to 5 μmol/L TCC exhibited more cardiotoxic effect.

Next, we conducted transcriptomic analysis on cardiac organoids exposed to 2 μmol/L TCC for 24 h. TCC exposure resulted in significantly upregulated expression of *SLC7A11*, *SLC7A5*, and *SLC3A2* (Fig. 1j and Fig. S2b online), members of the solute carrier (SLC) family of membrane transporters that are associated with amino acid transport [11], suggesting that TCC exposure may affect amino acid transport in cardiac organoids. Furthermore, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis showed that TCC exposure affected amino acid synthesis at the level of metabolic pathways (Fig. 1k). RT-PCR further confirmed the increased mRNA expression of *SLC7A11*, *SLC7A5*, *SLC3A2*

and arginase II (*ARG2*) in cardiac organoids (Fig. S2c online). WB analysis showed that the protein expression of amino acid transport marker SLC7A11 increased in a dose-dependent manner after 1 and 2 μmol/L TCC exposure (Fig. S2d,e online). Taken together, these results suggest that TCC exposure induced reprogrammed amino acid metabolism in cardiac organoids.

To evaluate the effect of TCC exposure on cardiac organoid metabolism, we performed a global metabolic profile analysis using ^{1}H NMR and validated it with LC/MS-based targeted metabolomics. Multivariate statistical analysis of ^{1}H NMR data showed that TCC exposure induced dose-dependent metabolic alterations in cardiac organoids (Fig. S3, Table S3 online). Compared to the control, TCC treatment for 24 h induced significant metabolic changes including lactate, myo-inositol and amino acids (Figs. S3 and S4b online) in cardiac organoids. In particular, TCC exposure led to a significant increase in dimethylamine (DMA) levels and a significant depletion of myoinositol, arginine and glutamate (Fig. 2a–d) with the increase in exposure concentration. Previous studies suggest DMA is a downstream metabolite of methylated arginine (Fig. 2e). Its precursor, asymmetric dimethylarginine (ADMA), is an endogenous nitricoxide synthase (NOS) inhibitor that affects nitricoxide (NO) function and is elevated in the serum

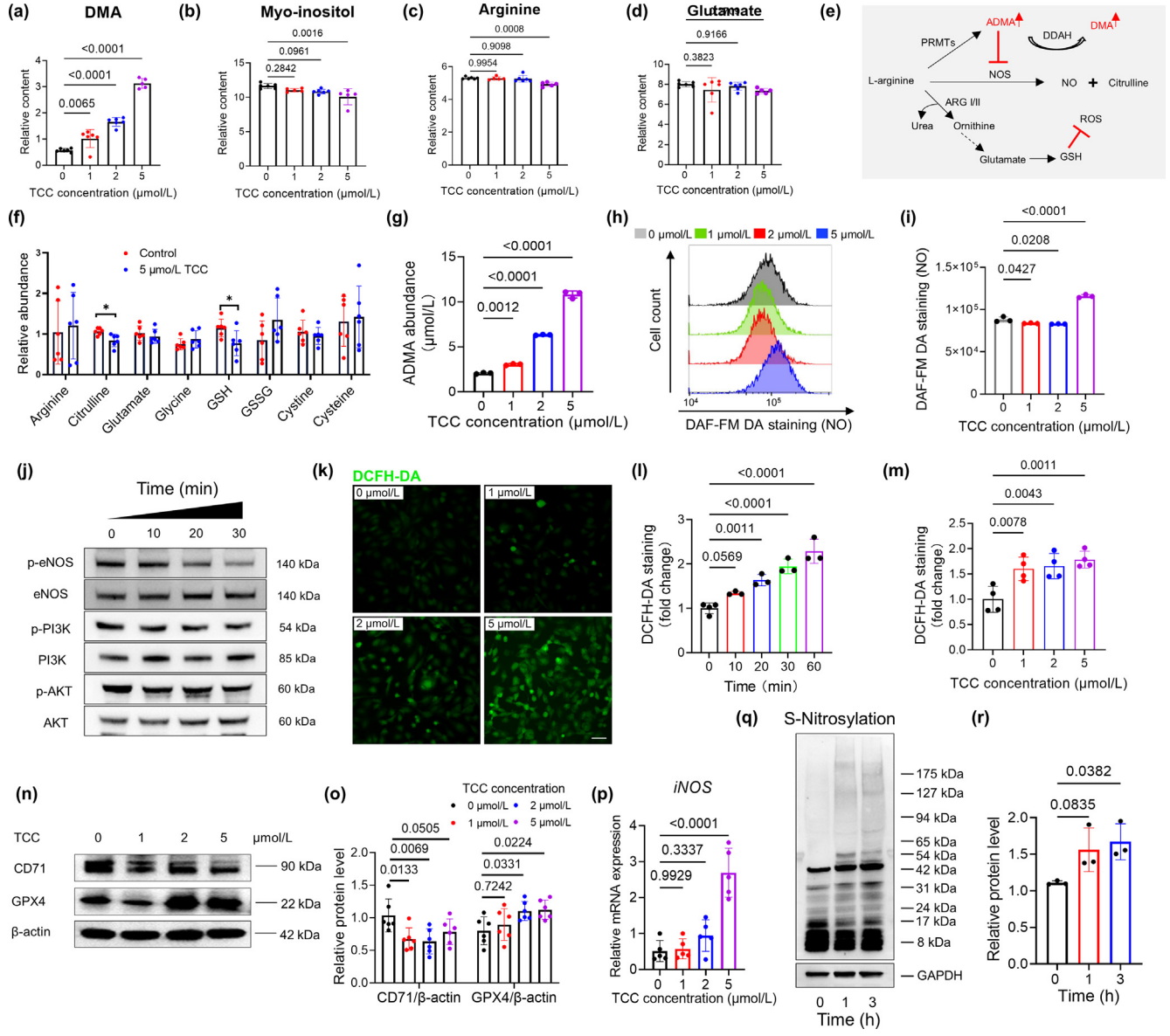

Fig. 2. Metabolic profiles of amino acids are altered in cardiac organoids and HUVECs. (a-d) Relative abundance of (a) DMA, (b) myoinositol, (c) arginine and (d) glutamate in cardiac organoids from ^{1}H NMR metabolomics analysis. (e) Arginine and dimethylamine metabolism-related pathways. TCC exposure affects NO balance of arginine metabolism in HUVECs. (f) LC-MS quantitative detection of changes in metabolites related to arginine metabolism pathway in HUVECs after TCC exposure, two-tailed unpaired student's *t*-test. (g) Quantitative detection of metabolite ADMA in HUVECs; (h,i) Flow cytometry quantitative analysis of NO content stained with DAF-FM DA in HUVECs; (j) Proteins expression of the PI3K/AKT-eNOS pathway activation in TCC-stimulated HUVECs; (k) Representative image of ROS fluorescence staining with DCFH-DA fluorescent probe on HUVECs treated with TCC (scale bar = 50 μm); (l,m) Fluorescence intensity of ROS was analyzed by flow cytometry DCFH-DA staining at 2 μmol/L TCC (l) for 4 h (m); (n,o) Representative western blotting and quantitive analysis of band intensity of CD71 and GPX4 expression after 48 h of TCC exposure; The intensity of the band for all the proteins mentioned above was calculated as a ratio to β-actin. (p) mRNA levels of *iNOS* in HUVECs exposed by TCC for 24 h. (q) Immunoblots of nitrosylated cysteines (Cys-SNO) and GAPDH in HUVECs samples after 0, 1 and 3 h of 2 μmol/L TCC exposure; (r) Densitometric analysis of total protein Cys-SNO bands in each group. Multiple comparison tests were conducted by ordinary one-way ANOVA except special instructions. Numbers above lines show *P* values. ARG: Arginase; DCFH-DA: dichlorodihydro-fluorescein diacetate; DDAH: dimethylarginine dimethylamine hydrolase; DMA: dimethylamine; eNOS: endothelial nitric oxide synthase; GSH: glutathione; GSSG: oxidized glutathione; iNOS: inducible nitric oxide synthase; LC-MS: liquid chromatography-mass spectrometry; NMR: nuclear magnetic resonance; PRMTs: arginine methyltransferases; ROS: reactive oxygen species.

of patients with heart failure [12]. Upon exposure to increasing doses of TCC, an increase in the concentration of ADMA was detected (Fig. S4a online). These findings indicate that TCC exposure affects amino acid metabolism in cardiac organoids, particularly in the arginine metabolism pathway.

Since cardiomyocytes and endothelial cells are the two most abundant types of cells in cardiac organoids, based on the above transcriptomic and metabolomic results, we validated the effects of TCC on cardiomyocytes and endothelial cells, respectively. In human umbilical vein endothelial cells (HUVECs), the mRNA expression of *SLC7A11*, *SLC3A2*, *SLC7A5*, and *ARG2* significantly increased with the increase of TCC exposure dose (Fig. S4c online), and the expression level of SLC7A11 protein was also significantly increased (Fig. S4d,e online), which was also consistent with the transcriptomic results of organoids. However, the mRNA expression level of these genes in myocardial cells AC16 was not significantly changed (Fig. S5a–i online). Absolute quantitative detection of ADMA (DMA precursor) showed a significant increase in HUVECs (Fig. 2g) but not in AC16 (Fig. S5j online). Targeted metabolomics for amino acids-related metabolites in HUVECs after TCC exposure showed that citrulline and the endogenous antioxidant metabolite glutathione (GSH) were significantly reduced (Fig. 2f). In addition, TCC treatment of AC16 cells alone did not result in changes in cell viability nor *BNP* levels (Fig. S5k–m online). However, supernatants from endothelial cells after TCC treatment resulted in elevated *BNP* levels in AC16 cells. *BNP* was also elevated after TCC treatment when AC16 was co-cultured with HUVECs (Fig. S5n–r online). It can therefore be speculated that it is the endothelial cell metabolites that cause the cardiomyocyte response. In the previous section, we found that TCC treatment resulted in a characteristic elevation of ADMA in endothelial cells (Fig. 2g). Then we treated cardiomyocytes and cardiac organoids with ADMA, which led to a hypertrophic phenotype (Fig. S6 online), suggesting that ADMA is likely one of the metabolites in the crosstalk between endothelial cells and cardiomyocytes.

These results indicated that the metabolic changes in cardiac organoids induced by low-dose TCC exposure are primarily due to its action on endothelial cells, and mainly affect the arginine metabolic pathway. As a substrate of NOS, arginine plays an important role in the synthesis of NO [12]. Subsequently, we investigated the effects of TCC exposure on endothelial cell function. DAF-FM DA (4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate) probe staining was used to detect intracellular NO production. Flow cytometry showed that TCC (1 and 2 μmol/L) exposure resulted in decreased NO production (Fig. 2h,i). In addition, the protein phosphorylation levels in the PI3K/AKT-eNOS pathway showed a significant decrease in time-dependent way (Fig. 2j and Fig. S4h–j online), suggesting that 2 μmol/L TCC exposure affected NO synthesis. However, 5 μmol/L TCC exposure induced high expression of pro-inflammatory mediator iNOS (Fig. 2p), accompanied by increased levels of NO (Fig. 2h,i) and inflammatory cytokines (Fig. S4g online). Overall, TCC exposure affected arginine metabolism and NO balance in endothelial cells and caused endothelial dysfunction and inflammation.

Oxidative stress is the major inducer of endothelial dysfunction and reduction in NO bioavailability. The imbalance of nitro-redox signals between endothelial and myocardial cells affects myocardial cell hypertrophy and angiogenesis [13]. Next, we characterized ROS levels in HUVECs treated with different concentrations of TCC by DCFH-DA (dichloro-dihydro-fluorescein diacetate) staining. An increase in intracellular ROS content was observed from 10 min to 1 h after TCC stimulation (Fig. 2k–m), indicating that TCC induced significant ROS production in time-dependent and dose-dependent way. Cysteine/glutamate transporter (xCT system) is an important subtype of amino acid transporter family [14]. As a functional subunit of the xCT system, SLC7A11 is involved in extracellular uptake and release of cysteine, promotes the synthesis of GSH, protects cells from oxidative stress, and maintains cell redox balance. We observed that the expression of *SLC7A11* and *SLC3A2* genes was simultaneously significantly increased in cardiac organoids exposed to TCC (Fig. S2 c–e online). Glutathione peroxidase (GPX4) mRNA and protein expression levels were also significantly increased (Fig. 2n-o and Fig. S4k online). Meanwhile, the expression of transferrin receptor (CD71) significantly decreased with increasing TCC exposure dose (Fig. 2n,o), suggesting that TCC exposure may affect iron homeostasis, cause the xCT-GPX4 system to be activated to protect cells from oxidative stress damage and maintain cell redox balance. The heightened iNOS activity and nitrosative stress can lead to the S-nitrosylation of cysteine residues in many proteins, which in turn disrupts their function. Previous studies have shown that nitrosative stress of cardiomyocytes drives heart failure with preserved ejection fraction (HFpEF) [15]. Accordingly, iNOS was activated and highly expressed as the TCC exposure dose increases (Fig. 2p). We also observed an increase in total protein nitrosylation (Fig. 2q,r) in TCC-treated HUVECs, and these were also validated in human cardiac microvascular endothelial cells (Fig. S7 online). Together, these data suggest that TCC exposure caused oxidative stress and a high expression of pro-inflammatory mediator iNOS in HUVECs, which drives total protein nitrosylation, thereby affecting endothelial function.

In summary, our findings indicated that exposure to environmental contaminant antimicrobial agent TCC can induce hypertrophy and metabolic remodeling of hCOs, and confirmed the early promoting role of endothelial cell metabolism in pathological cardiac hypertrophy, with doses that induce changes in endothelial metabolism preceding myocardial injury. Mechanistically, TCC exposure interferes with NO balance by altering arginine metabolism in cardiac organoid endothelial cells. As the dose increases, ROS production and iNOS activation lead to nitrosative stress and inflammation in endothelial cells, causing endothelial dysfunction and ultimately promoting cardiac organoids myocardial hypertrophy. These results suggest that endothelial arginine metabolism pathway and nitrosative stress may be a new treatment target for myocardial hypertrophy.

Heart failure is the terminal stage of various cardiovascular diseases, usually characterized by pathological myocardial hypertrophy. So far, the pathogenesis of heart failure is not fully understood. The global burden of cardiovascular disease and epidemiological evidence indicate that in addition to traditional risk factors such as genetic inheritance and hypertension, exposure to exogenous environmental pollutants is a new risk factor. In recent years, the use of antimicrobials has increased, resulting in more exposure of these substances to humans, raising concerns about potential risks to human and environmental health [1]. According to the findings of our study, the exposure to antibacterial agent TCC may be a new risk factor for metabolic cardiovascular diseases. This conclusion is supported by physiological indicator tests and a combined analysis of metabolomics and transcriptomics in cardiac organoids.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

This work was supported by the National High Technology Research and Development Program of China (2020YFA0803700 and 2023YFA1800904), the Natural Science Foundation of Beijing, China (L232031 and J230039), the Postdoctoral Fellowship Program of CPSF (GZC20240068), the China Postdoctoral Science

Foundation (2021M690252 and 2021TQ0019), and the National Natural Science Foundation of China (82371363). The graphical abstract was drawn by Figdraw. We would like to express our sincere gratitude to Dr. Yang Li (Peking University, Beijing, China) for his generous contribution to the iPSCs. We thank the Facility Center of Metabolomics and Lipidomics of China National Center for Protein Sciences (Tsinghua University) for the metabolomics profiling.

Author contributions

Lemin Zheng, Manyuan Dong, Nana Yang, Jiahong Chen and Yujie Zhu conceived of the presented idea. Manyuan Dong, Jiahong Chen, Yujie Zhu and Nana Yang carried out the experiment and wrote the manuscript. Wenxin Shan contributed to the graphical abstract. Zheng Cao, Yukun Xiang, Shusi Ding, Yaobo Zhao, Liang Ji and Zhaomeng Wang helped with metabonomics and LC-MS analysis. Yiwen Fu helped with manuscript improvement. Huanhuan Cao, Rui Zhan and Yufei Wu provided critical feedback. Lemin Zheng supervised the project with the help of Nana Yang. All authors discussed the results and contributed to the final manuscript.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scib.2024.11.037.

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