BPB Reports

Regular Article

The Antibiotic Cefotaxime Works as Both an Activator of Nrf2 and an Inducer of HSP70 in Mammalian Cells

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Received November 27, 2019; Accepted January 23, 2020

Both NF-E2-related factor 2 (Nrf2) and heat shock protein 70 (HSP70) contribute to cellular defense to various stresses, and have emerged as candidates of therapeutic targets to improve or prevent tissue damage. Cefotaxime (CTX), a third-generation cephalosporin antibiotic, is conceived as a safe drug largely free from side effects. CTX exhibits broad-spectrum antimicrobial activity, and thereby, is most commonly prescribed for the treatment of infectious diseases induced by Gram-positive or Gram-negative bacteria. In this study, we unexpectedly found the beneficial properties of CTX that upregulate both Nrf2 and HSP70 to the extent that stress-induced damage is ameliorated. Non-toxic levels of reactive oxygen species (ROS) induced by CTX activated the Nrf2 pathway without cytotoxicity, which in turn upregulated HSP70. Interestingly, the cytotoxicity of Fas/CD95 ligand (FasL), a cytotoxic cytokine that strongly induces apoptosis, was significantly ameliorated by pretreatment with CTX, most likely because of the upregulation of Nrf2 and HSP70. Our results therefore show novel properties of CTX, which raise the possibility that CTX works as a non-toxic therapeutic agent for preventing and repairing tissue damage.

Key words Nrf2, HSP70, ROS, cefotaxime, cephalosporin antibiotic

INTRODUCTION

Antibacterial cephalosporins are a class of β-lactam antibiotics that contain β-lactam and hetero six-membered rings as basic skeletons, and constitute a subgroup called cephems together with cephamycins. The first generation of the cephalosporins is predominantly active against Gram-positive bacteria, whereas the successive generations have increased activity against Gram-negative bacteria. Therefore, the cephalosporins are broad-spectrum antibiotics used to control various infectious diseases. Cefotaxime (CTX), a third-generation cephalosporin listed on the World Health Organization's List of Essential Medicines, is one of the most commonly prescribed antibiotics for the treatment of infectious diseases.[1,2)]

Reactive oxygen species (ROS) are continuously generated by aerobic metabolism. Since excessive ROS accumulation induces oxidative stress that brings about severe cellular dysfunctions and cell death, intracellular ROS are immediately eliminated by various antioxidant proteins.[3)] NF-E2-related factor-2 (Nrf2), a transcription factor, is critical for the upregulation of antioxidant proteins.[4)] Under resting conditions, Nrf2 is continually ubiquitinated by the Keap1-Cullin3 ubiquitin ligase complex and is routinely degraded by the 26s proteasome. However, upon oxidative stress conditions, the activity of the ubiquitin ligase is blocked through the oxidative modification of cysteine residues in Keap1, which allows stabilization and nuclear translocation of Nrf2. Then, Nrf2 exerts its transcriptional activity through binding to the antioxidant response element (ARE) that governs the antioxidant gene expression.[5)] Nrf2 is therefore a master regulator of the oxidative stress response, and it has been suggested that pharmaceutical activation of Nrf2 could be a potential therapeutic approach for tissue damage caused by oxidative stress. On the other hand, heat shock protein 70 (HSP70), an inducible molecular chaperone, has also emerged as a candidate therapeutic target to improve oxidative stress-induced tissue damage.[6)] Moreover, previous reports have demonstrated the effectiveness of the Nrf2 activators and HSP70 inducers as mucosal protectants for reflux esophagitis and gastric lesions, respectively.[7–10)] However, there are few non-toxic agents that strongly activate Nrf2 or induce HSP70.

In this study, we found novel and unexpected properties of CTX that may contribute to tissue repair and protection, indicating the possibility of CTX as a therapeutic agent for tissue damage.

MATERIALS AND METHODS

Cell Culture and Reagents Human fibrosarcoma cell line HT1080 and mouse embryonic fibroblasts (MEF) were grown in Dulbecco's Modified Eagle Medium (DMEM), 10% heat-inactivated fetal bovine serum (FBS), and 1% penicillin-streptomycin solution, at 37°C under a 5% CO_2 atmosphere. All reagents were obtained from commercial sources; CTX, NAC (Wako, Tokyo, Japan), FasL (Enzo Life Science, Farmingdale, NY, USA). The antibodies used were against Nrf2

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(C20), HSP70 (W27), Lamin A/C (636) (Santa Cruz, Dallas TX, USA), p38 (#9212), p-p38 (#9211), JNK (#9252), p-JNK (#9251) (Cell Signaling, Danvers, USA), and β-actin (Wako, Tokyo, Japan).

Trypan Blue Dye Exclusion Assay Cells were seeded on 24-well plates. After indicated stimulation, cells were harvested with trypsin followed by staining with 0.5% trypan blue dye. The trypan blue-positive cells were counted by using a hemocytometer under phase-contrast microscopy as described previously.[11]

Immunoblot Cells were lysed with the 1% Triton X-100 buffer [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton-X100, 10% Glycerol, and 1% protease inhibitor cocktails (Nacalai Tesque, Kyoto, Japan)]. After centrifugation, the cell extracts were resolved by SDS-PAGE, and analyzed by immunoblotting as described previously.[12] The blots enhanced by chemiluminescence ECL (Merck Millipore, Burlington, USA) were developed using ChemiDoc Touch (Bio-Rad, Hercules CA, USA).

Generation of Knockout Cell Lines Knockout cells were generated using Clustered Regularly Interspaced Short Palindromic Repeat/CRISPR-associated protein-9 nuclease (CRISPR/Cas9) system. Guide RNAs (gRNAs) were designed to target a region in the exon 3 of *Nrf2* gene (5'-CTGGGCTCTCGATGTGACC-3') using CRISPRdirect. gRNA-encoding oligonucleotide was cloned into gRNA cloning vector (Addgene, Watertown, NY, USA), and knockout cells were established. To determine the mutations of Nrf2 in cloned cells, genomic sequence around the target region was analyzed by PCR-direct sequencing using extracted DNA from each clone as a template and the following primers: 5'-AGCTCCTCCCAAACTTGCTC-3' and 5'-CCCCTGTTGGTGGAAGACTC-3'.

Bioimaging and Quantification of ROS HT1080 cells were seeded on glass plates. After stimulation, cells were treated with 10 μM DCFH-DA (Merck Millipore, Burlington, USA) for 30 min at 37 °C. After washing with PBS, the intracellular ROS generation was observed using a Zeiss LSM800 laser confocal microscope (Carl Zeiss, Oberkochen, Germany), and the images were processed with Zen software (Carl Zeiss, Oberkochen, Germany). The fluorescence images were obtained from three different fields of view. Data shown are the mean ± SD of three images.

Quantitative Real-Time PCR Total RNA was extracted using Sepasol-RNA I Super G (Nacalai Tesque, Kyoto, Japan) and reverse transcribed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City CA, USA) according to the manufacturer's instructions. Template cDNA was sampled by using Luna Universal qPCR Master Mix (New England Biolabs, Ipswich, USA) according to the manufacturer's instructions, and amplified by quantitative real-time PCR (qRT-PCR) using UBI prism 7000 (Applied Biosystems, Foster City CA, USA) as described previously.[13] Primers used for qRT-PCR; 5'-CCGTGGATCCCTTGCAGAGA-3' and 5'- AGGACCCTTCCGGAGTAAGA-3' for human *Nqo1*, 5'- CGTGCTCATCTTTGACCTG -3' and 5'-TGTTTTCTCTTGAACTCCTCCAC -3' for human *HSP70*, 5'-AACAGCCTCAAGATCATCAGC -3' and 5'-GGATGATGTTCTGGAGAGCC -3' for human *GAPDH*. Each gene expression levels were normalized to that of *GAPDH*.

Nuclear Extraction Cells were lysed in ice-cold lysis buffer containing 10 mM HEPES (pH 7.5), 10 mM KCl, 0.1 mM EGTA, 0.1 mM EDTA, 1 mM DTT, and 1% protease inhibitor cocktails (Nacalai Tesque, Kyoto, Japan) for 15 min. Cell lysates were added 1% NP-40, and then centrifuged at 4 °C at 2,500 rpm for 3 min. After the supernatants containing cytoplasmic fraction were removed, the pellets were suspended in ice-cold lysis buffer containing 20 mM HEPES (pH 7.5), 400 mM NaCl, 1 mM EGTA, 1 mM DTT, and 1% protease inhibitor cocktails for 15 min with vortexed every 5 min. Cell lysates were then centrifuged at 4 °C at 15,000 rpm for 15 min, and then the supernatants were collected as nuclear fractions.

Colorimetric Cell Viability Assay Cell viability assay was performed as described previously.[14] Cells were seeded on 96-well plates. After indicated stimulation, cell viability was measured by phenazine methosulfate (PMS)/3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay using Cell Titer 96 Cell Proliferation Assay kit (Promega, Madison, USA), according to the manufacturer's protocol. The absorbance was read at 492 nm using a microplate reader. Data are normalized to control (100%) without stimulus.

RESULTS AND DISCUSSION

Nontoxic Concentration of CTX Induces ROS Generation CTX is conceived as a safe drug largely free from side effects, and hence its effects on human cells have not been well studied. We have recently demonstrated that CTX has an ability to induce cell death by promoting mitochondrial reactive oxygen species (ROS) generation in human fibrosarcoma HT1080 cells.[15] However, the experimental concentrations of CTX were much higher than clinical concentrations, and then we examined its cellular effects at lower concentrations that are clinically attainable.[16] The cell viability assay assessed by counting cells revealed that treatment with CTX for 48 h reduced viability of HT1080 cells in a dose-dependent manner (Fig. 1A). To determine whether the reduction of cell viability is responsible for cell death, we performed trypan blue dye-exclusion assay to calculate the number of dead cells, and found that little trypan blue positive cells (dead cells) are observed in cells treated with lower concentrations of CTX (200 or 400 μg/mL) at least until 72 h treatment, indicating that the reduction of cell number is caused by other factors including growth inhibition rather than cell death (Fig. 1B). On the other hand, microscopic analysis using the ROS indicator 2', 7'-dichlorodihydrofluorescein diacetate (DCFH-DA) revealed that CTX promotes ROS generation at the nontoxic concentrations of CTX (200 or 400 μg/mL) (Fig. 1C and 1D).

Nontoxic Concentration of CTX Activates the Nrf2 Signaling Pathways As a representative of ROS-inducible transcription factor, NF-E2-related factor-2 (Nrf2) plays essential roles in the maintenance of cellular redox homeostasis and the protection against ROS-mediated cytotoxicity.[4] Indeed, in HT1080 cells, our recent study has demonstrated that the pharmacological inhibition of Nrf2 exacerbates the cytotoxicity induced by polymyxin B, a polypeptide antibiotic that causes undesirable cytotoxic side effects.[17] In a manner similar to ROS generation, we observed that CTX promotes nuclear translocation of Nrf2, a hallmark of its activation, even if the nontoxic concentrations of CTX (200 or 400 μg/mL) are treated (Fig. 2A). Moreover, consistent with the dose-dependent activation of Nrf2, mRNA of NAD(P)H: quinone acceptor oxidoreductase 1 (Nqo1), a well-known target of Nrf2, was upregulated in a dose-dependent manner, which was signifi-

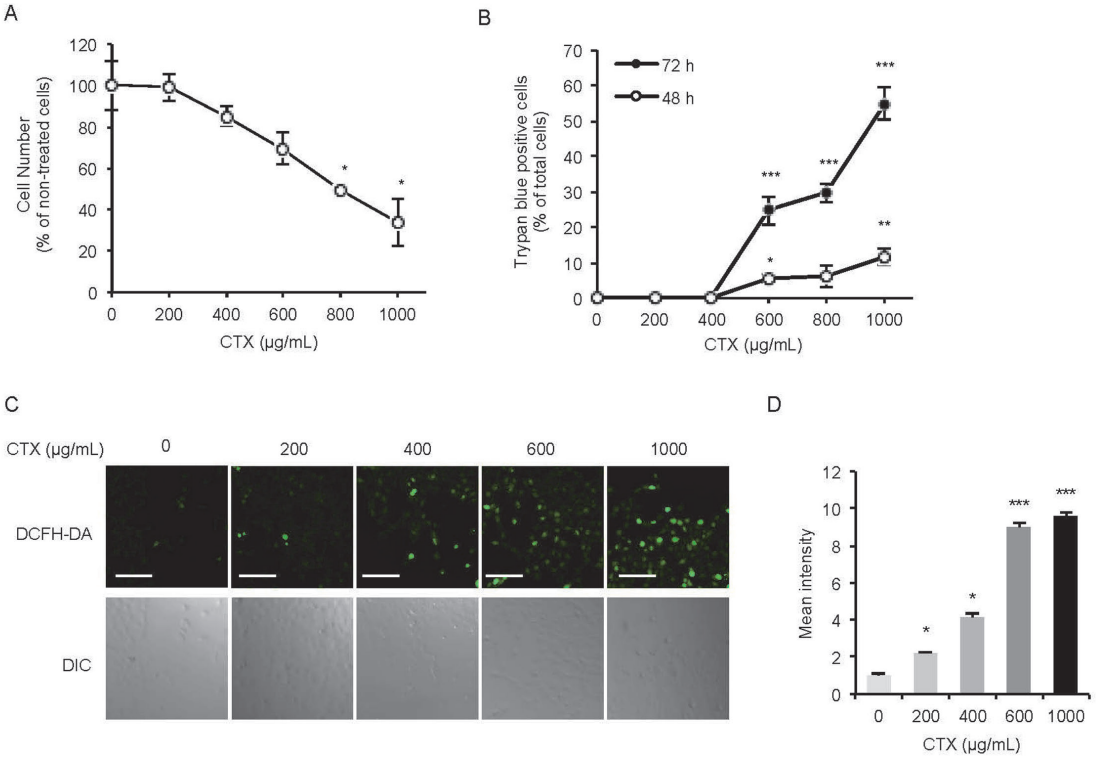

Fig. 1. Nontoxic Concentration of CTX Induces ROS Generation

(A)(B) HT1080 cells were treated with the indicated concentrations of CTX, and then subjected to cell counting assay (A) and trypan blue dye-exclusion assay (B). The vertical axis shows % of non-treated cells (C)(D) HT1080 cells were treated with the indicated concentrations of CTX for 24 h, and then treated with 10 μM DCFH-DA. Fluorescence images (C) and intensity (D) of HT1080 cells were acquired as described in supporting information. Cell morphology was determined by Nomarski differential interference contrast (DIC) microscopy. Scale bar, 200 μm. (A)(B)(D) All data shown are the mean ± SD. Significant differences were determined by one-way ANOVA, followed by Tukey-Kramer test; *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. All data are representative of at least three independent experiments.

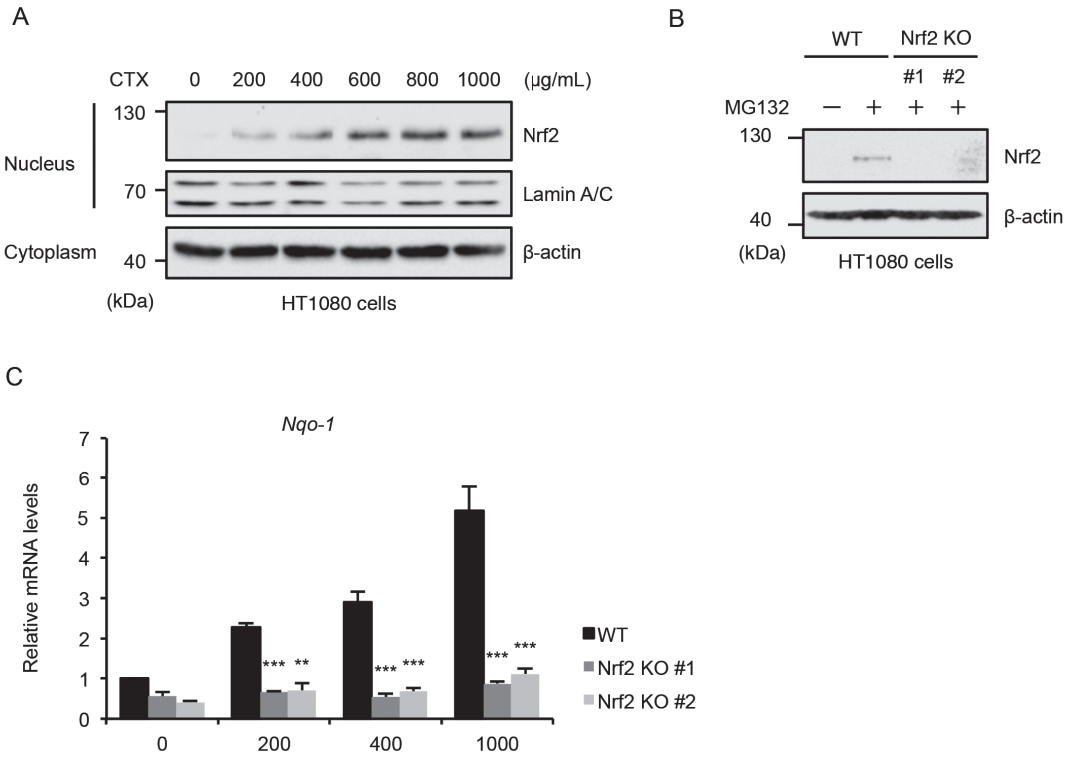

Fig. 2. Nontoxic Concentration of CTX Activates the Nrf2 Signaling Pathways

(A) HT1080 cells were treated with the indicated concentrations of CTX for 16 h, and then the nuclear and cytoplasmic extracts were subjected to immunoblotting with the indicated antibodies. Lamin A/C is loading control for nuclear proteins. (B) Since Nrf2 is routinely degraded by the 26s proteasome, the expression levels of Nrf2 were examined in the presence of proteasome inhibitor MG132. After treatment with proteasome inhibitor 10 μM MG132 for 3 h in order to stabilize Nrf2 protein, whole cell extracts of HT1080 cells were subjected to immunoblotting with the indicated antibodies. (C) HT1080 cells were treated with the indicated concentrations of CTX for 24 h, and then the mRNA levels were measured by quantitative real-time PCR using. Data shown are the mean ± SD. Significant differences were determined by one-way ANOVA, followed by Tukey-Kramer test; *** $p < 0.001$, ** $p < 0.01$. All data are representative of at least three independent experiments.

cantly attenuated in Nrf2 KO cells (Fig. 2B and 2C). Collectively, these observations indicate that the cellular responses to CTX correlate well with its concentration, and notably, at lower concentrations, CTX generates low levels of ROS that stimulate the Nrf2 activation but not cell death mechanisms.

Nontoxic Concentration of CTX Fails to Activate MAPK Signaling Pathways It is well known that mitogen-activated protein kinase (MAPK) cascades, such as the c-Jun N-terminal kinase (JNK) and p38 MAPK pathways, mediate a wide variety of ROS-dependent cellular responses including cell death, and we have previously demonstrated that a high-molecular mass complex, designated ASK1 signalosome, works as the redox-sensing signaling complex, which determines the threshold for ROS-dependent MAPK activation, and particularly, subsequent cell death.[18–20] As shown in Fig. 3A, hydrogen peroxide (H_2O_2), a representative ROS inducer, clearly promoted the phosphorylation of both JNK and p38, hallmarks of their activation. On the other hand, the nontoxic concentrations of CTX failed to promote the activation of JNK and p38, whereas 1 mg/mL CTX robustly activated JNK, as previously demonstrated (Fig. 3B).[15] Therefore, ROS generation induced by treatment with the lower concentrations of CTX does not seem to reach a threshold concentration that elicits activation of the ASK1 signalosome, which enables to escape from ASK1-medated cytotoxicity. This property of CTX is distinct from other ROS inducers that cause the ASK1-mediated cell death.[12,21,22]

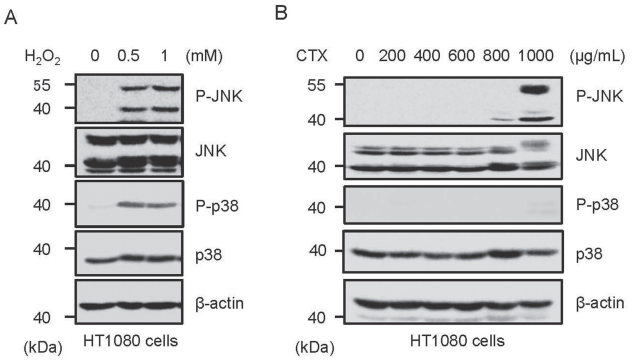

Fig. 3. Nontoxic Concentration of CTX Fails to Activate MAPK Signaling Pathways

(A)(B) HT1080 cells were treated with the indicated reagents for 1 h (A) or 36 h (B), and then immunoblot analysis was performed with the indicated antibodies. P-JNK and P-p38 antibodies specifically detect the phosphorylation required for its activation.

Nontoxic Concentration of CTX Promotes the HSP70 Expression Under oxidative stress conditions, not only Nrf2 but also heat shock factor (Hsf) 1 plays a pivotal role in cellular responses.[23] Interestingly, recent evidence has demonstrated the importance of crosstalk between Nrf2 and Hsf1 to organize cytoprotective transcriptional programs.[24] HSP70 is a transcript induced by both Nrf2 and Hsf1, and functions as a molecular chaperone that protects cells from various stress-

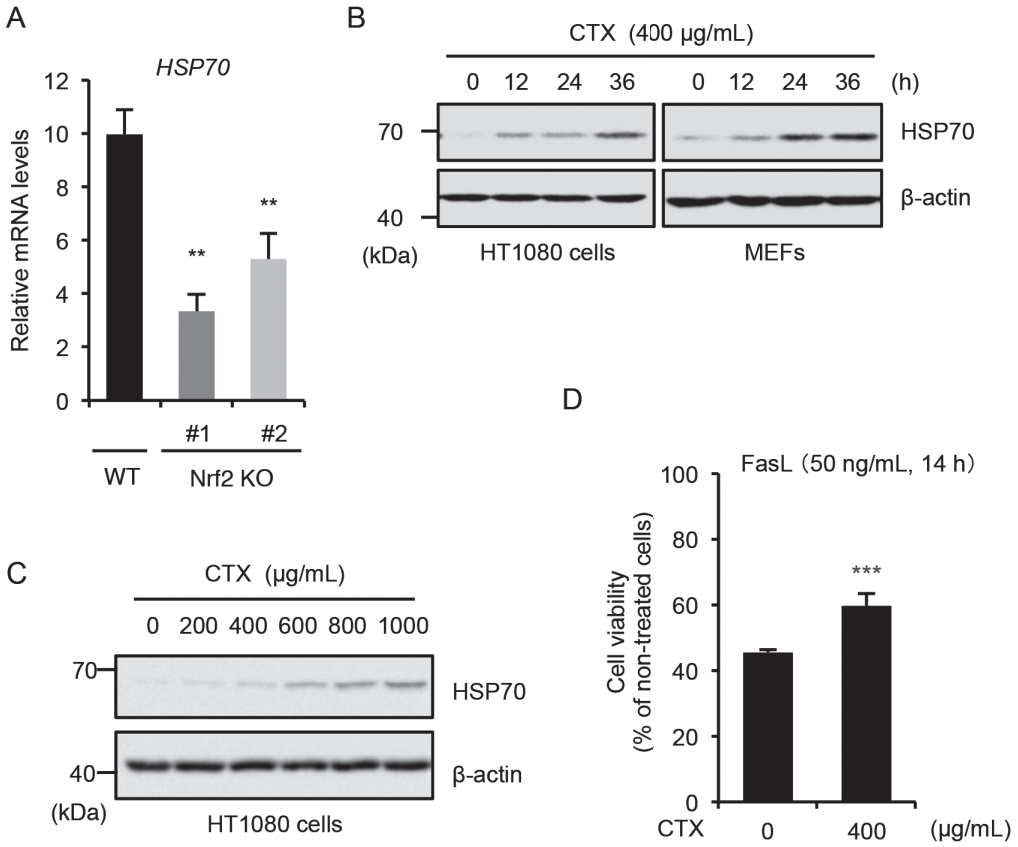

Fig. 4. Nontoxic Concentration of CTX Promotes the HSP70 Expression

(A) HT1080 cells were treated with 400 μg/mL CTX for 24 h, and then the mRNA levels were measured by quantitative real-time PCR. (B) HT1080 cells or MEF cells were treated with 400 μg/mL CTX for the indicated periods, and then immunoblot analysis was performed with the indicated antibodies. (C) HT1080 cells were treated with the indicated concentrations of CTX for 24 h, and then immunoblot analysis was performed with the indicated antibodies. (D) HT1080 cells pretreated with or without 400 μg/mL CTX for 24 h were treated with 50 ng/mL FasL for 14 h, and then subjected to cell viability assay as described in supporting information. (A)(D) Data shown are the mean ± SD. Significant differences were determined by one-way ANOVA, followed by Tukey-Kramer test; *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. All data are representative of at least three independent experiments.

es including drug toxicities.[23,24] We next tested whether CTX upregulates HSP70. As expected, CTX clearly upregulated the mRNA of HSP70, which was partially attenuated in Nrf2 KO cells (Fig. 4A). Moreover, immunoblot analysis revealed that CTX induces HSP70 protein in a time- and dose-dependent manner, indicating that CTX-driven ROS stimulate not only the Nrf2 activation but also the upregulation of HSP70 (Fig. 4B and 4C). In addition, similar result was observed in MEF, another type of cell line, suggesting that CTX upregulates HSP70 in various cell types (Fig. 4B). We next tested whether the Nrf2 activation and the upregulation of HSP70 induced by CTX contribute to the protection from cellular damage. In regard to the protective effect of HSP70, we have recently demonstrated that the downregulation of HSP70 by anti-cancer drug gefitinib accelerates cytotoxicity of Fas/CD95 ligand (FasL),[25] a cytotoxic cytokine associated with liver damage including fulminant hepatitis.[26] Moreover, loss of Nrf2 increased the susceptibility to FasL-mediated cytotoxicity.[27] We therefore tested whether CTX has an ability to protect cells from FasL-mediated cytotoxicity. Interestingly, when cells were pretreated with 400 μg/mL CTX for 24 h, FasL-mediated cytotoxicity was significantly ameliorated, suggesting that CTX-triggered upregulation of Nrf2 and HSP70 exerts its protective effects against FasL-mediated cytotoxicity (Fig. 4D).

Antibiotics greatly contribute to human health by exerting its antibacterial activity. However, little attention has been paid to host cellular responses induced by antibiotics, which may be responsible for the adverse reactions to antibiotics. Unexpectedly, in the present study, we found the beneficial properties of CTX that upregulate both Nrf2 and HSP70 to the extent that stress-induced damage is ameliorated (Fig. 4D), which raises the possibility that CTX works as a nontoxic therapeutic agent for preventing and repairing tissue damage. Accordingly, to investigate the cellular responses induced by antibiotics may lead to not only the elucidation of the molecular mechanisms involved in the adverse reactions, but also the discovery of novel biochemical properties that may provide novel therapeutic strategies.

Acknowledgments This work was supported by JSPS KAKENHI Grant Numbers JP18H02567 and JP18K06622, and by MEXT KAKENHI JP17H05518 and JP19H05282. This work was also supported by the Fugaku Trust for Medicinal Research, the Takeda Science Foundation, and the Division for Interdisciplinary Advanced Research and Education (DIARE) Tohoku University.

Author contributions Author contributions: M.Y., M.S., T.N., and A.M. conceived and designed research; M.Y., M.S., T.Y., Y.S., and N.M. performed research; T.N., T.T., Y.H., G.W.H., and A.M. analyzed data; T.N. and A.M. wrote the paper.

Conflict of interest The authors declare no conflict of interest.

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