Epigallocatechin-3-gallate protects against cisplatin-induced nephrotoxicity by inhibiting endoplasmic reticulum stress-induced apoptosis

Binbin Chen¹, Guangyi Liu^{1,2}, Peimei Zou¹, Xing Li¹, Qiufa Hao¹, Bei Jiang¹, Xiangdong Yang¹ and Zhao Hu¹

¹Department of Nephrology, Qi Lu Hospital of Shandong University, Jinan, Shandong 250012, China; ²Ministry of Education Key Laboratory of Experimental Teratology and Institute of Molecular Medicine and Genetics, Shandong University School of Medicine, Jinan, Shandong 250013, China

Corresponding author: Zhao Hu. Email: huzhaoql@sina.com

The first two authors contributed equally to this work

Abstract

Cisplatin (CP)-induced nephrotoxicity hampers its application in clinic. Green tea, particularly its predominant polyphenolic constituent epigallocatechin-3-gallate (EGCG), possesses anti-inflammatory, antioxidant, and anti-apoptotic properties. The present study was designed to investigate the protective effects of EGCG against CP-induced nephrotoxicity in mice. Male C57/BL6 mice in different groups received single injection of CP (20 mg/kg) and EGCG (100 mg/kg) in various sets and kidney tissues and blood were collected after killing. Then, samples were used for biochemical and immunohistochemical assay. Our results showed EGCG decreased biochemical factors and immunohistochemical damage induced by CP. Besides, expression of phosphorylated-extracellular signal-regulated kinase (p-ERK), glucose-regulated protein 78 (GRP78), caspase-12, and apoptosis of kidney were decreased by EGCG via inhibition of endoplasmic reticulum (ER) stress-induced apoptosis.

Keywords: Cisplatin, apoptosis, endoplasmic reticulum stress, epigallocatechin-3-gallate, nephrotoxicity

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Introduction

Cisplatin (cis-diammine-dichloroplatinum(II) [CP]) is widely accepted as a treatment for various types of malignancies, including gastric, testicular, urologic, and ovarian cancer.¹ Its serious adverse effects, especially nephrotoxicity, limit high-dose therapy with CP. CP induces renal tubular dysfunction and leads to acute renal injury (AKI) in a large proportion of patients.² The CP concentration in proximal tubular epithelial cells is several fold higher than the serum concentration,³ where it triggers DNA damage, apoptosis, and inflammation.^{4,5} Several apoptotic pathways such as endoplasmic reticulum (ER) stress pathway, the extrinsic pathway activated through death receptors, and the intrinsic mitochondrial pathway have been researched.^{4,6}

The imbalance in ER homeostasis, as multifunctional organelle, caused disturbance of calcium homeostasis, glucose deprivation, increasing of Reactive oxygen species (ROs) formation and oxidative stress induction.^{7–9} The primary mitigating mechanism for ER stress is the unfolded

protein response (UPR), which is an intracellular system to reduce the buildup of unfolded proteins and restore ER's normal function.^{10–13} If ER stress is severe, caspase-12-mediated apoptosis might be stimulated by UPR.^{14,15} Accumulating evidence suggests that ER stress or UPR has been associated with many human diseases, including ischemia, heart disease, vascular disease, liver disease, neurodegenerative diseases, diabetes, hyperhomocysteinemia, and cancer.^{10,12,16}

Epigallocatechin-3-gallate (EGCG), the major ingredient of green tea, has potent anti-apoptotic, antioxidant, and anti-inflammatory properties. Several studies have demonstrated the EGCG can inhibit apoptosis in neurological and cardiovascular disease.^{17,18} In addition, EGCG has protective effects against chronic diseases including cancer, neurodegenerative disease, diabetes, and heart disease.^{19–22} Herein, the present study was designed to investigate the protective effects of EGCG against CP-induced nephrotoxicity and examine whether these effects are mediated by the inhibition of ER stress-induced apoptosis.

Materials and methods

Materials

From Sigma-Aldrich (St. Louis, MO) EGCG and CP were bought for research. A variety of antibodies were supplied, including β -actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), ERK and p-ERK (Cell Signaling Technology, Inc., Danvers, MA), and caspase-12 and GRP78 (Abcam Ltd., Hong Kong, China). Anti-rabbit and anti-mouse secondary antibodies used in our study were provided by Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). TUNEL staining kit was purchased from Roche Diagnostics (Indianapolis, IN).

Animals

Male C57/BL6 mice (6–8 weeks old, weighing 20–25 g) from the Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) were housed under a controlled temperature (18–22°C), humidity (50–70%), and a 12/12 h light/dark cycle, with free access to food and water. The experimental procedures involving mice were performed in strict compliance with the NIH Guide for the Care and Use of Laboratory Animals.

Experimental procedures

Kidney nephrotoxicity was triggered by a single intra-peritoneal (i.p.) injection of CP. Mice were randomly divided in the following groups: (1) control group (N; $n=7$) were treated with 0.9% saline (10 mL/kg, i.p.); (2) EGCG group (E; $n=7$) were treated with EGCG (100 mg/kg, i.p.) in 0.9% saline (10 mL/kg); (3) CP group (C; $n=7$) were treated with CP (20.0 mg/kg, i.p.) in 0.9% saline (10 mL/kg); (4) CP + EGCG group (C + E; $n=7$) were pretreated with EGCG (100 mg/kg, i.p.) at 30 min before i.p. injection of CP (20.0 mg/kg). After 48 h of CP injection, mice in this group were treated with EGCG (100 mg/kg, i.p.). Five days (120 h) after injection of CP or 0.9% saline, all animals were weighed and killed. Kidney tissues and blood were collected after killing. The serum was separated from blood by centrifugation (3000 rpm, 15 min, at 4°C) and stored at -80°C . Serum levels of blood urea nitrogen (BUN) and creatinine (Scr) were measured using Cobas®8000 modular analyzer (Roche Diagnostics) in Qilu Hospital, Shandong University. We immediately weighed the kidneys, cut them into two coronal sections, and calculated the renal index (RI) as both kidneys' weight (g)/animal's weight (g) $\times 100$. One section of kidney was stored at -80°C . The remaining section was stored in 4% paraformaldehyde (PFA) at 4°C, then dehydrated, and embedded in paraffin.

Histological evaluation of kidney

The histological examination was conducted by using periodic acid-Schiff (PAS) reagents to stain the sections (4 μm). The experiment was performed as described previously.²³

Immunohistochemical study

Immunohistochemical study was conducted as described previously.²³ The primary antibodies used in this

experiment were anti-caspase-12 (1:100), anti-p-ERK (1:100), and anti-GRP78 (1:100).

TUNEL assay

The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining kit was used to detect cell apoptosis. TUNEL assay was conducted according to the manufacturer's instructions.

Western blot analysis

Western blot was conducted as described previously.²³ The primary antibodies used in this experiment were anti- β -actin (1:5000), anti-caspase-12 (1:500), anti-p-ERK (1:1000), anti-ERK (1:1000), and anti-GRP78 (1:250).

Statistical analysis

Data are expressed as mean $\pm$ standard deviation (SD). These data were analyzed by analysis of variance (ANOVA) or Student's *t*-tests. $P < 0.05$ was considered statistically significant.

Results

EGCG attenuates the CP-induced renal dysfunction

Compared to the control group, the levels of BUN, Scr, and RI increased dramatically in the CP group as shown in Table 1 ($P < 0.05$), which were significantly decreased by EGCG treatment in the CP + EGCG group ($P < 0.05$).

EGCG attenuates CP-induced kidney tubular damage

In the CP group, renal proximal tubule cells revealed vacuolation, necrosis, and desquamation, while in the control and EGCG groups, the proximal tubule cells maintained normal morphology as shown in Figure 1a. CP treatment resulted in severe tubular damage, which was attenuated by EGCG pretreatment in the CP + EGCG group. The scores of tubular damage were calculated and shown in Figure 1b. CP + EGCG group scored 1.7, while the CP group had a higher score of 3.7. The difference of them was significant ($P < 0.05$), suggesting that EGCG can attenuate renal injury induced by CP.

Table 1 The effect of EGCG on renal function and renal index^a

Group	No.	RI	Scr	BUN
N	7	1.45 $\pm$ 0.1	28.43 $\pm$ 6.55	10.1 $\pm$ 0.97
E	7	1.47 $\pm$ 0.09	28.14 $\pm$ 3.85	10.24 $\pm$ 1.45
C	7	1.82 $\pm$ 0.08*	149.14 $\pm$ 16.53*	35.16 $\pm$ 2.6*
C + E	7	1.67 $\pm$ 0.08 [#]	64.71 $\pm$ 6.02 [#]	15.09 $\pm$ 0.97 [#]

No.: number of mice in the group; RI: renal index; BUN: blood urea nitrogen level; Scr: serum creatinine level.

^aEach value is expressed as mean $\pm$ SD. Animals were divided into four groups: the control group (N), the EGCG group (E), the CP group (C), and the CP + EGCG group (C + E).

* $P < 0.05$ versus N group; [#] $P < 0.05$ versus C group.

Figure 1 EGCG attenuates CP-induced kidney tubular damage. Animals were randomly arranged into four groups: the control group (N), the EGCG group (E), the CP group (C), and the CP + EGCG group (C + E). (a): Histological examination showed vacuolation, necrosis, and desquamation of epithelial cells in the renal proximal tubules of the C group, while the C + E group showed very little structural damage. (b) Scores of tubular damage. Data are presented as means $\pm$ SD. * $P < 0.05$ versus the N group; # $P < 0.05$ versus the C group. (A color version of this figure is available in the online journal.)

EGCG attenuates the CP-induced apoptosis of tubular epithelial cells

We used TUNEL assay to evaluate apoptosis of renal tubular epithelial cells. As shown in Figure 2, TUNEL-positive apoptotic cell number raised significantly in the CP group compared to the control group ($P < 0.05$) and were reduced in the CP + EGCG group ($P < 0.05$). TUNEL-positive cells in the control and the EGCG group were very limited as shown in Figure 2.

EGCG attenuates expression of caspase-12, p-ERK, and GRP78 in kidney

We examined the expression of ER stress-related markers (GRP78, caspase-12 and p-ERK) so as to study whether EGCG protects against CP-induced nephrotoxicity by suppressing ER stress-induced apoptosis. The results showed that CP injection significantly increased the expression of GRP78, caspase-12, and p-ERK in the CP group compared to the control group. However, the expression of these proteins was significantly attenuated with EGCG treatment in the CP + EGCG group ($P < 0.05$) (Figures 3 and 4). The difference between the control group and the EGCG group did not show any significance.

Discussion

CP is an effective anti-cancer drug with the main side effect of nephrotoxicity.³⁻⁶ CP-induced nephrotoxicity results in renal dysfunction.²⁴ In our study, the levels of BUN and Scr showed a significant decrease in the CP + EGCG group compared to the CP group, indicating improved renal function (Table 1).

Much importance has been attached to the direct toxic effects of CP on proximal renal tubular epithelial cells. In this part, CP induces inflammation, oxidative stress, and apoptosis.^{4,5,25} Accumulating evidence indicates that EGCG is a potent antioxidant, anti-inflammatory, and anti-apoptotic agent.^{17,18,26-30} EGCG is considered to be

100 times as potent as vitamin C and 25 times as potent as vitamin E,³¹ and appears to protect renal injury by reducing oxidative stress and inflammation.³² It has been also shown that EGCG can attenuate cigarette smoke-induced cardiomyocyte apoptosis through the inhibition of mitochondrial dependent apoptotic pathway.¹⁷ Renal tubular apoptosis plays an important role in CP-induced nephrotoxicity.^{6,33} In the present study, apoptosis of renal tubular epithelial cells was significantly ameliorated by EGCG treatment, indicating the renal protective effect of EGCG on CP-induced apoptosis (Figure 2).

Several pathways have been studied in CP-induced apoptosis, including the extrinsic pathway, the ER stress pathway and the intrinsic mitochondrial pathway. ER stress pathway has been implicated to play an important role in CP-induced apoptosis.^{34,35} In cells, ER dysfunction can trigger ER stress, resulting in the accumulation and aggregation of unfolded proteins in ER lumen, and prolonged ER stress will initiate the apoptotic pathway.^{14,15} Three ER transmembrane receptors including PERK, inositol-requiring enzyme 1 (IRE1), and activating transcription factor 6 (ATF6) regulate the complicated network of physiological responses to ER stress. These three ER stress receptors maintained inactive through their association with GRP78.¹⁰ GRP78, the ER chaperone, has been used widely as a marker for the induction of ER stress.^{8,36} On accumulation of unfolded proteins, GRP78 dissociates from the three receptors to promote ER protein folding. Caspase-12 is located primarily on the cytoplasmic face of the ER and is highly expressed in the renal proximal tubular epithelial cells.⁵ Caspase-12 can be specially activated via ER stress, but not via membrane or mitochondrial signals. It was previously demonstrated that caspase-12-deficient mice do not respond to ER stress-induced apoptosis.¹⁴ Therefore, GRP78 is the marker of ER stress and caspase-12 is a featured marker of ER stress-induced apoptosis. In our study, ER stress and apoptosis were triggered in CP-induced

Figure 2 EGCG attenuates the CP-induced apoptosis of tubular epithelial cells. Animals were randomly divided into four groups: the control group (N), the EGCG group (E), the CP group (C), and the CP + EGCG group (C + E). The superpositions of nuclear staining to TUNEL (red) staining on the merge panel represent TUNEL-positive cells. An obvious increase in the number of TUNEL-positive cells occurred in the C group and was reduced in the CP + EGCG group. Data are expressed as means $\pm$ SD. * $P < 0.05$ versus the N group; # $P < 0.05$ versus the C group. (A color version of this figure is available in the online journal.)

Figure 3 Western blot analyses of GRP78, p-ERK, and caspase-12. Animals were randomly arranged into four groups: the control group (N), the EGCG group (E), the CP group (C), and the CP + EGCG group (C + E). Ratios of GRP78, p-ERK, and caspase-12 to β -actin are presented. * $P < 0.05$ versus the N group; # $P < 0.05$ versus the C group.

Figure 4 Immunohistochemical staining of GRP78, p-ERK, and caspase-12 in the kidney. Animals were randomly arranged into four groups: the control group (N), the EGCG group (E), the CP group (C), and the CP + EGCG group (C + E). GRP78, p-ERK, and caspase-12 are highly expressed in the C group compared to the N group. However, the expression of these proteins was significantly attenuated with EGCG treatment in the C + E group. Data are expressed as means $\pm$ SD. * $P < 0.05$ versus the N group; # $P < 0.05$ versus the C group. (A color version of this figure is available in the online journal.)

Figure 5 The mechanism of EGCG in mice with cisplatin-induced kidney injury. (A color version of this figure is available in the online journal.)

nephrotoxicity as demonstrated by the significant elevation of GRP78 and caspase-12. By contrast, the CP + EGCG group showed a significant reduction in the levels of GRP78 and caspase-12. These results indicated that EGCG could attenuate ER stress-induced apoptosis (Figure 5). As we know, this is the first study to demonstrate that CP-induced nephrotoxicity is alleviated by EGCG through the ER stress-mediated apoptosis.

In summary, the results of this study showed that EGCG could protect against CP-induced nephrotoxicity by suppression of ER stress-mediated apoptosis in renal tubular epithelial cells of mouse. This study may provide supporting evidence for the efficacy of EGCG in the CP-induced AKI. Although EGCG showed its protection in our study, it warrants further research for use as therapeutic potential in clinic.

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REFERENCES

1. Bottone MG, Soldani C, Veneroni P, Avella D, Pisu M, Bernocchi G. Cell proliferation, apoptosis and mitochondrial damage in rat B50 neuronal cells after cisplatin treatment. *Cell Prolif* 2008;**41**:506–20
2. Luke DR, Vadiel K, Lopez-Berestein G. Role of vascular congestion in cisplatin-induced acute renal failure in the rat. *Nephrol Dial Transplant* 1992;**7**:1–7
3. Kuhlmann MK, Burkhardt G, Kohler H. Insights into potential cellular mechanisms of cisplatin nephrotoxicity and their clinical application. *Nephrol Dial Transplant* 1997;**12**:2478–80
4. Miller RP, Tadagavadi RK, Ramesh G, Reeves WB. Mechanisms of cisplatin nephrotoxicity. *Toxins* 2010;**2**:2490–518
5. Ramesh G, Reeves WB. TNF- α mediates chemokine and cytokine expression and renal injury in cisplatin nephrotoxicity. *J Clin Invest* 2002;**110**:835–42
6. Pabla N, Dong Z. Cisplatin nephrotoxicity: mechanisms and renoprotective strategies. *Kidney Int* 2008;**73**:994–1007
7. Anelli T, Sitia R. Protein quality control in the early secretory pathway. *EMBO J* 2008;**27**:315–27
8. Groenendyk J, Sreenivasiah PK, Kim do H, Agellon LB, Michalak M. Biology of endoplasmic reticulum stress in the heart. *Circ Res* 2010;**107**:1185–97
9. Kaufman RJ. Stress signaling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls. *Genes Dev* 1999;**13**:1211–33
10. Szegezdi E, Logue SE, Gorman AM, Samali A. Mediators of endoplasmic reticulum stress-induced apoptosis. *EMBO Rep* 2006;**7**:880–5
11. Marciniak SJ, Ron D. Endoplasmic reticulum stress signaling in disease. *Physiol Rev* 2006;**86**:1133–49
12. Schroder M, Kaufman RJ. The mammalian unfolded protein response. *Ann Rev Biochem* 2005;**74**:739–89
13. Schroder M, Kaufman RJ. ER stress and the unfolded protein response. *Mutat Res* 2005;**569**:29–63
14. Nakagawa T, Zhu H, Morishima N, Li E, Xu J, Yankner BA, Yuan J. Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid- β . *Nature* 2000;**403**:98–103
15. Xu C, Bailly-Maitre B, Reed JC. Endoplasmic reticulum stress: cell life and death decisions. *J Clin Invest* 2005;**115**:2656–64
16. Kaufman RJ. Orchestrating the unfolded protein response in health and disease. *J Clin Invest* 2002;**110**:1389–98
17. Adikesavan G, Vinayagam MM, Abdulrahman LA, Chinnasamy T. (-)-Epigallocatechin-gallate (EGCG) stabilize the mitochondrial enzymes and inhibits the apoptosis in cigarette smoke-induced myocardial dysfunction in rats. *Mol Biol Rep* 2013;**40**:6533–45
18. Nie G, Jin C, Cao Y, Shen S, Zhao B. Distinct effects of tea catechins on 6-hydroxydopamine-induced apoptosis in PC12 cells. *Arch Biochem Biophys* 2002;**397**:84–90
19. Higdon JV, Frei B. Tea catechins and polyphenols: health effects, metabolism, and antioxidant functions. *Crit Rev Food Sci Nutr* 2003;**43**:89–143
20. Siddiqui IA, Afaq F, Adhami VM, Ahmad N, Mukhtar H. Antioxidants of the beverage tea in promotion of human health. *Antioxid Redox Signal* 2004;**6**:571–82
21. Grove KA, Lambert JD. Laboratory, epidemiological, and human intervention studies show that tea (*Camellia sinensis*) may be useful in the prevention of obesity. *J Nutr* 2010;**140**:446–53
22. Yang CS, Maliakal P, Meng X. Inhibition of carcinogenesis by tea. *Ann Rev Pharmacol Toxicol* 2002;**42**:25–54
23. Zou P, Song J, Jiang B, Pei F, Chen B, Yang X, Liu G, Hu Z. Epigallocatechin-3-gallate protects against cisplatin nephrotoxicity by inhibiting the apoptosis in mouse. *Int J Clin Exp Pathol* 2014;**7**:4607–16
24. Shimeda Y, Hirotani Y, Akimoto Y, Shindou K, Ijiri Y, Nishihori T, Tanaka K. Protective effects of capsaicin against cisplatin-induced nephrotoxicity in rats. *Biol Pharm Bull* 2005;**28**:1635–8
25. Leibbrandt ME, Wolfgang GH, Metz AL, Ozobia AA, Haskins JR. Critical subcellular targets of cisplatin and related platinum analogs in rat renal proximal tubule cells. *Kidney Int* 1995;**48**:761–70
26. Takano K, Nakaima K, Nitta M, Shibata F, Nakagawa H. Inhibitory effect of (-)-epigallocatechin 3-gallate, a polyphenol of green tea, on neutrophil chemotaxis in vitro and in vivo. *J Agric Food Chem* 2004;**52**:4571–6
27. Lambert JD, Elias RJ. The antioxidant and pro-oxidant activities of green tea polyphenols: a role in cancer prevention. *Arch Biochem Biophys* 2010;**501**:65–72
28. Katiyar SK, Matsui MS, Elmets CA, Mukhtar H. Polyphenolic antioxidant (-)-epigallocatechin-3-gallate from green tea reduces UVB-induced inflammatory responses and infiltration of leukocytes in human skin. *Photochem Photobiol* 1999;**69**:148–53
29. Tipoe GL, Leung TM, Hung MW, Fung ML. Green tea polyphenols as an anti-oxidant and anti-inflammatory agent for cardiovascular protection. *Cardiovasc Hematol Disord Drug Targets* 2007;**7**:135–44
30. Cao G, Chen M, Song Q, Liu Y, Xie L, Han Y, Liu Z, Ji Y, Jiang Q. EGCG protects against UVB-induced apoptosis via oxidative stress and the JNK1/c-Jun pathway in ARPE19 cells. *Mol Med Rep* 2012;**5**:54–9
31. Clark J, You M. Chemoprevention of lung cancer by tea. *Mol Nutr Food Res* 2006;**50**:144–51
32. El-Mowafy AM, Salem HA, Al-Gayyar MM, El-Mesery ME, El-Azab MF. Evaluation of renal protective effects of the green-tea (EGCG) and red grape resveratrol: role of oxidative stress and inflammatory cytokines. *Nat Prod Res* 2011;**25**:850–6
33. Yao X, Panichpisal K, Kurtzman N, Nugent K. Cisplatin nephrotoxicity: a review. *Am J Med Sci* 2007;**334**:115–24

34. Liu H, Baliga R. Endoplasmic reticulum stress-associated caspase 12 mediates cisplatin-induced LLC-PK1 cell apoptosis. *J Am Soc Nephrol* 2005;**16**:1985-92
35. Kong D, Zhuo L, Gao C, Shi S, Wang N, Huang Z, Li W, Hao L. Erythropoietin protects against cisplatin-induced nephrotoxicity by attenuating endoplasmic reticulum stress-induced apoptosis. *J Nephrol* 2013;**26**:219-27
36. Liu G, Sun Y, Li Z, Song T, Wang H, Zhang Y, Ge Z. Apoptosis induced by endoplasmic reticulum stress involved in diabetic kidney disease. *Biochem Biophys Res Commun* 2008;**370**:651-6

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