Impact of epigallocatechin-3-gallate on expression of nuclear factor erythroid 2-related factor 2 and γ-glutamyl cysteine synthetase genes in oxidative stress-induced mouse renal tubular epithelial cells

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Abstract. The aim of the present study was to investigate the antioxidant response mechanism of epigallocatechin-3-gallate (EGCG) in H_2O_2-induced mouse renal tubular epithelial cells (MRTECs). The cultured MRTECs were divided into normal, H_2O_2 (control) and EGCG treatment groups. The MTT assay was used to assess cell viability, and reverse transcription-quantitative polymerase chain reaction (RT-qPCR), immunocytochemical and western blot analyses were performed to detect the expression of nuclear factor erythroid 2-related factor 2 (Nrf2) and γ-glutamyl cysteine synthetase (γ-GCS). EGCG was able to mitigate H_2O_2-mediated cell damage. The RT-qPCR results demonstrated that EGCG was able to upregulate the gene expression of Nrf2 and γ-GCS in MRTECs in a dose-dependent manner. The immunocytochemistry and western blot analyses demonstrated that EGCG was able to increase the protein expression of Nrf2 and γ-GCS in MRTECs in a dose-dependent manner. Oxidative stress may lead to a decrease in the viability of MRTECs, while EGCG was able to promote the expression of Nrf2 and γ-GCS in MRTECs, thereby improving the antioxidant capacity of the cells and promoting the repair of oxidative stress injury.

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Introduction

Oxidative stress occurs when the body is subjected to various harmful stimuli, leading to overproduction *in vivo* of active molecules, including reactive oxygen species (ROS) and reactive nitrogen species. When the amount of oxidation exceeds the removal of oxide material, the oxidation and antioxidant systems are imbalanced, which leads to tissue damage. The interaction between inflammation and ROS is a principal mechanism of multiple renal diseases (1-3). The kidney is an organ with high perfusion, rich blood flow and oxygen supply, and is therefore predisposed towards the production of ROS (4-7). The inhibition of ROS and ROS-mediated oxidative damage is an important consideration in clinical practice for kidney disease.

Nuclear factor erythroid 2-related factor 2 (Nrf2) and its cytoplasmic adapter protein Kelch-like ECH-associated protein 1 (Keap1) are the central regulators of the cellular antioxidant response. Nrf2 is able to interact with other elements of the antioxidant response, inducing the expression of antioxidant proteins and phase II detoxification enzymes, and serves an important role in cellular defense. The Keap1-Nrf2/antioxidant responsive element (ARE) signaling pathway serves a range of protective functions against cellular stress, apoptosis and inflammation (8). However, the function of the Keap1-Nrf2/ARE pathway in renal antioxidation remains unclear.

Catechins are polyphenol compounds extracted from green tea, and are important natural antioxidants produced by plants (9). Epigallocatechin-3-gallate (EGCG) is the most potent antioxidant among the catechins (10). The present study investigated the protective effect of EGCG on oxidative injury in mouse renal tubular epithelial cells (MRTECs).

Materials and methods

Materials. MRTECs were purchased from the cell bank of the Type Culture Collection of the Chinese Academy of

Sciences (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM) with high glucose and fetal bovine serum were purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The EGCG was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). The Nrf2 (cat no. bs-1074R) and γ-GCS (cat no. YSRIBIO-068461) rabbit anti-rat immunoglobulin (Ig)G primary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The streptavidin peroxidase immunocytochemical staining kit and 3,3'-diaminobenzidine(DAB) staining reagent were purchased from ZSGB-Bio (Beijing, China). The TRIzol total RNA extracting solution was purchased from Shanghai Sangon Biological Engineering Technology and Services Co., Ltd (Shanghai, China). The PrimeScript™ RT Reagent kit was purchased from Takara Biotechnology Co., Ltd. (Dalian, China). The RT-qPCR SYBR Premix Ex Taq™ kit was purchased from Takara Biotechnology Co., Ltd. The RT-qPCR primers were designed and synthesized by Takara Biotechnology Co., Ltd.

Culturing of MRTECs and grouping. MRTECs were digested with 0.25% trypsin to form a single cell suspension and cultured in DMEM containing 100 ml/l fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin dual anti-solution, at 37˚C in an atmosphere containing 5% CO_2. The medium was replaced every 24 h and 0.25% trypsin was used for digestion every 3 days; passaging was performed at a ratio of 1:3. The passaged cells were inoculated uniformly and, when the cells had reached the sub-integration state, serum-free medium was added for a 12-h synchronization, followed by the addition of 1% fetal bovine serum-containing medium for the experiments. The cells were seeded on coverslips, 6-well plates or flasks. When the cells had achieved ~80% fusion, the serum-free medium was replaced and, according to the experimental group, different dosages of drugs were added. Each experiment was performed with ≥3 different generations of cells.

The cultured cells were divided into the untreated normal group (N); the control group incubated with 250 μmol/l H_2O_2 for 6 h (C); and the EGCG groups incubated with 250 μmol/l H_2O_2, and co-cultured with EGCG at concentrations of 5 mg/l (T1), 10 mg/l (T2) and 20 mg/l (T3).

Detection of cell viability. The MTT chromogenic assay was used to assess the cell viability of each group. The cells were transfected into 96-well plates, and cultured for 72 h. A total of 50 μl MTT (5 g/l) was added to each well and incubated for 4 h. Subsequently, the supernatant was discarded and 100 μl dimethyl sulfoxide was added for the detection of the absorbance at 570 nm (A570) using an automatic microplate reader. The number of viable cells was proportional to the value of A570: Live cell rate=A570 of the experimental group/A570 of the control group. Trypan blue staining was performed on the EGCG groups prior to the assay and the living cell rates were observed to be >90%, indicating that EGCG was non-toxic to the cells.

Detection of Nrf2 and γ-GCS mRNA expression in MRTECs using RT-qPCR. TRIzol was used to extract the total RNA and reverse transcription was performed to synthesize cDNA. The primer sequences for the RT-qPCR were as follows: The upstream and downstream primers of Nrf2 were 5'-TTG ATTGACATCCTTTGG-3' and 5'-GTTCCTTCTGGAGTT GCT-3', respectively, with an amplified product of 142 bp; the upstream and downstream primers of γ-GCS were 5'-ATC TACCACGCAGTCAAG-3' and 5'-CCGCCATTCAGTAAC AAC-3', respectively, with an amplified product of 119 bp; and the upstream and downstream primers of GAPDH-F were 5'-GCACCGTCAAGGCTGAGAAC-3' and 5'-ATGGTGGTG AAGACGCCAGT 3', respectively, with an amplified product of 142 bp. The PCR composition, reaction conditions (94˚C for 2 min for 1 cycle, 94˚C for 40 sec, 50-65˚C for 40 sec and 72˚C for 1 min for 25-35 cycles, and 72˚C for 5 min for 1 cycle) and dissolution profile conditions were in accordance with the manufacturer's protocol.

The SYBR-Green 1 fluorescent dye embedment method was used to prepare the standard curves of the target genes, Nrf2 and γ-GCS, and the housekeeping gene GAPDH. The standard curves were used to quantifying the target gene and housekeeping gene levels in the samples. Through the correction of the housekeeping gene, the relative gene expression of Nrf2 and γ-GCS was measured in each group (11).

Detection of Nrf2 and γ-GCS protein expression in MRTECs using immunocytochemical analysis. The coverslips containing cultured cells were prepared according to the streptavidin-biotin complex test kit protocol. The primary antibodies were rabbit anti-Nrf2 and γ-GCS polyclonal antibodies (both 1:200), which was incubated at 4˚C overnight. After washing with PBS for 5 min 3 times, the secondary antibody was added, which was a biotinylated goat anti-rabbit IgG (1:200; Agrisera AB, Vännäs, Sweden; cat no. AS09608), which was incubated at 37˚C for 30 min prior to washing with PBS as before. DAB staining was subsequently performed, and the DAB developer was added and incubated for 3 min at room temperature and then the reaction was stopped by subjecting cells to running water for 1 min. Cells were then stained with hematoxylin for 15 sec. In group N, PBS was used to replace the primary antibody and all subsequent steps were the same. The existence of black/dark grey particles inside the nucleus and/or cytoplasm was considered to be positive. The optical density values were calculated using MetaMorph image analysis software.

Detection of Nrf2 and γ-GCS protein expression in MRTECs using western blot analysis. Total Nrf2 nucleoprotein and γ-GCS cytoplasmic protein was extracted using radioimmunoprecipiation assay lysis buffer (BioTeke Corporation, Beijing, China), and the assay was performed according to the manufacturer's protocol. 3% SDS-PAGE 3% gel and 10% separation gel was used, and 10 μl protein was loaded per lane. The protein was then transfered to nitrocellulose membrane and blocked with 5% skimmed milk powder at 4˚C overnight. The primary antibodies (rabbit anti-Nrf2 and γ-GCS polyclonal antibody) were diluted 1:1,000, incubated at 4˚C overnight and the reaction was stopped with washing solution, then the goat anti-rabbit IgG secondary antibody was added (1:2,000) at room temperature for 1 h and stopped with washing solution as before. Staining was performed using the illuminating kit (BestBio, ShangHai; http://bestbio.biogo.net). Quantitative

Table I. Effects of EGCG dose on the cytoactivity of H_2O_2-induced mouse renal tubular epithelial cells 6 h post-treatment.

			EGCG treatment		
Group	N	C	T1	T2	T3
Cell survival rate, %	93.51±2.27	56.54±5.42[a]	77.87±5.86[a,b]	82.41±5.66[a,b]	90.22±2.48[a,b]

[a]P<0.01 vs. N; [b]P<0.01 vs. C. N, normal control; C, H_2O_2 control; EGCG, epigallocatechin-3-gallate; T1, treated with 5 mg/l EGCG; T2, treated with 10 mg/l EGCG; T3, treated with 20 mg/l EGCG.

Table II. Effects of EGCG doses on relative expression of Nrf2 and γ-GCS mRNA in mouse renal tubular epithelial cells (n=10).

			EGCG treatment		
Group	N	C	T1	T2	T3
Nrf2	0.0018±0.0002	0.0036±0.0004[a]	0.0042±0.0005[a,b]	0.0063±0.0005[a,c]	0.0067±0.0006[a,c]
γ-GCS	0.0023±0.002	0.0046±0.0004[a]	0.0049±0.0004[a,b]	0.0071±0.0005[a,c]	0.0074±0.0005[a,c]

[a]P<0.01 vs. N; [b]P<0.05, [c]P<0.01 vs. C. N, normal control; C, H_2O_2 control; EGCG, epigallocatechin-3-gallate; Nrf2, nuclear factor erythroid 2-related factor 2; γ-GCS, γ-glutamyl cysteine synthetase; T1, treated with 5 mg/l EGCG; T2, treated with 10 mg/l EGCG; T3, treated with 20 mg/l EGCG.

analysis was performed using Fluor Chem software (version 2.0; ProteinSimple; Bio-Techne, Minneapolis, MN, USA) and β-actin (1:200; cat no. A5441; Sigma-Aldrich; Merck KGaA) was used as the internal reference for the analysis, and was incubated at room temperature for 1 h.

Statistical analysis. All of the experiments were repeated ≥3 times and the results are expressed as the mean ± standard deviation. Statistical analysis was performed using SPSS software (version 13.0; SPSS, Inc., Chicago, IL, USA). One-way analysis of variance was used for the comparisons of overall and intergroup differences. P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of different EGCG doses on H_2O_2-induced MRTEC cytoactivity. Subsequent to 6 h treatment, in the control group, the cytoactivity of MRTECs was reduced. Among the treatment groups, at an EGCG concentration of 5 mg/l, the cytoactivity was significantly increased compared with the control group (P<0.01); at an EGCG concentration of 10 or 20 mg/l, cytoactivity was significantly increased compared with the control group (P<0.01). EGCG protected against the H_2O_2-mediated decrease in cytoactivity in a dose-dependent manner (Table I).

Effects of EGCG on Nrf2 and γ-GCS mRNA expression in MRTECs following oxidative stress. The standard curves of Nrf2, γ-GCS and GAPDH were prepared, and the correlation coefficients were 0.998, 0.990 and 0.995, respectively. The linearities were good, and the fusion curves demonstrated that the specificity was good.

The standard curve was used to quantify the expression of Nrf2, γ-GCS and GAPDH in the samples, and it was observed that, compared with the N group, the Nrf2 and γ-GCS mRNA in the C group was increased; compared with the C group, the expression of Nrf2 and γ-GCS mRNA was increased in the T1 group (P<0.05), and in the T1 and T3 groups (P<0.01), indicating that EGCG was able to upregulate the gene expression of Nrf2 and γ-GCS in MRTECs in a dose-dependent manner (Table II; Fig. 1).

Impact of EGCG on the expression of Nrf2 and γ-GCS protein in MRTECs following oxidative stress. Nrf2-positive expression is exhibited as brown particles in the MRTECs. When stimulated with 250 μmol/l H_2O_2 for 6 h, the Nrf2-positive expression increased. At increasing concentrations of EGCG, the expression of Nrf2 in the nucleus increased markedly, exhibited by deeper staining, most notably in the T3 group (Fig. 2). The γ-GCS-positive expression is exhibited as brown particles, and also located in the cytoplasm of normal MRTECs. When stimulated with H_2O_2 for 6 h, the γ-GCS-positive expression increased. At increasing concentrations of EGCG, the expression of γ-GCS in the nucleus increased markedly, exhibited by deeper staining, most notably in the T3 group (Fig. 3). Compared with the normal control group, the average optical density values in the control group and each treatment group were significantly increased (P<0.01); compared with the H_2O_2 control group, the average optical density values in the T1 (P<0.05), and the T2 and T3 groups (P<0.01) significantly increased (Table III).

The western blotting analysis results demonstrated that, compared with the normal group, Nrf2 and γ-GCS protein expression in the H_2O_2 control group significantly increased (P<0.01). Compared with the H_2O_2 control group, Nrf2 and

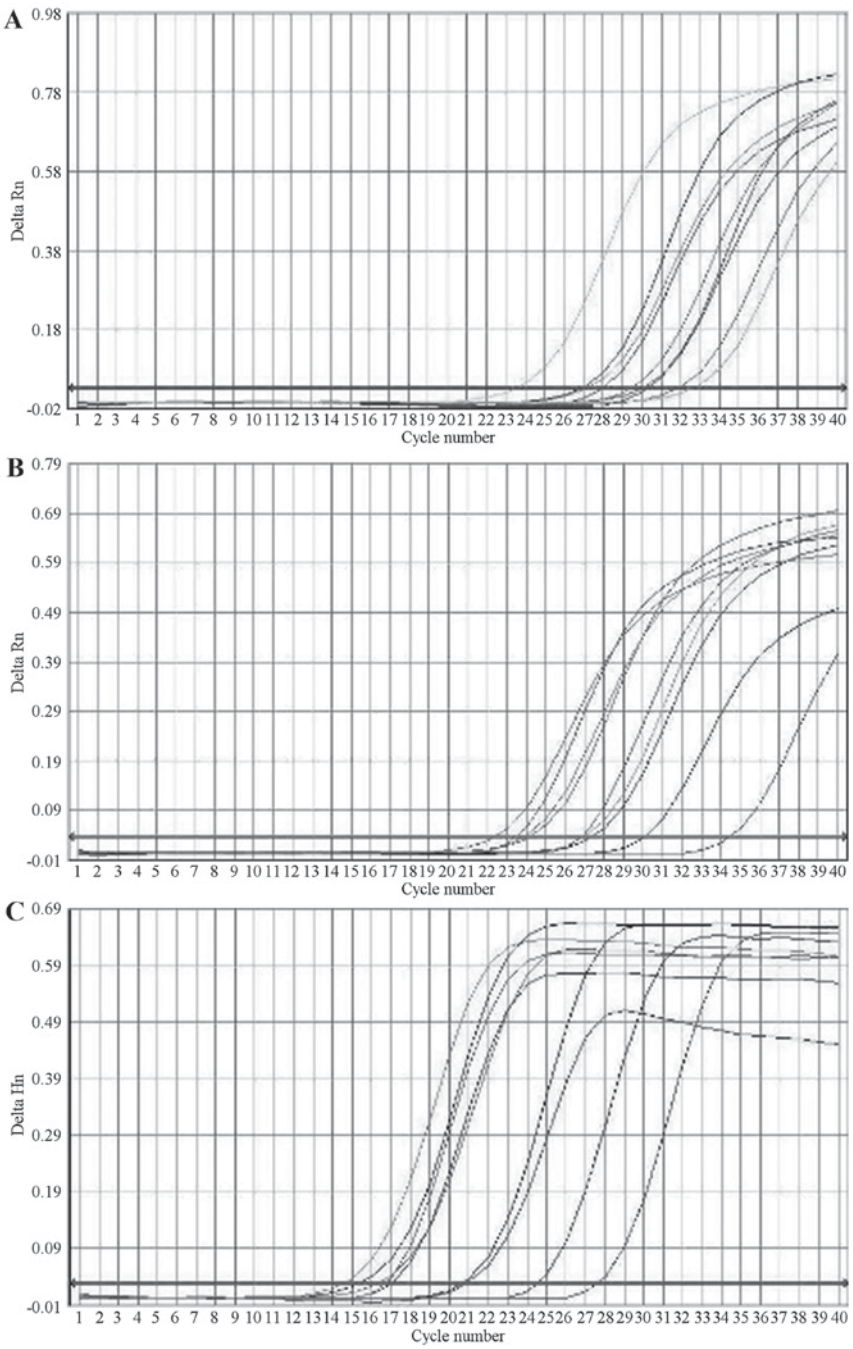

Figure 1. Effects of EGCG on (A) Nrf2, (B) γ-GCS and (C) GAPDH mRNA expression in MRTECs under oxidative stress. EGCG was able to upregulate the expression of Nrf2 and γ-GCS genes in MRTECs in a dose-dependent manner. MRTECs, mouse renal tubular epithelial cells; EGCG, epigallocatechin-3-gallate; Nrf2, nuclear factor erythroid 2-related factor 2; γ-GCS, γ-glutamyl cysteine synthetase.

γ-GCS protein expression increased in the T1 ($P<0.05$), and the T2 and T3 groups ($P<0.01$), suggesting that EGCG was able to increase Nrf2 and γ-GCS protein expression in MRTECs in a dose-dependent manner (Table IV; Fig. 4).

Discussion

Studies have demonstrated that catechins exhibit therapeutic effects in immune nephritis (C-BSA nephritis and Masugi nephritis), diabetic nephropathy, renal dysfunction, chronic renal failure, and antibiotic- and heavy metal-induced acute kidney disease (12-16). Catechins are efficient scavengers of ROS, and enhance the activity and complexation of antioxidant enzymes (17,18). Although H_2O_2 is not a free radical, it is a highly-reactive ROS and may promote the generation of free radicals, thereby causing lipid peroxidation of biological membranes, and resulting in necrosis and apoptosis (19,20). In the present study, H_2O_2 was used in MRTECs to replicate oxidative stress. The potential protective mechanism of EGCG following oxidative stress was investigated. The results of the present study demonstrated that oxidative stress led to a decrease in cell viability and an increase in apoptosis, while EGCG was able to ameliorate the H_2O_2-mediated cell damage, increasing cell viability and decreasing apoptosis.

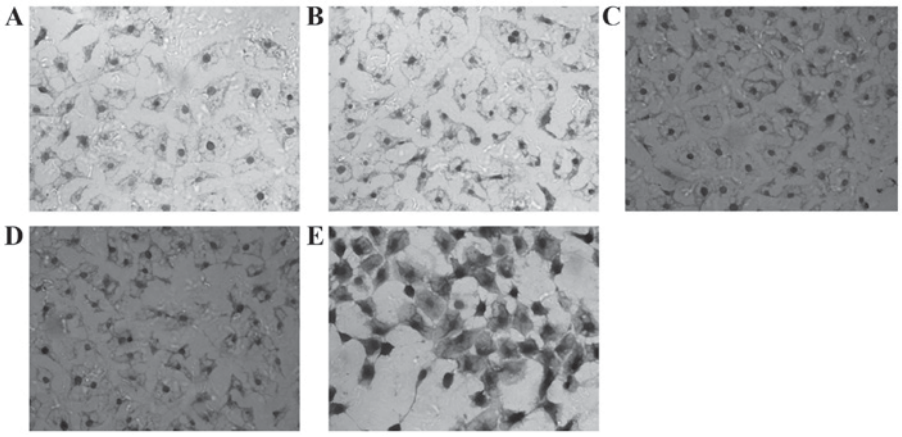

Figure 2. Immunocytochemical staining of Nrf2 in mouse renal tubular epithelial cells (magnification, x400). (A) normal group; (B) H_2O_2 control group; (C) T1 group; (D) T2 group; (E) T3 group. Nrf2 positive expression is exhibited as black/dark grey particles. Nrf2 expression increased in the H_2O_2 control group, and increased further in the EGCG treatment groups in a dose-dependent manner, exhibited by deeper staining. Nrf2, nuclear factor erythroid 2-related factor 2.

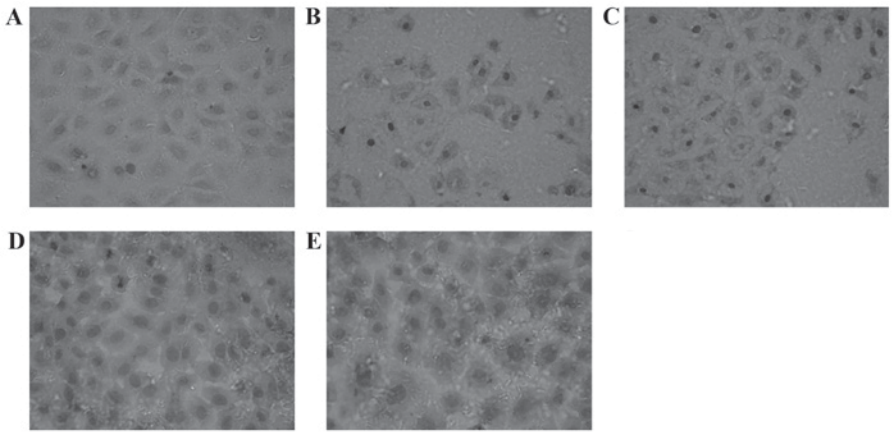

Figure 3. Immunocytochemical staining of γ-GCS in mouse renal tubular epithelial cells (magnification, x400). (A) normal group; (B) H_2O_2 control group; (C) T1 group; (D) T2 group; (E) T3 group. γ-GCS positive expression is exhibited as brown particles. γ-GCS expression increased in the H_2O_2 control group, and increased further in the EGCG treatment groups in a dose-dependent manner, exhibited by deeper staining. γ-GCS, γ-glutamyl cysteine synthetase.

Following the EGCG intervention, the antioxidant capacity of the MRTECs was increased, as was the capacity of the cells to protect against and repair H_2O_2-mediated damage.

The receptor Nrf2 regulates the expression of certain target genes under conditions of oxidative stress. Studies have demonstrated that, when exposed to oxidative and/or electrophilic stress, overexpression of Nrf2 is able to upregulate the expression of target genes, and Nrf2 is more effective at activating gene expression compared with Nrf1 (21). In addition, Nrf2-knockout mice exhibit decreased expression of target genes (22). These previous results indicated that the Nrf2 serves an important role in the regulation of antioxidative genes and the expression of oxidative stress-induced genes.

The metabolism of foreign compounds or the pathological production of active may directly or indirectly interfere with the physiological functions of biological macromolecules, including DNA, proteins and lipids, and therefore serve an important role in the pathology of cancer, neurodegenerative diseases, atherosclerosis and aging. In order to combat this, cells have evolved defensive capabilities against toxic substances; for example, detoxifying enzymes and antioxidant stress protein genes are synergistically induced following exposure to nucleophilic substances and ROS (23,24). This antioxidant response involves cis-acting regulatory regions of target genes, also termed the ARE or electrophile response element (25,26). Previous studies have demonstrated that Nrf2 and its cytoplasmic adapter protein Keap1 are the central regulators of the cellular antioxidant response (27). Experiments have demonstrated that Nrf2 is able to interact with ARE to regulate the expression of antioxidant proteins and phase II detoxifying enzymes (28). A number of studies have demonstrated that the Keap1-Nrf2/ARE pathway exhibits broad cytoprotective effects in antitumor, neuroprotective and anti-inflammatory responses (29,30). These previous results suggested that the Keap1-Nrf2/ARE pathway served an important role in resisting the cell damage caused by foreign compounds, drugs and ultraviolet radiation. Nrf2 is highly expressed in detoxification organs, including the liver and kidneys, and other organs which are exposed to the outside environment, including the skin, lungs and digestive tract (31). The Nrf2-Keap1 system is an essential part of the mechanisms of resisting environmental and endogenous stress.

γ-GCS is able to catalyze the reaction between glutamic acid and cysteine to generate γ-glutamyl cysteine, which

Table III. Effects of EGCG on expression of Nrf2 and γ-GCS protein in mouse renal tubular epithelial cells measured by immunocytochemical assay (OD value).

			EGCG treatment		
Group	N	C	T1	T2	T3
Nrf2	0.291±0.022	0.421±0.023[a]	0.441±0.030[a,b]	0.523±0.024[a,c]	0.545±0.031[a,c]
γ-GCS	0.340±0.028	0.482±0.023[a]	0.507±0.017[a,b]	0.548± 0.029[a,c]	0.596±0.028[a,c]

[a]P<0.01 vs. N; [b]P<0.05, [c]P<0.01 vs. C. N, normal control; C, H_2O_2 control; EGCG, epigallocatechin-3-gallate; Nrf2, nuclear factor erythroid 2-related factor 2; γ-GCS, γ-glutamyl cysteine synthetase; T1, treated with 5 mg/l EGCG; T2, treated with 10 mg/l EGCG; T3, treated with 20 mg/l EGCG.

Table IV. Effects of EGCG on expression of Nrf2 and γ-GCS protein in mouse renal tubular epithelial cells measured by quantified western blotting.

			EGCG		
Group	N	C	T1	T2	T3
Nrf2	0.044±0.005	0.083±0.005[a]	0.091±0.006[a,b]	0.116±0.007[a,c]	0.121±0.006[a,c]
γ-GCS	0.051±0.006	0.095±0.006[a]	0.099±0.007[a,b]	0.126±0.005[a,c]	0.132±0.0.006[a,c]

[a]P<0.01 vs. N; [b]P<0.05, [c]P<0.01 vs. C. N, normal control; C, H_2O_2 control; EGCG, epigallocatechin-3-gallate; Nrf2, nuclear factor erythroid 2-related factor 2; γ-GCS, γ-glutamyl cysteine synthetase; T1, treated with 5 mg/l EGCG; T2, treated with 10 mg/l EGCG; T3, treated with 20 mg/l EGCG.

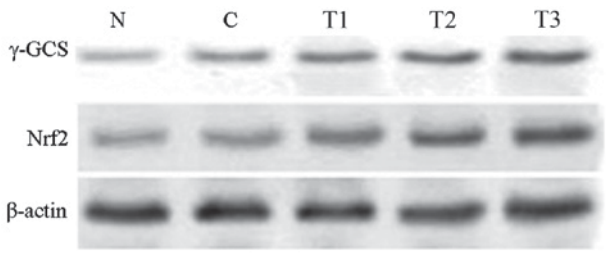

Figure 4. Expression of Nrf2 and γ-GCS protein in mouse renal tubular epithelial cells using western blot analysis. N, normal group; C, H_2O_2 control group; T1, 5 mg/l EGCG group; T2, 10 mg/l EGCG group; T3, 20 mg/l EGCG group; EGCG, epigallocatechin-3-gallate; Nrf2, nuclear factor erythroid 2-related factor 2; γ-GCS, γ-glutamyl cysteine synthetase.

further reacts with glycine to form glutathione (GSH). γ-GCS is the rate-limiting enzyme in the synthesis of GSH and is the target gene of Nrf2 regulation. A previous study has demonstrated that antioxidants and exogenous toxic substances may induce the overexpression of Nrf2, and upregulate the expression of γ-GCS (32). Nrf2 is therefore able to regulate the expression of γ-GCS, consequently affecting the synthesis of GSH.

Expression of γ-GCS is regulated by a variety of transcription factors, which are enhanced by ARE, heavy metal response element, transcription factor AP-1 and nuclear factor-κB. Previous studies have demonstrated that the majority of antioxidant genes are enhanced by ARE, and that Nrfs, particularly Nrf2, are trans-acting factors of ARE, which are able to regulate the expression of antioxidant genes (33,34). The present study further investigated the association between the Nrf2 and γ-GCS signaling pathway, and the effect of EGCG on oxidative stress-induced tubular epithelial cell injury. The results of the present study demonstrated that H_2O_2-induced oxidative stress in MRTECs led to increased gene and protein expression of Nrf2, which further increased following treatment with EGCG. The results of the present study indicated that the regulation of Nrf2 in MRTECs primarily occurred at the transcriptional and post-transcriptional levels.

The present study additionally investigated the gene and protein expression of γ-GCS in MRTECs, and the results suggested that oxidative stress promoted the expression of γ-GCS. Treatment with EGCG was able to further promote the increase in γ-GCS expression, indicating that oxidative stress in MRTECs may upregulate the expression of Nrf2, thereby upregulating its downstream target gene, γ-GCS.

In the present study, the upregulated expression of the downstream target gene γ-GCS during oxidative stress, through upregulation of the transcription factor Nrf2, was demonstrated to occur via the Keap1-Nrf2/ARE signaling pathway. Following treatment with EGCG, the expression of γ-GCS and Nrf2 increased further. Therefore, the results of the present study demonstrated that EGCG was able to increase the expression of Nrf2 in a dose-dependent manner, and improve the antioxidant activity of MRTECs. In conclusion, EGCG exhibited antioxidant effects in oxidative stress-induced MRTECs in a dose-dependent manner.

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