Free Radical Research, 2003 Vol. 37 (5), pp. 561–571

Taylor & Francis
healthsciences

Protective Activity of (−)-Epicatechin 3-*O*-gallate against Peroxynitrite-mediated Renal Damage

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Accepted by Professor E. Niki

(Received 10 October 2002; In revised form 2 December 2002)

The protective effect of (−)-epicatechin 3-*O*-gallate (ECg) against peroxynitrite ($ONOO^-$)-mediated damage was examined using an animal model and a cell culture system. In rats subjected to lipopolysaccharide (LPS) administration plus ischemia-reperfusion, the plasma 3-nitrotyrosine level, an indicator of $ONOO^-$ production *in vivo*, was elevated, whereas it declined significantly and dose-dependently after the oral administration of ECg at doses of 10 and 20 μmoles/kg body weight/day for 20 days prior to the process. Moreover, oral administration of ECg significantly enhanced the activities of the antioxidant enzymes, superoxide dismutase, catalase and glutathione peroxidase, and the antioxidant glutathione, showing enhancement of the biological defense system against the damage induced by $ONOO^-$. In addition, the significant increase in the renal mitochondrial thiobarbituric acid-reactive substance level of LPS and ischemic-reperfused control rats was attenuated in rats given ECg. Furthermore, the elevations in the plasma urea nitrogen and creatinine (Cr) levels and the urinary methylguanidine/Cr ratio induced by the procedure were attenuated markedly after oral administration of ECg, implying amelioration of renal impairment. The addition of ECg (25 or 125 μM) prior to 3-morpholinosydnonimine (SIN-1, 800 μM) exposure reduced $ONOO^-$ formation and increased the viability of cultured renal epithelial (LLC-PK_1) cells in a dose-dependent manner. In particular, ECg inhibited $ONOO^-$-mediated apoptotic cell death, which was confirmed by decreases in the DNA fragmentation rate and the presence of apoptotic morphological changes, i.e. small nuclei and nuclear fragmentation. Furthermore, adding ECg before SIN-1 treatment regulated the cell cycle by enhancing G_2/M phase arrest. This study provides evidence that ECg has protective activity against the renal damage induced by excessive $ONOO^-$ in cellular and *in vivo* systems.

Keywords: (−)-Epicatechin 3-*O*-gallate; Peroxynitrite; Lipopolysaccharide; Ischemia-reperfusion; LLC-PK_1 cell; Renal damage

INTRODUCTION

In a variety of clinical situations, several renal diseases, including glomerulonephritis, vasculitis, pyelonephritis, acute renal failure and others, are mediated, at least in part, by oxidant injury.[1–4] Such oxidative alterations of proteins, DNA and basement membranes lead to cell and organ dysfunction.[5] Evidence for a role of reactive oxygen and nitrogen metabolites in the pathogenesis of renal diseases has accumulated and peroxynitrite ($ONOO^-$) formed *in vivo* from superoxide anion (O_2^-) and nitric oxide (NO) has been suggested to be an important pathogenic causative agent of cellular damage and renal dysfunction.[6,7]

Experimental evidence has demonstrated that reactions between $ONOO^-$ and biological molecules mediate toxic oxidative and nitrosative reactivity, leading to impaired function, toxicity and alterations in signaling pathways that are implicated in diverse forms of free radical-induced tissue injury.[8,9] In addition, exogenous and endogenous $ONOO^-$ caused alterations in the structure and function of mitochondrial proteins, resulting in mitochondrial dysfunction and organ injury.[10] Such results suggest that protection against $ONOO^-$ is an important defense against various pathological diseases as well as under normal conditions. Therefore, antioxidant therapy to reduce the toxicity of $ONOO^-$ and its metabolites is considered as a new avenue of therapeutic intervention and may have beneficial effects by ameliorating the damage

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2003 Taylor & Francis Ltd
DOI: 10.1080/1071576031000083134

and lesions involved in various pathological conditions.

In order to find compounds with curative properties to treat diseases in which oxidative damage is involved, we have searched for novel agents with protective activity against toxic $ONOO^-$ and, in previous studies, we found that Wen-Pi-Tang, a traditional medicine for renal failure, has antioxidant properties that ameliorate renal oxidative injury under *in vivo* and *in vitro* experimental conditions.[11–14] Moreover, we demonstrated that the most active crude drug ingredient of Wen-Pi-Tang in improving metabolism under conditions of renal failure is Rhei Rhizoma, and its beneficial antioxidative effect is mainly attributable to (−)-epicatechin 3-*O*-gallate (ECg).[15–17] Other studies have also demonstrated the free radical-scavenging properties of plant polyphenols such as ECg and epigallocatechin gallate[18,19] and the results suggest that ECg can thereby protect the kidney against damage caused by excessive $ONOO^-$ levels.

In the present study, we used a lipopolysaccharide (LPS) plus ischemia-reperfusion animal model and a 3-morpholinosydnonimine (SIN-1)-induced $ONOO^-$-generating cell culture system to investigate whether ECg protects against the renal damage induced by $ONOO^-$.

MATERIALS AND METHODS

Reagents and Medium

LPS (from *Escherichia coli* serotype 055:B5), SIN-1, dihydrorhodamine 123, 3-nitro-L-tyrosine and Hoechst 33342 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Propidium iodide (PI) was purchased from Molecular Probes (Eugene, Oregon, USA). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) was obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Dulbecco's modified Eagle medium/nutrient mixture F-12 (DMEM/F-12) and fetal calf serum (FCS) were purchased from Life Technologies Inc. (Grand Island, NY, USA) and Cell Culture Laboratories (Cleveland, OH, USA), respectively.

Isolation of ECg

Roots of *Rheum officinale* BAILLON (3.2 kg) grown in China were finely powdered and extracted 5 times with 80% aqueous acetone at room temperature. After removal of the acetone by evaporation under reduced pressure, the extract was subjected to Sephadex LH-20 chromatography. Elution with H_2O containing increasing proportions of methanol (MeOH) yielded six fractions (I–VI), which consisted of relatively low-molecular-weight phenolics.

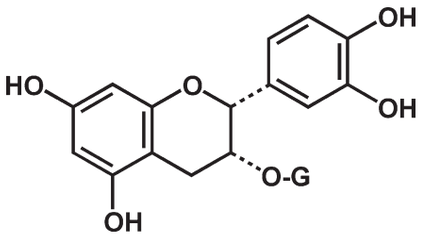

FIGURE 1 Chemical structure of (−)-epicatechin 3-*O*-gallate.

Fraction IV (140 g) was rechromatographed using Sephadex LH-20 with ethanol (EtOH) and MCI-gel CHP 20P with H_2O–MeOH (1:0 → 1:1) to yield ECg (11.0 g).[20] The chemical structure of ECg is shown in Fig. 1.

Animal Experiment

The "Guidelines for Animal Experimentation" approved by Toyama Medical and Pharmaceutical University were followed in these experiments. Male Wistar rats aged 5 weeks (120–130 g) were obtained from Japan SLC Inc. (Hamamatsu, Japan). They were kept in wire-bottomed cages and exposed to a 12-h light/dark cycle. The room temperature and humidity were maintained automatically about 25°C and 60%, respectively. Laboratory commercial chow (CLEA Japan Inc., Tokyo, Japan; comprising 24.0% protein, 3.5% lipid and 60.5% carbohydrate) and water were given *ad libitum*. Following several days of adaptation, the rats were divided into four groups, avoiding any intergroup differences in body weight. Two groups (normal and sham) were given water, while the other two groups were given ECg dissolved in their drinking water orally. The dose and period of administration were determined on the basis of our previous report.[16] The ECg dose was adjusted to 10 or 20 μmoles/kg body weight/day for 20 consecutive days by regulating the concentration in response to water consumption. To generate $ONOO^-$ enough in biological system, the established animal model, LPS plus ischemia-reperfusion model, was employed.[21] Operative procedures were performed under general anesthesia induced by 50 mg/kg sodium pentobarbital administered intraperitoneally. Using aseptic technique, bilateral flank incisions were made, the renal artery and vein of each kidney were occluded with microvascular clamps for 60 min, the clamps were released and then the kidneys were subjected to reperfusion for 350 min. Fifty minutes after the ischemia started, the rats received an intravenous injection of LPS (5 mg/kg body weight). The sham group underwent sham surgery (incisions were made to expose the kidneys, but the renal pedicles were not clamped). Six hours after the LPS challenge,

blood was collected by cardiac puncture and centrifuged immediately to prepare plasma. Subsequently, the renal arteries of each rat were perfused with ice-cold perfusion buffer comprising 50 mM sodium phosphate, 10 mM EDTA-2Na and 120 mM NaCl, and the kidneys were removed, quickly frozen and kept at −80°C until analysis.

Plasma 3-Nitrotyrosine Level

The plasma concentration of 3-nitrotyrosine was determined by HPLC, following the methods of both van der Vliet *et al.*[22] and Kaur *et al.*[23] with slight modifications. Briefly, the blood samples were centrifuged for 15 min at 14,000 rpm, the resulting plasma samples were incubated with proteinase K (1 U/10 mg protein) for 18 h at 55°C, centrifuged for 15 min at 14,000 rpm and passed through a 10,000-Da molecular mass cut-off filter. The samples were loaded onto a reversed-phase column (Nucleosil 5 μC-18, 250 × 46 mm) at 25°C and eluted with 50 mM KH_2PO_4–H_3PO_4 (pH 3.01) in 10% MeOH (v/v) at a flow rate of 0.8 ml/min. Detection of the amino acid derivatives was accomplished by monitoring ultraviolet absorbance at 365 nm. The peaks were identified by comparing their retention times with those of authentic standards added to additional samples and quantified according to their peak areas relative to known amounts of the external standards.

Antioxidant Enzyme Activities

Superoxide dismutase (SOD) activity was determined according to the nitrous acid method described by Elstner and Heupel[24] and Oyanagui,[25] which is based on the inhibition of nitrite formation by hydroxylamine in the presence of O_2^- generators. Catalase activity was evaluated by following the decomposition of hydrogen peroxide (H_2O_2) directly by monitoring the decrease in extinction at 240 nm.[26] Glutathione peroxidase (GSH-Px) activity was measured by a colorimetric assay that determined the concentration of 2-nitro-5-thiobenzoic acid, a compound produced by the reaction between glutathione (GSH) and 5,5′-dithiobis (2-nitrobenzoic acid).[27] Protein levels were determined by the microbiuret method with bovine serum albumin as the standard.[28]

Oxidative Damage in Renal Mitochondria

Renal mitochondria were prepared essentially by following the procedure of Jung and Pergande.[29] Briefly, renal tissue was homogenized in three volumes of ice-cold preparation medium comprising 210 mM mannitol, 70 mM sucrose, 500 μM EDTA and 10 mM Tris–HCl at pH 7.4. The homogenate was centrifuged for 10 min at 800*g* in a refrigerated centrifuge and the resulting supernatant was centrifuged for a further 5 min at 12,000*g*. The pellet was then resuspended in preparation medium to produce a concentration of about 10 μg mitochondrial protein/ml and stored on ice. Throughout the isolation procedure, the tissue and all solutions were kept at 0–4°C. The level of lipid peroxidant was estimated by determining the thiobarbituric acid (TBA)-reactive substance concentration according to the method of Mihara and Uchiyama.[30] GSH levels were measured by the method of Floreani *et al.*,[31] using *o*-phthalaldehyde as the fluorescent reagent.

Plasma and Urinary Renal Function Parameters

Plasma urea nitrogen and creatinine (Cr) levels were determined using the commercial reagents BUN Kainos and CRE-EN Kainos (Kainos Laboratories, Inc., Tokyo, Japan). Urinary methylguanidine (MG) and Cr levels were measured using a Japan Spectroscopic liquid chromatograph employing a step-gradient system, according to the method of Higashidate *et al.*[32] A fluorescence spectrometer (model FP-210, excitation 365 nm, emission 495 nm; Japan Spectroscopic Co., Tokyo, Japan) was used to detect MG and Cr on the column.

Cell Culture Experiment

The porcine kidney cell line LLC-PK_1 was maintained in culture plates containing 5% FCS-supplemented DMEM/F-12 medium at 37°C in a humidified atmosphere of 5% CO_2 in air. The cells were subcultured weekly with 0.05% trypsin-EDTA in calcium- and magnesium-free phosphate buffered saline (PBS). In order to measure $ONOO^-$ formation and DNA fragmentation rates, LLC-PK_1 cells were seeded at a density of 10^5 cells/well in 24-well culture plates and incubated for 48 h. In addition, to determine cell viability, the same number of cells were seeded in 96-well plates and incubated for 2 h to enable them to adhere, whereas 5×10^5 cells/well were seeded in 6-well culture plates and incubated for 48 h to observe the morphological changes and cell cycle distribution. Subsequently, 25 or 125 μM (final concentration) ECg or control vehicle was added to the wells, incubation was continued for 24 h and then all the cells were treated with 800 μM SIN-1 for 4 h before the various parameters were measured.

Cellular $ONOO^-$ Formation

The $ONOO^-$-dependent oxidation of dihydrorhodamine 123 to rhodamine 123 was measured based on the principles of the method described by Haddad *et al.*[33] An 1.25 μM aliquot of dihydrorhodamine 123

was added to each well of a 24-well plate, which was incubated for 4 h at 37°C, and the absorbance at 500 nm of rhodamine 123 in the medium was measured.

Cell Viability

Cell viability was assessed using the MTT colorimetric assay.[34] A 50 μl aliquot of MTT solution (1 mg/ml) was added to each well of a 96-well culture plate, incubated for 4 h at 37°C and the medium containing MTT was removed. The incorporated formazan crystals in the viable cells were solubilized with 100 μl dimethyl sulfoxide and the absorbance at 540 nm of each well was read using a Microplate Reader (Model 3550-UV, Bio-Rad, Tokyo, Japan).

DNA Fragmentation Assay

According to the method of Sellins and Cohen,[35] the cells were lysed in an ice-cold hypotonic lysis buffer (10 mM Tris, 1 mM EDTA, pH 7.5) containing 0.2% Triton X-100 and the lysates were centrifuged at 13,000*g* for 10 min to separate intact from fragmented chromatin. The supernatant, which contained fragmented DNA, was placed in a separate microfuge tube and both the pellet and supernatant were mixed with 12.5% trichloroacetic acid (TCA) and left to stand overnight at 4°C. The resulting precipitates were sedimented at 13,000*g* for 5 min, the DNA in the precipitates was hydrolyzed by heating to 90°C for 10 min in 80 μl 5% TCA and then the DNA was quantified by the method of Burton[36] with a modification. Briefly, 100 μl diphenylamine solution (mixture of acetic acid, diphenylamine and sulfic acid = 1:15:0.15) was treated at 25–30°C in the dark overnight and the amount of DNA was determined colorimetrically at 600 nm. The percentage of DNA fragmentation was calculated as the ratio of the amount of DNA in the 13,000*g* supernatant to the total amount of DNA recovered in the 13,000*g* pellet and supernatant.

Morphological Changes

To observe the SIN-1-induced morphological changes in non-fixed cells, the dye Hoechst 33342 (10 μg/ml) and PI (10 μg/ml) were added to unfixed monolayer cells and kept on ice (Stapper *et al.*, 1995). Other monolayer cells were fixed with PBS containing 2% paraformaldehyde for 30 min, washed twice with PBS, and then reacted with Hoechst 33342 (10 μg/ml) in PBS at 4°C for 30 min.[37] The fixed and unfixed cell nuclei were viewed under a fluorescence microscope.

Flow Cytometric Cell Cycle Analysis

LLC-PK$_1$ cells cultured in 6-well plates were collected by centrifugation, fixed for at least 30 min at 4°C in 3 ml 70% ice-cold EtOH,[38,39] washed twice with PBS, incubated with RNase solution for 30 min at 37°C, and then treated with PI solution at 4°C for 30 min. The cells were analyzed on a FACSCalibur (Becton Dickinson, CA, USA) with laser excitation at 488 nm using a 639 nm band pass filter to collect the red fluorescence due to PI. The percentages of cells at various phases of the cell cycle, namely G_0/G_1, S and G_2/M, were assessed and the data were analyzed using ModFit LT software (Verity Software House, Topsham, ME, USA).

Data Analysis

The results for each group are expressed as mean ± S.E. values. The statistical significance of differences among the four groups was evaluated using Dunnett's test. In all cases, the level for a statistically significant difference was set at $p < 0.05$.

RESULTS

Animal Experiment

Plasma Concentrations of 3-Nitrotyrosine

Figure 2 shows the plasma levels of 3-nitrotyrosine, an indicator of $ONOO^-$ formation *in vivo*, of rats subjected to the LPS plus ischemia-reperfusion

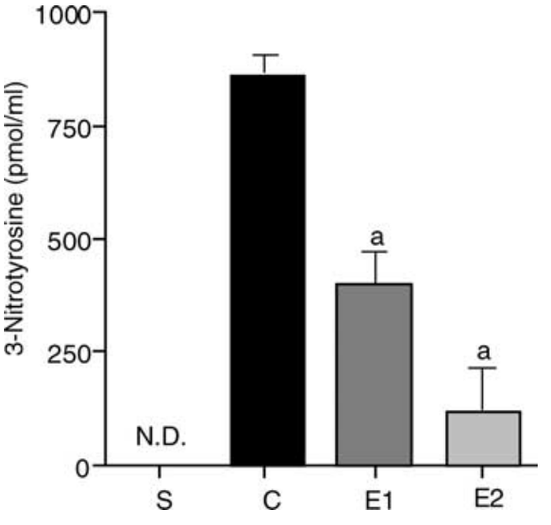

FIGURE 2 Effect of (−)-epicatechin 3-*O*-gallate on plasma 3-nitrotyrosine levels. S, sham treatment; C, LPS plus ischemia-reperfusion; E1, LPS plus ischemia-reperfusion after (−)-epicatechin 3-*O*-gallate (10 μmoles/kg B.W./day); E2, LPS plus ischemia-reperfusion after (−)-epicatechin 3-*O*-gallate (20 μmoles/kg B.W./day). N.D., not detectable; [a]$p < 0.001$ vs. LPS plus ischemic-reperfused control values.

TABLE I Effect of (−)-epicatechin 3-*O*-gallate on oxygen species-scavenging enzymes in renal tissue

Group	SOD (U/mg protein)	Catalase (U/mg protein)	GSH-Px (U/mg protein)
Sham treatment	31.82 ± 2.29	255.3 ± 35.0	138.7 ± 10.3
LPS plus ischemic-reperfused			
Control	16.67 ± 2.52[c]	146.8 ± 19.3[c]	79.5 ± 7.2[c]
(−)-Epicatechin 3-*O*-gallate (10 μmoles/kg B.W./day)	18.18 ± 1.70[c]	176.0 ± 15.3[c]	105.7 ± 8.0[c,e]
(−)-Epicatechin 3-*O*-gallate (20 μmoles/kg B.W./day)	21.45 ± 3.67[c,d]	194.4 ± 22.6[b,d]	118.7 ± 11.0[a,f]

[a]$p < 0.05$, [b]$p < 0.01$, [c] $p < 0.001$ vs. sham treatment values, [d]$p < 0.05$, [e]$p < 0.01$, [f]$p < 0.001$ vs. LPS plus ischemic-reperfused control values.

process. The 3-nitrotyrosine concentration of control rats subjected to LPS plus ischemia-reperfusion increased markedly to 859 pmol/ml, whereas no 3-nitrotyrosine was detected in the plasma of the rats that underwent the sham operation. In contrast, the oral administration of ECg at daily doses of 10 and 20 μmoles/kg body weight to rats for 20 days significantly reduced the formation of 3-nitrotyrosine to 401 and 117 pmol/ml, respectively.

Antioxidant Enzymes in Renal Tissues

As shown in Table I, the activities of the antioxidant enzymes, SOD, catalase and GSH-Px in renal tissues were reduced significantly by LPS plus ischemia-reperfusion, whereas after the oral administration of 10 and 20 μmoles of ECg, the reduced enzyme activities were elevated dose-dependently. The SOD activity increased significantly to 18.18 and 21.45 U/mg protein (9 and 29% increases, respectively) from the control value of 16.67 U/mg protein, and the catalase activity increased from 146.8 to 176.0 and 194.4 U/mg protein (20 and 32% increases, respectively). The GSH-Px activity, which was 79.5 U/mg protein in the LPS plus ischemic-reperfused control rats, increased by 33 and 49% to 105.7 and 118.7 U/mg protein in rats given 10 and 20 μmoles/day ECg, respectively.

Oxidative Damage in Renal Mitochondria

As shown in Table II, the renal mitochondrial GSH content of the LPS plus ischemic-reperfused control rats was significantly lower (a 38% decrease), whereas the TBA-reactive substance level was significantly higher (a 36% increase), than the levels of the rats subjected to the sham operation. However, the decrease in GSH was reversed and the level was elevated from 2.75 to 3.72 and 3.77 nmol/mg protein after ECg administration at doses of 10 and 20 μmoles/day, respectively. In addition, ECg reversed the decrease in the TBA-reactive substance level, which increased from 0.165 to 0.147 and 0.144 nmol/mg protein, respectively, after the above dosages.

Plasma Urea Nitrogen and Cr Levels, and the Urinary MG/Cr Ratio

Table III shows the effects of ECg on plasma and urinary parameters of renal function. The plasma urea nitrogen and Cr levels, and the urinary MG/Cr ratio were increased significantly (4.3-, 4.9- and 1.4-fold, respectively) by LPS plus ischemia-reperfusion. After the administration of ECg at a dose of 20 μmoles/kg body weight/day, the plasma urea nitrogen level declined from 64.5 to 56.4 mg/dl. The plasma Cr level also exhibited a significant decrease from 1.38 to 1.25 mg/dl. In addition, the oral administration of ECg 10 and 20 μmoles/day reduced the urinary MG/Cr ratio markedly from 3.28 to 1.32 (a 60% decrease) and 1.25 (a 62% decrease), respectively.

Cell Culture Experiment

Cellular $ONOO^-$ Formation, Cell Viability and DNA Fragmentation after SIN-1 Treatment

Figure 3 represents the effects of ECg on $ONOO^-$ production, viability and DNA fragmentation in cultured LLC-PK_1 renal epithelial cells treated with the $ONOO^-$ donor SIN-1. The addition of 800 μM SIN-1 to the routine culture medium stimulated cellular production of $ONOO^-$ (1000 pmol/ml), whereas no $ONOO^-$ was detected in the medium of cells cultured without SIN-1. When SIN-1-treated

TABLE II Effect of (−)-epicatechin 3-*O*-gallate on the oxidative damages of renal mitochondria

Group	GSH (nmol/mg protein)	TBA-reactive substance (nmol/mg protein)
Sham treatment	4.42 ± 0.09	0.121 ± 0.001
LPS plus ischemic-reperfused		
Control	2.75 ± 0.14[a]	0.165 ± 0.007[a]
(−)-Epicatechin 3-*O*-gallate (10 μmoles/kg B.W./day)	3.72 ± 0.18[a,b]	0.147 ± 0.003[a,b]
(−)-Epicatechin 3-*O*-gallate (20 μmoles/kg B.W./day)	3.77 ± 0.21[a,b]	0.144 ± 0.007[a,b]

[a]$p < 0.001$ vs. sham treatment values, [b]$p < 0.001$ vs. LPS plus ischemic-reperfused control values.

TABLE III Effect of (−)-epicatechin 3-*O*-gallate on renal function

Group	Plasma urea nitrogen (mg/dl)	Plasma Cr (mg/dl)	Urinary MG/Cr ratio ($\times 10^{-3}$)
Sham treatment	15.1 ± 0.5	0.28 ± 0.01	2.30 ± 0.02
LPS plus ischemic-reperfused			
Control	64.5 ± 2.7[b]	1.38 ± 0.02[b]	3.28 ± 0.93[a]
(−)-Epicatechin 3-*O*-gallate (10 μmoles/kg B.W./day)	64.3 ± 1.6[b]	1.28 ± 0.04[b,c]	1.32 ± 0.16[a,d]
(−)-Epicatechin 3-*O*-gallate (20 μmoles/kg B.W./day)	56.4 ± 1.9[b,d]	1.25 ± 0.07[b,d]	1.25 ± 0.18[a,d]

[a]$p < 0.05$, [b]$p < 0.001$ vs. sham treatment values, [c]$p < 0.01$, [d]$p < 0.001$ vs. LPS plus ischemic-reperfused control values.

LLC-PK$_1$ cells were cultured with 25 and 125 μM ECg, $ONOO^-$ was substantially scavenged in a concentration-dependent manner, and eventually it was not detected in the presence of 125 μM dose ECg. In response to the addition of 800 μM SIN-1, the viability of LLC-PK$_1$ cells decreased significantly to 68%, whereas DNA fragmentation increased 3-fold, compared with cells not treated with SIN-1. In contrast, ECg at concentrations of 25 and 125 μM increased the viability of SIN-1-treated cells markedly to 104 and 129%, respectively, of the SIN-1-free values. Moreover, treatment with 25 and 125 μM ECg reduced DNA fragmentation significantly to 72 and 76% of the SIN-treated levels.

Morphological Analysis

The morphological changes in fixed and non-fixed cells induced by SIN-1 are shown in Fig. 4. The nuclei of fixed cells treated with SIN-1 were small and showed nuclear fragmentations with homogenous Hoechst dye staining, representing a characteristic morphological feature of apoptosis (Fig. 4—upper panel (A)). Non-fixed cell damage was observed as red coloration in the nuclei due to SIN-1-induced infiltration by the PI staining probe (Fig. 4—lower panel (A)). However, the addition of ECg before SIN-1 exposure reduced the apoptotic morphological changes induced by SIN-1 in a concentration-dependent manner in both fixed and non-fixed cells.

Cell Cycle

Table IV shows the effects of ECg on cell cycle disturbance caused by SIN-1. The addition of 800 μM SIN-1 significantly reduced the proportion of LLC-PK$_1$ cells in the G_2/M phase from 6.7 to 1.2%. However, pretreatment with 25 and 125 μM ECg increased the proportion of SIN-1-treated cells in the G_2/M phase from 1.2 to 2.4 and 5.7%, respectively.

DISCUSSION

Numerous pieces of evidence confirm that catechins such as ECg and epigallocatechin gallate have a variety of pharmacologic properties, i.e. antioxidative,[40] antimutagenic,[41] anticarcinogenic,[42,43] anti-inflammatory,[44] antimicrobial[45] and hypolipidemic effects.[46] However, the beneficial effects, particularly against renal oxidative damage, of the catechins have rarely been studied. Therefore, in this study, we focused on the activity of ECg purified from Rhei Rhizoma, which is the main and most effective free radical-scavenging ingredient of the Chinese traditional prescription Wen-Pi-Tang, against renal oxidative injury induced by $ONOO^-$.

Under several experimental conditions of ischemia-reperfusion of the heart, lung or kidney, or endotoxin-induced renal oxidant injury, there were induction of NO synthase, increased O_2^- production, and formation of $ONOO^-$ as the potent oxidizing agent.[47–50] In addition, ischemia/reperfusion of kidney led to tubular dysfunction supported

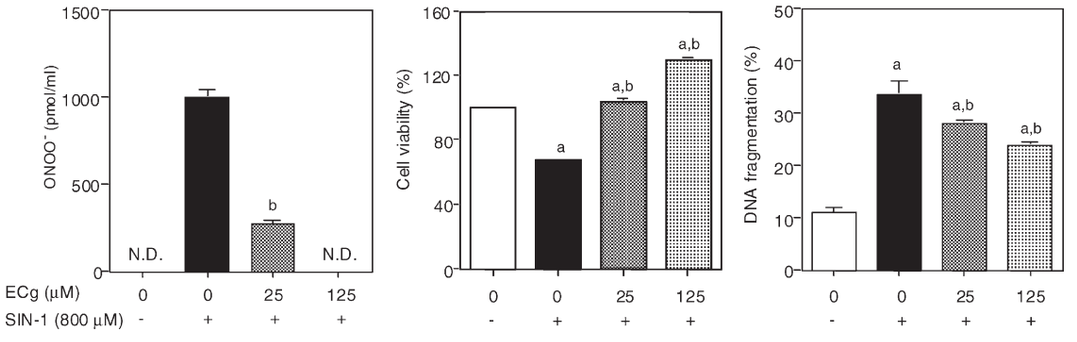

FIGURE 3 Effects of epicatechin 3-*O*-gallate on SIN-1-induced $ONOO^-$ formation, viability and DNA fragmentation in renal epithelial cells, LLC-PK$_1$. N.D., not detectable; [a]$p < 0.001$ vs. no treatment values, [b]$p < 0.001$ vs. SIN-1 treatment values.

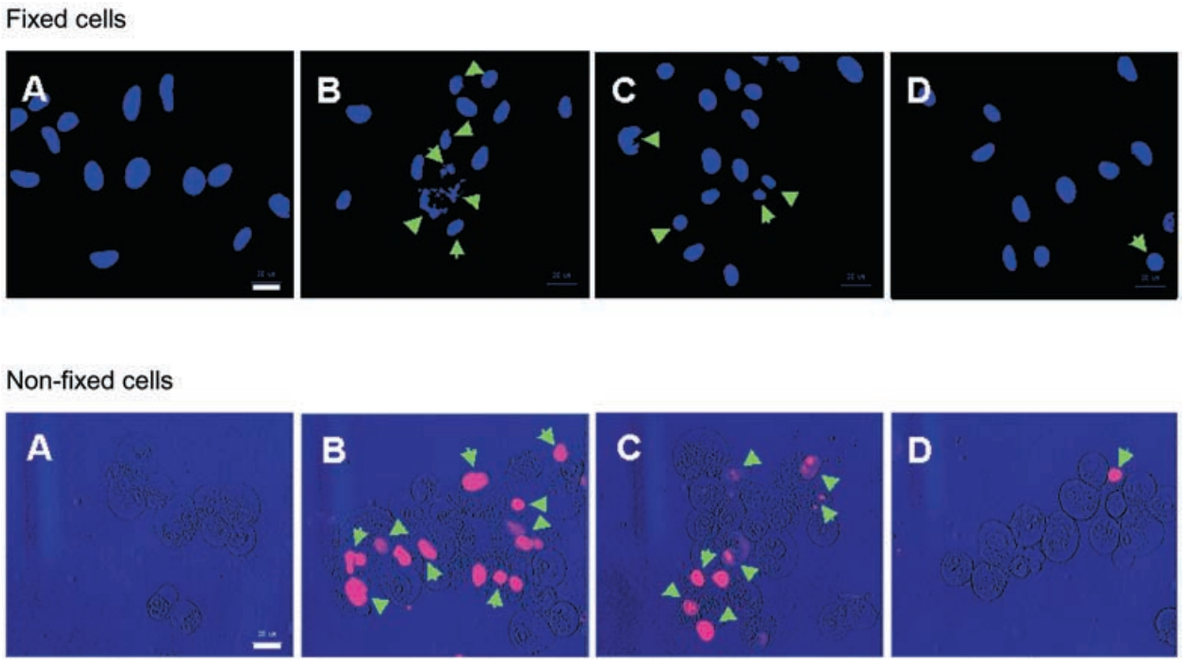

FIGURE 4 Morphological changes of fixed (upper panel) and non-fixed (lower panel) cells. After incubation with epicatechin 3-*O*-gallate for 24 h, SIN-1 was added and the cells were incubated for a further 4 h. A, No treatment; B, SIN-1 (800 μM) treatment; C, SIN-1 (800 μM) and epicatechin 3-*O*-gallate (25 μM) treatment; D, SIN-1 (800 μM) and epicatechin 3-*O*-gallate (125 μM) treatment. Arrows indicate apoptotic cells. Magnification, × 800. Bar represents 20 μm.

by the histological scoring of renal injury.[51] However, due to the instability and reactivity of $ONOO^-$ in biological systems, the formation of enough $ONOO^-$ to enable its toxicity under conditions of renal failure to be evaluated should be accompanied by the simultaneous and excessive generation of NO and O_2^-. Hence, we employed a LPS plus ischemia-reperfusion model *in vivo* in which renal damage is severer than ischemia-reperfusion model without LPS injection and SIN-1 as an $ONOO^-$ donor to evaluate the protective activity of ECg against $ONOO^-$ in a cellular system *in vitro*. $ONOO^-$ reacts with numerous targets, including protein tyrosine residues, to form nitrotyrosine and nitration of target proteins alters the structure or function of molecules, leading to cell dysfunction. The existence of nitrotyrosine and its relationship to cell or tissue damage in pathological conditions have been demonstrated in various lesions, such as atherosclerotic plaque, and in ischemia-reperfusion and acute renal failure models.[3,52] Thus, the ability to inhibit $ONOO^-$-dependent nitration provides a useful assay to screen various compounds for their ability to scavenge $ONOO^-$ and the nitrating species derived from it.[53] Our present study showed significant increases in plasma 3-nitrotyrosine levels occurred in response to the pathological process of LPS plus ischemia-reperfusion (Fig. 2) and that cellular formation of $ONOO^-$ was increased by SIN-1 treatment (Fig. 3). Furthermore, the viability of LLC-PK_1 renal epithelial cells, which are derived from proximal tubules that are known to be susceptible to ischemic renal failure, decreased as $ONOO^-$ formation increased. Proximal tubular epithelial cell death was observed under various pathological conditions of renal failure.[54,55] Therefore, the central role of the proximal tubular cells in renal injury and dysfunction has attracted considerable attention. The magnitudes of the significant elevations of $ONOO^-$ production *in vivo* and in the cellular system were

TABLE IV Effect of (−)-epicatechin 3-*O*-gallate on the cell cycle

	Percentage of cells in each phase of cell cycle (%)		
Treatment	G_0/G_1	S	G_2/M
None	61.1 ± 3.0	32.3 ± 1.9	6.7 ± 1.1
SIN-1 (800 μM)	64.4 ± 0.6	34.5 ± 1.6	1.2 ± 1.2[c]
SIN-1 (800 μM) and epicatechin 3-*O*-gallate (25 μM)	60.9 ± 1.9	36.7 ± 2.7[a]	2.4 ± 0.7[b]
SIN-1 (800 μM) and epicatechin 3-*O*-gallate (125 μM)	63.4 ± 1.8	30.9 ± 1.2	5.7 ± 0.8[d]

[a]$p < 0.05$, [b]$p < 0.01$, [c]$p < 0.001$ vs. none treatment values, [d]$p < 0.01$ vs. SIN-1 treatment values.

decreased by ECg treatment prior to the processes without affecting NO levels (data on NO levels not shown). From the results of both the *in vivo* and cell culture experiments, ECg was considered to improve $ONOO^-$-mediated renal failure by acting as a direct scavenger of $ONOO^-$.

$ONOO^-$-mediated oxidant injury resulted from the shift between the oxidant and antioxidant balance, indicating depletion of the antioxidant defense mechanisms had occurred.[6] Our study also showed that the antioxidative defense system was significantly suppressed by the excessive increase of $ONOO^-$ resulting from the LPS plus ischemia-reperfusion process. To counteract the damaging effect of oxidative stress, biological systems have developed two important defense mechanisms that prevent or limit oxidative stress.[56] One is the antioxidative enzymatic system, including SOD, catalase and GSH-Px, which decomposes reactive oxygen species. The other is a thiol reducing buffer containing small proteins, such as GSH and thioredoxin, with a redox-active sulfhydryl group. Our present investigation showed that the administration of ECg resulted in concentration-dependent elevations of the activities of the antioxidative enzymes, SOD, catalase and GSH-Px, and the cellular antioxidant, GSH (Tables I and II). These findings suggest that ECg exerts protective activity against $ONOO^-$-mediated oxidative damage. SOD plays a role as an O_2^- scavenger before O_2^- reacts with NO to form $ONOO^-$. In addition, catalase is involved in the dismutation of H_2O_2 and inhibition of •OH production, a decomposition product of $ONOO^-$ that is a highly toxic and reactive oxygen free radical mainly responsible for the oxidative damage. Moreover, GSH-Px detoxifies lipid peroxides as well as H_2O_2.

$ONOO^-$ promotes mitochondrial oxidative damage by directly reacting with various mitochondrial targets[10] and mitochondria can partially detoxify $ONOO^-$ as a result of the contribution of one or more scavenging systems, including cytochrome *c* oxidase, GSH and ubiquinol.[57–59] In particular, the redox state of GSH plays an important role in the protection from oxidative stress. Therefore, we investigated the effect of ECg on $ONOO^-$-mediated oxidative stress by measuring the mitochondrial GSH level. The result showed that the process of LPS plus ischemia-reperfusion resulted in a decrease of the GSH level in renal mitochondria, but the depletion of GSH caused by this process was reversed significantly by the administration of ECg (Table II). Lizasoain *et al.*[60] reported that GSH prevented $ONOO^-$-mediated oxidation and nitration in mitochondria. The reaction of $ONOO^-$ with GSH modulates potentially deleterious reactions caused by $ONOO^-$.[61] Moreover, Yen and Lai[62] demonstrated that the inhibition of $ONOO^-$-mediated oxidative damage is attributed to the redox state of GSH and antioxidative enzyme activity such as GSH-Px, GSH reductase and catalase. In addition, renal GSH is well known to protect against renal failure by acting as an important cellular antioxidant, •OH scavenger and cofactor for GSH-Px.[63] Therefore, GSH depletion increases the susceptibility of cells to oxidative stress and is involved in the chronic pathogenesis of several conditions, including nephropathy.[60,63,64] The result that ECg increases the mitochondrial GSH level indicates that ECg makes an effect on GSH redox state, resulting in the alleviation of mitochondrial oxidative damage through inhibition of $ONOO^-$-mediated oxidation and nitration, and improvement of renal failure through the enhancement of antioxidative defense systems.

$ONOO^-$ has been reported to induce membrane lipid peroxidation.[6] Subjecting the kidneys to LPS plus ischemia-reperfusion, which generated excessive $ONOO^-$, increased lipid peroxidation of renal mitochondria (Table II) and we confirmed the mitochondrial oxidative damage caused by $ONOO^-$. In contrast, the administration of ECg reduced the magnitude of the lipid peroxidation level elevation caused by the experimental process. Several studies have demonstrated that catechins suppress the occurrence of lipid peroxidation in biological tissues and subcellular fractions through the elimination of oxidative stress.[18,65–67] The inhibition of lipid peroxidation exerts various pharmacological effects that prevent oxygen radical-induced pathological events. In the light of these reports, ECg is expected to ameliorate the oxidative damage of renal mitochondria induced by $ONOO^-$ through the inhibition of lipid peroxidation.

Under conditions of renal failure, the excretion of final metabolites from the kidney is reduced because of the decreased renal function, resulting in accumulation of uremic toxins in the body.[68] Uremic toxins accumulating in the blood and tissue as renal failure progresses have an impact on several biochemical and physiological functions. Our present study showed that LPS plus ischemia-reperfusion elevated the levels of uremic toxins such as urea nitrogen and Cr, leading to renal damage and dysfunction (Table III). In addition, the procedure resulted in an increase of the urinary MG/Cr, which has been implicated in •OH generation,[69] indicating that •OH, a powerful oxidant formed during the decomposition of $ONOO^-$, was involved in the renal failure resulting from the process. We reported that ECg markedly reduced the serum urea nitrogen, Cr, MG and guanidinosuccinic acid levels, and increased the glomerular filtration rate and renal blood flow in rats with adenine-induced renal failure, thereby improving metabolism under conditions of renal failure.[16,70,71] This

study also implies that the administration of ECg would ameliorate the renal injury in the LPS plus ischemia-reperfusion animal model through the decreases in the level of uremic toxins and the MG/Cr. In particular, the MG/Cr decrease suggests two possible protective mechanisms of ECg against damage caused by the procedure. One is that ECg scavenges $ONOO^-$, thereby inhibiting •OH formation resulting from the decomposition of $ONOO^-$. The other is that •OH produced from $ONOO^-$ is itself scavenged by ECg. Furthermore, we hypothesize that these two mechanisms are involved simultaneously in the inhibition of •OH generation.

Our *in vivo* results confirmed that $ONOO^-$ production resulted in renal failure in rats due to oxidative stress and the formation of uremic toxins. In addition, $ONOO^-$ production led to renal epithelial cell death in a SIN-1-induced $ONOO^-$-generating cell culture system. However, ECg improved the renal injury induced by $ONOO^-$. Therefore, we hypothesize that several cellular mechanisms are involved in the protective activity of ECg against renal failure. Thus, to investigate the mechanisms of its action against $ONOO^-$, the effects of ECg on $ONOO^-$-mediated apoptosis and cell cycle regulation in a cell culture system were observed. The cytotoxic effects of $ONOO^-$ are ascribed to DNA damage, inhibition of DNA repair, and induction of cell death either by apoptosis or necrosis.[72–77] In particular, apoptosis has been regarded to contribute to extensive cell loss in many pathological states.[74,78] Our present results also indicate that $ONOO^-$ generated by SIN-1 causes apoptotic cell death. The LLC-PK_1 cells exposed to SIN-1 displayed DNA fragmentation and morphological changes, such as small nuclei and nuclear fragmentation, indicative of apoptosis (Figs. 3 and 4). However, the presence of ECg prior to SIN-1 exposure resulted in significant decreases in the DNA fragmentation rate and characteristic apoptotic morphological changes, implying that ECg would prevent cell death resulting from $ONOO^-$-mediated apoptosis.

The oxidative stress resulting from free radicals disturbed the cell cycle, eventually causing inhibition of cell proliferation.[79,80] Most organisms respond to biological damage by regulating the cell cycle, apoptosis and DNA repair pathway. In particular, regulation of the cell cycle is of obvious importance for the proper structure and function of a biological system. As shown in this study, $ONOO^-$ disordered the cell cycle by reducing the proportion of cells in the G_2/M phase (Table IV). However, the addition of ECg before SIN-1 treatment increased the proportion significantly and concentration-dependently, which implies that ECg regulated the cell cycle by promoting G_2/M phase arrest. We consider that the protective activity of ECg against $ONOO^-$ involved a decrease in apoptosis-mediated cell death and regulation of the cell cycle, although the specific mechanisms responsible are unclear and need to be elucidated.

The results of our present study suggest that the renal damage mediated by excessive $ONOO^-$ under *in vivo* and cell culture system would be improved by the administration of ECg.

Acknowledgements

This work was supported in part by a grant from the Japan Foundation for Aging and Health.

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