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(–)-Epicatechin 3-*O*-gallate ameliorates the damages related to peroxynitrite production by mechanisms distinct from those of other free radical inhibitors

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Abstract

This study was carried out to elucidate whether the protective activity of (–)-epicatechin 3-*O*-gallate (ECg) against excessive peroxynitrite ($ONOO^-$) production, is distinct from the activity of several well-known free radical inhibitors, the $ONOO^-$ inhibitors ebselen and uric acid, the superoxide anion (O_2^-) scavenger copper zinc superoxide dismutase (CuZnSOD) and the selective inducible nitric oxide synthase inhibitor L-N^6-(1-iminoethyl)lysine hydrochloride (L-NIL). To generate $ONOO^-$, male Wistar rats (n = 6/group) were subjected to ischaemia–reperfusion process together with lipopolysaccharide (LPS) injection. Although ECg did not scavenge the $ONOO^-$ precursors nitric oxide (NO) and O_2^-, it reduced the 3-nitrotyrosine level, a property similar to that of uric acid, but distinct from L-NIL. In addition, the elevation in myeloperoxidase activity was reversed by the administration of ECg, uric acid and SOD, but not by that of L-NIL. Furthermore, ECg was the more potent scavenger of the $ONOO^-$ decomposition product, the hydroxyl radical (·OH), than any other free radical inhibitor tested. The LPS plus ischaemia–reperfusion process resulted in renal dysfunction, estimated by measuring the parameters of renal function – serum urea nitrogen and creatinine levels. However, administration of ECg ameliorated renal dysfunction more than that of the other free radical inhibitors. Moreover, ECg reduced the excessive uric acid level, while the others did not, suggesting a property of ECg distinct from the others. Furthermore, proteinuria, which was demonstrated by the low- and high-molecular weight (LMW and HMW) protein bands of the sodium dodecyl sulfate-polyacrylamide gel electrophoresis pattern, caused by LPS plus ischaemia–reperfusion, was attenuated by administration of ECg and L-NIL, after which the HMW band intensities decreased and LMW protein bands were absent. This study indicates that, in an in-vivo model of $ONOO^-$ generation, ECg, L-NIL and uric acid exert stronger protective activity against $ONOO^-$-induced oxidative damage than SOD and ebselen, and that the mechanism whereby ECg protects against $ONOO^-$ is distinct from that of L-NIL or uric acid.

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Introduction

Evidence for the role of reactive oxygen and nitrogen metabolites in the pathogenesis of renal diseases has accumulated and peroxynitrite ($ONOO^-$) formed in-vivo from the superoxide anion (O_2^-) and nitric oxide (NO) has been suggested to be an important causative agent in pathogenesis of cellular damage and renal dysfunction (Radi et al 1991; Douki et al 1996). The pathological effects of $ONOO^-$ and its decomposition product, the hydroxyl radical (·OH), contribute to the antioxidant depletion, alterations of protein structure and function by tyrosine nitration and oxidative damage observed in human diseases and animal models of diseases (Fukuyama et al 1997; Ischiropoulos 1998; Nakazawa et al 2000; Ceriello et al 2001; Cuzzocrea & Reiter 2001). Therefore, reactive nitrogen species should be considered as potential targets for therapeutic intervention in the prevention and treatment of oxidative stress-related diseases, including renal failure. Several workers have reported that the effective use of $ONOO^-$ inhibitors and oxygen radical scavengers ameliorates oxidative stress-mediated pathological changes (Bartlett et al 1995; Chatterjee et al 2000, 2002; Noiri et al 2001).

As the result of great effort to search for novel and safe $ONOO^-$ scavengers, we found that (–)-epicatechin 3-*O*-gallate (ECg) attenuated the $ONOO^-$-induced renal

damage observed in a cellular system and an in-vivo animal model, suggesting that it acts as an $ONOO^-$ inhibitor and antioxidant (Yokozawa et al 2003). Other results also showed that polyphenols, such as catechin, ECg and epigallocatechin gallate, are well known as natural agents that protect against $ONOO^-$-induced nitration and oxidation reactions (Chung et al 1998; Arteel & Sies 1999; Bors & Michel 1999). However, further understanding of the mechanisms whereby ECg attenuates $ONOO^-$-induced renal damage, that are distinct from those of the well-known free radical scavengers, is needed to develop an efficient and safe therapeutic agent for the disorders caused by $ONOO^-$. Therefore, in this study, we used an in-vivo lipopolysaccharide (LPS) plus ischaemia–reperfusion model, in which excessive $ONOO^-$ is produced, to compare the protective activity of ECg against $ONOO^-$-induced renal damage with that of the well-known free radical inhibitors ebselen and uric acid ($ONOO^-$ inhibitors), copper zinc superoxide dismutase (CuZnSOD; O_2^- scavenger) and L-N^G-(1-iminoethyl)lysine hydrochloride (L-NIL; inducible nitric oxide synthase (iNOS) inhibitor).

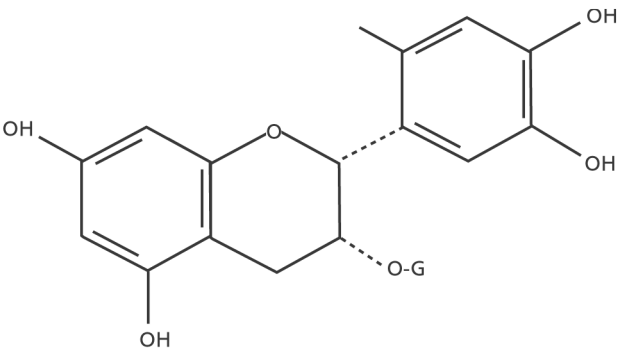

Figure 1 Chemical structure of (−)-epicatechin 3-*O*-gallate (ECg).

Materials and Methods

Reagents

LPS (from *Escherichia coli* serotype 055: B5), 3-nitro-L-tyrosine, nitrate reductase, hexadecyltrimethylammonium bromide (HETAB) and 3,3′,5,5′-tetramethylbenzidine were purchased from Sigma Chemical Co. (St Louis, MO). Uric acid, CuZnSOD, L-NIL, phenazine methosulfate (PMS), nitro blue tetrazolium (NBT), 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) and CBB R 250 were purchased from Wako Pure Chemical Industries Ltd (Osaka, Japan). Ebselen was purchased from CN Biosciences Inc. (Darmstadt, Germany).

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. Fraction IV (140 g) was rechromatographed using Sephadex LH-20 with ethanol and MCI-gel CHP 20P with H_2O–MeOH (1:0→1:1 v/v) to yield ECg (11.0 g) (Nonaka et al 1981). The chemical structure of ECg is shown in Figure 1.

Animals and treatments

The Guidelines for Animal Experimentation approved by Toyama Medical and Pharmaceutical University were followed for these experiments. Male Wistar rats, 220∼230 g, were obtained from Japan SLC Inc. (Hamamatsu, Japan). They were kept in wire-bottomed cages at constant temperature (about 25 °C) and humidity (about 60%) with a 12-h light–dark cycle and allowed free access to a commercial chow (24.0% protein, 3.5% lipid and 60.5% carbohydrate; CLEA Japan Inc., Tokyo, Japan) and water. Following several days of adaptation, the rats were divided into eight groups, avoiding any inter-group differences in body weight. Six rats were used for each experimental group. Using aseptic technique, bilateral flank incisions were made, the renal artery and vein of each kidney were occluded with microvascular clamps for 60 min and then the incisions were closed with skin staples. Fifty minutes after starting the ischaemia, the rats received an intravenous injection of LPS (5 mg kg^{-1} body weight) and then the microvascular clamps were released for 360 min. The sham group underwent sham surgery in which incisions were made to expose the kidneys, but the renal pedicles were not clamped. Subsequently, the rats were returned to clean cages and allowed free access to water. Groups of rats were injected intraperitoneally twice with ECg, ebselen, uric acid or L-NIL, 10 or 20, 5, 62.5 and 3 mg kg^{-1} body weight for a single injection, respectively. The first injection was given 6 h before surgery and the second injection was given 5 min after clamp release, according to the method reported previously by other researchers (Yu et al 1998; Walker et al 2000; Noiri et al 2001). The CuZnSOD group (10 000 U kg^{-1} body weight) was injected intravenously at the same time as LPS was injected (Shutenko et al 1999). Six hours after the LPS challenge, blood was collected by cardiac puncture and the blood samples were centrifuged immediately to prepare plasma. Subsequently, the renal arteries of each rat were perfused with ice-cold perfusion buffer (pH 7.4) comprising 50 mM sodium phosphate, 10 mM ethylenediamine-*N,N,N′,N′*-tetraacetic acid, disodium salt (EDTA-2Na) and 120 mM NaCl, and the kidneys were removed, quickly frozen and kept at −80 °C until analysis.

Plasma 3-nitrotyrosine levels

The plasma concentration of 3-nitrotyrosine was determined by high-performance liquid chromatography (HPLC) using the methods of both van der Vliet et al (1994) and Kaur et al (1998) with slight modifications. Briefly, the blood samples were centrifuged for 15 min at 17 300 *g*, 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 17 300 *g* and passed through a 10 000-Da molecular mass cut-off filter. The samples were loaded onto a reverse-phase column (Nucleosil 5 μm C-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^{-1}. 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 the peak areas of known amounts of the external standards.

Myeloperoxidase (MPO) activity in renal tissue

The renal MPO activity was determined by the tetramethylbenzidine method, as described by Suzuki et al (1983) and Laight et al (1994). Renal tissue was homogenized in five volumes of a solution comprising 0.5% HETAB (w/v) dissolved in 50 mM potassium phosphate buffer (pH 6.0) and then centrifuged for 30 min at 20 000 *g* at 4 °C. This supernatant was incubated for 2 h at 60 °C and then centrifuged once more at 4000 *g* for 12 min. A sample of supernatant (40 μL) was removed and added to a reaction mixture (160 μL), comprising 1.6 mM 3,3′,5,5′-tetramethylbenzidine and 3 mM hydrogen peroxide (H_2O_2) in 80 mM phosphate buffer (pH 5.4), in a 96-well microplate. The rate of change of the absorbance was measured spectrophotometrically at 650 nm and the MPO activity was expressed as the absorbance per minute per 100 mg protein using change of absorbance. Protein levels were determined by the micro-biuret method with bovine serum albumin as the standard (Itzhaki & Gill 1964).

Plasma nitrite (NO_2^-)/nitrate (NO_3^-) and O_2^- levels

NO_2^-/NO_3^- levels were measured primarily by following the method of Misko et al (1993). Briefly, NO_3^- in the plasma samples (20 μL) was first reduced to NO_2^- by incubation with nitrate reductase (700 mU mL^{-1}), 200 μM NADPH solution was added to each sample and then the NO_2^-/NO_3^- levels were measured by a microplate assay method based on the Griess reaction (Green et al 1982). The levels were calculated by comparison with the levels of standard solutions of sodium nitrite.

Plasma O_2^- levels were measured by following the method described previously by Ewing & Janero (1995). For the assay, a sample (25 μL) of plasma was pipetted into a microplate well containing 200 μL freshly prepared 125 μM EDTA, 62 μM NBT and 98 μM NADH in 50 mM phosphate buffer, pH 7.4. The reaction was initiated by adding 25 μL freshly prepared 33 μM PMS in 50 mM phosphate buffer, pH 7.4. The absorbance at 540 nm was monitored continuously over 5 min, as an index of NBT reduction, using a Microplate Reader (Model 3550-UV; BIO-RAD, Tokyo, Japan).

·OH in renal tissue

Electron spin resonance (ESR) spectroscopy combined with spin-trapping with DMPO was used to determine the level of scavenging by renal tissue of ·OH generated by the Fenton reaction. Kidney tissue was homogenized with a 10-fold volume of ice-cold physiological saline. The homogenate (30 μL) was added to the Fenton reaction mixture (170 μL) containing 10% DMPO (v/v), 200 μM $FeSO_4$, 1 mM DTPA and 1 mM H_2O_2, and then stirred for 10 s. The ESR spectra of the mixtures were measured using a JEOL FE3X spectrometer (JEOL, Tokyo, Japan) at 25 °C for 5 min after the addition of DMPO. The microwave power, modulation amplitude and sweep time were set at 1 mW, 0.1 mT and 0.5 min, respectively. Two peaks of external manganese dioxide appearing at $g = 1.981$ and $g = 2.034$ were used to determine both the *g* value and the amount of each DMPO adduct.

Plasma uric acid, urea nitrogen and creatinine (Cr) levels

Plasma uric acid concentrations were measured using the commercial reagent Uric Acid (Wako Pure Chemical Industries Ltd, Osaka, Japan), which is based on the reaction of uricase, *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-*m*-toluidine sodium salt and 4-aminoantipyrine. Urea nitrogen and Cr concentrations were determined using the commercial reagents BUN Kainos and CRE-EN Kainos (Kainos Laboratories, Inc., Tokyo, Japan), respectively.

Electrophoretic pattern analysis of proteinuria

Samples (500 ng) of protein were loaded on to 10% acrylamide gel, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the protein bands were stained with CBB R 250. The molecular masses of the urinary protein bands were assessed by comparison with those of the bands of standard proteins.

Data analysis

Results are expressed as means ± s.e. One-way analysis of variance and Dunnett's test were employed to analyse the significant differences between the sham-operation group and LPS plus ischaemic–reperfused groups with or without ECg/free radical inhibitor treatment, and between LPS plus ischaemic–reperfused control group and ECg/free radical inhibitor-treated groups. A *P* value less than 0.05 was accepted as significant.

Results

Plasma 3-nitrotyrosine concentrations

Figure 2 shows the effects of LPS plus ischaemia–reperfusion and the free radical inhibitors on the plasma 3-nitrotyrosine level, an indicator of $ONOO^-$ formation in-vivo. The 3-nitrotyrosine level of the control group subjected to LPS plus ischaemia–reperfusion was 560 pmol mL^{-1}, whereas that of the group subjected to the sham operation

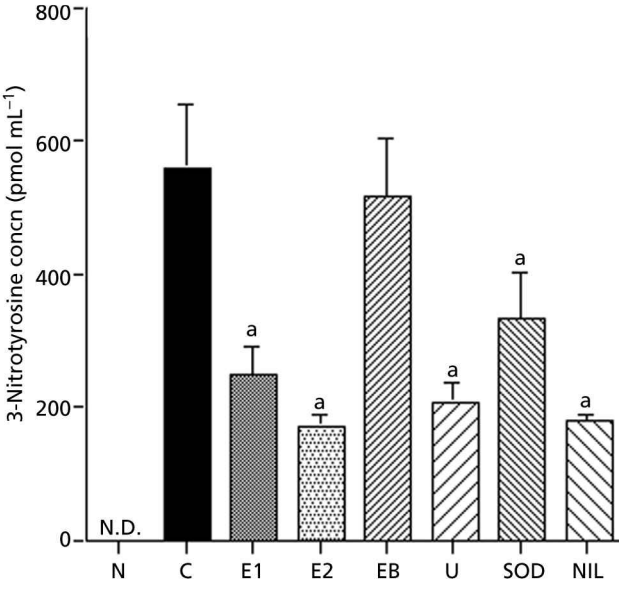

Figure 2 Effect of (−)-epicatechin 3-*O*-gallate (ECg) and free radical inhibitors on plasma 3-nitrotyrosine level in rats. N, sham operation; C, LPS plus ischaemia–reperfusion; E1, LPS plus ischaemia–reperfusion after ECg (10 mg kg^{-1}); E2, LPS plus ischaemia–reperfusion after ECg (20 mg kg^{-1}); EB, LPS plus ischaemia–reperfusion after ebselen (5 mg kg^{-1}); U, LPS plus ischaemia–reperfusion after uric acid (62.5 mg kg^{-1}); SOD, LPS plus ischaemia–reperfusion after CuZnSOD (10 000 U kg^{-1}); NIL, LPS plus ischaemia–reperfusion after L-NIL (3 mg kg^{-1}). N.D., not detectable. [a] $P < 0.001$ vs LPS plus ischaemic–reperfused control values.

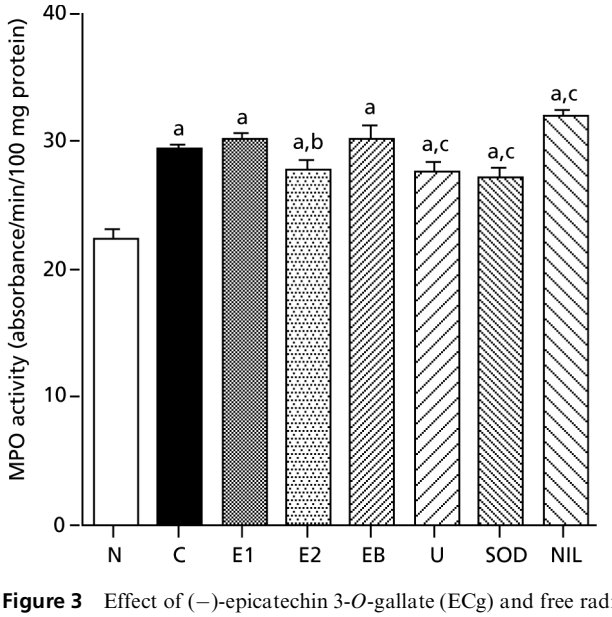

Figure 3 Effect of (−)-epicatechin 3-*O*-gallate (ECg) and free radical inhibitors on renal MPO activity in rats. N, sham operation; C, LPS plus ischaemia–reperfusion; E1, LPS plus ischaemia–reperfusion after ECg (10 mg kg^{-1}); E2, LPS plus ischaemia–reperfusion after ECg (20 mg kg^{-1}); EB, LPS plus ischaemia–reperfusion after ebselen (5 mg kg^{-1}); U, LPS plus ischaemia–reperfusion after uric acid (62.5 mg kg^{-1}); SOD, LPS plus ischaemia–reperfusion after CuZnSOD (10 000 U kg^{-1}); NIL, LPS plus ischaemia–reperfusion after L-NIL (3 mg kg^{-1}). [a] $P < 0.001$ vs sham operation values; [b] $P < 0.01$, [c] $P < 0.001$ vs LPS plus ischaemic–reperfused control values.

was undetectable. The administration of ECg at doses of 10 and 20 mg kg^{-1} led to dose-dependent low levels of 3-nitrotyrosine compared with the control group. In addition, uric acid, L-NIL and CuZnSOD also reduced the level significantly, while ebselen did not show significant effect on $ONOO^-$ formation.

Renal MPO activities

The renal MPO activity was significantly higher in LPS plus ischaemia–reperfusion groups of rats than in the rats that underwent a sham operation (Figure 3). Administration of ECg at the 20 mg kg^{-1} dose, uric acid and CuZnSOD resulted in inhibition of MPO activity, whereas ebselen did not inhibit MPO activity and L-NIL increased it.

Plasma NO and O_2^- concentrations

Table 1 shows the effects of ECg (10 and 20 mg kg^{-1}) and the free radical inhibitors ebselen, uric acid, CuZnSOD and L-NIL (5 mg, 62.5 mg, 10 000 U and 3 mg kg^{-1}, respectively) on the plasma NO and O_2^- levels in rats. The LPS plus ischaemia–reperfusion process elevated both the levels of NO and O_2^- significantly compared with the sham operation. However, the plasma levels of NO and O_2^- were significantly reduced by L-NIL and CuZnSOD, respectively, while ECg and the other free radical inhibitors had no effect on NO or O_2^- level.

·OH-Scavenging abilities in renal tissue

The ability of renal tissue to scavenge ·OH radicals generated by the Fenton reaction are summarized in Table 2. After the LPS plus ischaemia–reperfusion process, the height of the DMPO-OH peak of the ESR spectrum increased significantly by about 4-fold compared with the value for the rats that underwent the sham operation. In contrast, the administration of ECg at 10 and 20 mg kg^{-1}, CuZnSOD and L-NIL led to significant reductions of the ·OH level to 84 and 85, 81 and 83% decreases, respectively, while ebselen and uric acid exerted no effect on the ·OH-scavenging activity.

Plasma uric acid concentrations

A significantly high level of plasma uric acid was shown in the rats that underwent the LPS plus ischaemia–reperfusion

Table 1 Effect of (−)-epicatechin 3-*O*-gallate (ECg) and free radical inhibitors on plasma NO and O_2^- radicals in rats.

Group	NO (μM)	O_2^- (O.D.)
Sham operation	1.71 ± 0.18	0.315 ± 0.013
LPS plus ischaemia–reperfusion		
Control	15.33 ± 0.72[b]	0.371 ± 0.011[a]
ECg (10 mg kg^{-1})	15.02 ± 1.15[b]	0.377 ± 0.019[b]
ECg (20 mg kg^{-1})	14.24 ± 0.33[b]	0.401 ± 0.008[b]
Ebselen (5 mg kg^{-1})	15.98 ± 1.35[b]	0.345 ± 0.007
Uric acid (62.5 mg kg^{-1})	15.08 ± 1.15[b]	0.360 ± 0.026[a]
SOD (10 000 U kg^{-1})	19.04 ± 1.72[b,d]	0.336 ± 0.016[c]
L-NIL (3 mg kg^{-1})	3.39 ± 0.25[e]	0.363 ± 0.022[a]

[a] $P < 0.01$, [b] $P < 0.001$ vs sham operation values; [c] $P < 0.05$, [d] $P < 0.01$, [e] $P < 0.001$ vs LPS plus ischaemic–reperfused control values.

Table 2 Effect of (−)-epicatechin 3-*O*-gallate (ECg) and free radical inhibitors on renal hydroxyl radical in rats.

Group	Hydroxyl radical (DMPO-OH)
Sham operation	0.29 ± 0.07
LPS plus ischaemia–reperfusion	
Control	1.15 ± 0.15[a]
ECg (10 mg kg^{-1})	0.18 ± 0.01[b]
ECg (20 mg kg^{-1})	0.17 ± 0.00[b]
Ebselen (5 mg kg^{-1})	1.10 ± 0.18[a]
Uric acid (62.5 mg kg^{-1})	1.06 ± 0.07[a]
SOD (10 000 U kg^{-1})	0.22 ± 0.01[b]
L-NIL (3 mg kg^{-1})	0.20 ± 0.03[b]

[a] $P < 0.001$ vs sham operation values; [b] $P < 0.001$ vs LPS plus ischaemic–reperfused control values.

Table 3 Effect of (−)-epicatechin 3-*O*-gallate (ECg) and free radical inhibitors on plasma uric acid level in rats.

Group	Uric acid (mg dL^{-1})
Sham operation	1.53 ± 0.18
LPS plus ischaemia–reperfusion	
Control	1.95 ± 0.03[a]
ECg (10 mg kg^{-1})	1.64 ± 0.24
ECg (20 mg kg^{-1})	1.12 ± 0.11[c]
Ebselen (5 mg kg^{-1})	2.15 ± 0.37[b]
Uric acid (62.5 mg kg^{-1})	1.96 ± 0.35
SOD (10 000 U kg^{-1})	2.09 ± 0.09[a]
L-NIL (3 mg kg^{-1})	1.57 ± 0.25

[a] $P < 0.05$, [b] $P < 0.01$ vs sham operation values; [c] $P < 0.001$ vs LPS plus ischaemic–reperfused control values.

Table 4 Effect of (−)-epicatechin 3-*O*-gallate (ECg) and free radical inhibitors on plasma urea nitrogen and creatinine levels in rats.

Group	Urea nitrogen (mg dL^{-1})	Creatinine (mg dL^{-1})
Sham operation	14.11 ± 0.32	0.31 ± 0.01
LPS plus ischaemia–reperfusion		
Control	53.07 ± 0.71[a]	1.05 ± 0.04[a]
ECg (10 mg kg^{-1})	54.59 ± 7.09[a]	1.26 ± 0.09[a,c]
ECg (20 mg kg^{-1})	44.16 ± 1.28[a,b]	0.88 ± 0.03[a,b]
Ebselen (5 mg kg^{-1})	49.45 ± 0.48[a]	1.18 ± 0.07[a]
Uric acid (62.5 mg kg^{-1})	42.84 ± 3.48[a,c]	0.90 ± 0.17[a]
SOD (10 000 U kg^{-1})	51.90 ± 6.36[a]	1.26 ± 0.06[a,c]
L-NIL (3 mg kg^{-1})	45.04 ± 2.01[a,b]	1.06 ± 0.03[a]

[a] $P < 0.001$ vs sham operation values; [b] $P < 0.05$, [c] $P < 0.01$ vs LPS plus ischaemic–reperfused control values.

process compared with sham operation (Table 3). The administration of ECg 20 mg kg^{-1} reduced the uric acid level from 1.95 mg dL^{-1} to 1.12 mg dL^{-1} (43% decrease), but the other free radical inhibitors did not change the uric acid level.

Plasma urea nitrogen and Cr levels

Table 4 shows the effect on renal dysfunction in the rats, assessed by measuring plasma urea nitrogen and Cr levels, of LPS plus ischaemia–reperfusion. The levels of urea nitrogen and Cr were high, about 3.8 and 3.4 fold, after the LPS plus ischaemia–reperfusion process compared with the sham operation. While the treatment with ECg (20 mg kg^{-1} dose), uric acid and L-NIL significantly reduced the urea nitrogen level, the increase in the plasma Cr level was reversed significantly only by ECg at the dose of 20 mg kg^{-1}.

Electrophoretic patterns of proteinuria

Figure 4 shows the effects of ECg and the other free radical inhibitors on proteinuria induced by LPS plus ischaemia–reperfusion in rats. The bands were divided into low- and high-molecular-weight (LMW and HMW) proteins relative to the marker albumin (76 kDa). The SDS-PAGE pattern of the control group subjected to LPS plus ischaemia–reperfusion showed minor HMW and major LMW protein bands, but the group subjected to the sham operation did not show these bands. However, the groups treated with ECg and the other free radical inhibitors showed a tendency towards HMW bands of lower intensity and LMW protein bands were absent compared with the control group and, in particular, ECg and L-NIL showed greater amelioration of proteinuria than the others.

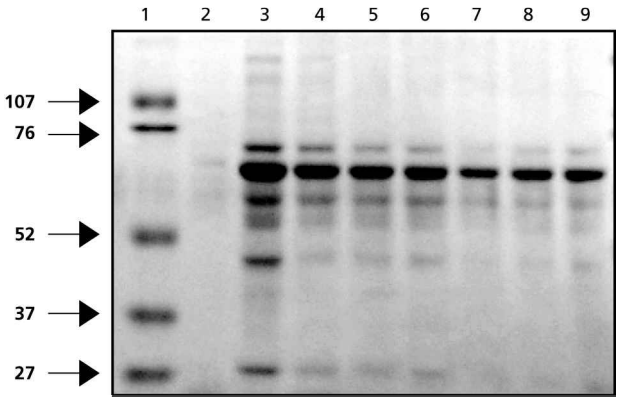

Figure 4 Effect of (−)-epicatechin 3-*O*-gallate (ECg) and free radical inhibitors on SDS-PAGE pattern of proteinuria in rats. 1, marker; 2, sham operation; 3, LPS plus ischaemia–reperfusion; 4, LPS plus ischaemia–reperfusion after ebselen ($5\,mg\,kg^{-1}$); 5, LPS plus ischaemia–reperfusion after uric acid ($62.5\,mg\,kg^{-1}$); 6, LPS plus ischaemia–reperfusion after CuZnSOD ($10\,000\,U\,kg^{-1}$); 7, LPS plus ischaemia–reperfusion after L-NIL ($3\,mg\,kg^{-1}$); 8, LPS plus ischaemia–reperfusion after ECg ($20\,mg\,kg^{-1}$); 9, LPS plus ischaemia–reperfusion after ECg ($10\,mg\,kg^{-1}$). Markers (kDa): 107, phosphorylase B; 76, bovine serum albumin; 52, ovalbumin; 37, carbonic anhydrase; 27, soybean trypsin inhibitor.

Discussion

Natural antioxidants with free radical-scavenging activity, such as polyphenols, have received much attention as potential, non-toxic, treatments for oxidative-stress-related pathological conditions. Our previous study suggested that ECg has great potential for ameliorating $ONOO^-$-induced renal damage (Yokozawa et al 2003). In the LPS plus ischaemia–reperfusion animal model, ECg attenuated the renal dysfunction induced by $ONOO^-$ through scavenging $ONOO^-$ and elevating the renal antioxidative capacity. Furthermore, in a cellular system in which $ONOO^-$ generation was induced by 3-morpholinosydnonimine (SIN-1), ECg showed protective activity against the effect of SIN-1. ECg reduced $ONOO^-$ formation and apoptotic cell death mediated by $ONOO^-$ and regulated the cell cycle by promoting G_2/M phase arrest. On the basis of these studies, the elucidation of the antioxidative properties of ECg that are distinct from the properties of well-known free radical inhibitors should provide evidence for the usefulness of ECg as a treatment for $ONOO^-$-induced oxidative damage.

In this study, we used ebselen and uric acid as $ONOO^-$ inhibitors, CuZnSOD as an O_2^- scavenger and L-NIL as a selective iNOS inhibitor. The major antioxidative property of ebselen is to act as a catalyst reducing both H_2O_2 and $ONOO^-$ levels and it was observed to afford protection against cisplatin-induced nephrotoxicity in both cellular and in-vivo systems and also delayed development of neurological deficits after acute middle cerebral artery occlusion (Baldew et al 1992; Masumoto & Sies 1996; Husain et al 1998; Ogawa et al 1999). Uric acid is a well-known natural antioxidant present in fluids and tissues throughout the body. It has been shown to be very effective in detoxifying both ·OH and $ONOO^-$ and suppressing lipid peroxidation. The role of uric acid as an effective inhibitor of tyrosine nitration by $ONOO^-$ has been shown to contribute to prevention of oxidative damage in the central nervous system through inhibition of certain $ONOO^-$-mediated chemical reactions (Whiteman & Halliwell 1996; Hooper et al 2000; Spitsin et al 2000; Kang et al 2002; Scott et al 2002). CuZnSOD has been well established as an antioxidative enzyme to scavenge O_2^-. In addition, after renal or focal cerebral ischaemic injury, treatment with SOD reduced membrane lipid peroxidation and protein nitration (Keller et al 1998; Noiri et al 2001). L-NIL has been reported to be a selective inhibitor of iNOS and has been used to evaluate the role of iNOS in a number of in-vivo studies (Connor et al 1995; Faraci et al 1996; Schwartz et al 1997). The experimental results showed that L-NIL reduced the renal injury and dysfunction associated with ischaemia–reperfusion of the kidney via inhibition of $ONOO^-$ generation from O_2^- and NO (Walker et al 2000; Noiri et al 2001; Chatterjee et al 2002).

In this study, the significant stimulation of NO and O_2^- generation in response to the LPS plus ischaemia–reperfusion process declined dramatically after treatment with L-NIL and CuZnSOD, respectively (Table 1). ECg, however, did not reverse the elevations in the plasma NO and O_2^- levels resulting from LPS plus ischaemia–reperfusion. This suggests that ECg does not act as a scavenger of the $ONOO^-$ precursors NO and O_2^-. In the light of these results, we hypothesized that the protective activity of ECg against $ONOO^-$ could be attributed to direct scavenging of $ONOO^-$, so we evaluated the levels of 3-nitrotyrosine and MPO activity as indicators of $ONOO^-$ formation.

3-Nitrotyrosine is a stable end-product of in-vivo $ONOO^-$ formed by the interaction of NO and O_2^- and its levels are measured to estimate $ONOO^-$ formation (Pryor & Squadrito 1995). The formation of 3-nitrotyrosine in human tissues and animal models of various diseases is a remarkable observation, since nitration has been viewed as a chemical modification that can be used to investigate the functional roles of tyrosine residues in enzymatic activity and protein function (Sokolovsky et al 1967). In addition, the increase in 3-nitrotyrosine content under certain pathogenic conditions has been widely assumed to arise from interactions between secondary oxidants spontaneously derived from $ONOO^-$ and reactions of nitrogen dioxide via MPO-catalysed oxidation of NO_2^-, indicating two alternative enzymatic and non-enzymatic pathways for 3-nitrotyrosine formation (Brennan et al 2002; Hurst 2002). Chatterjee et al (2000) suggested that the induction of MPO and nitrotyrosine production caused by renal ischaemia–reperfusion can be used as an indicator of increased nitrosative stress. The LPS plus ischaemia–reperfusion process led to elevation of the plasma 3-nitrotyrosine level in rats, suggesting that oxidative damage due to the formation of $ONOO^-$ had occurred (Figure 2). However, ECg reduced nitrotyrosine formation, in a dose-dependent

manner, markedly compared with ebselen and CuZnSOD. The activity of ECg was comparable with that of L-NIL, although ECg did not scavenge NO. Taken together, these findings indicate that ECg scavenges $ONOO^-$ directly, but not its precursors NO and O_2^-. In addition, the elevation of MPO activity was reversed by the administration of ECg, uric acid and SOD, but not by that of L-NIL (Figure 3). MPO utilizes H_2O_2 and a variety of substrates to generate an array of reactive oxidants and diffusible radical species, including nitrating intermediates (Hampton et al 1998; Mitra et al 2000; Podrez et al 2000). Therefore, it participates in the formation of NO-derived oxidants in-vivo and contributes to oxidative damage through the formation of lipid peroxidation products and $ONOO^-$-dependent nitration reactions. On the basis of these reports, we consider that reduction of MPO activity by ECg ameliorated $ONOO^-$-induced oxidative damage by inhibiting protein nitration and lipid peroxidation by a mechanism distinct from that of L-NIL, which actually increased MPO activity. In addition, uric acid acted in a similar way to ECg as a direct scavenger of $ONOO^-$ through the inhibition of 3-nitrotyrosine and MPO activity, not as a scavenger of $ONOO^-$ precursors.

Since $ONOO^-$ decomposes to form a strong and reactive oxidant, ·OH, the effects of free radical scavengers and ECg on ·OH also have to be evaluated to compare their protective actions against $ONOO^-$. In this study, we used the spin-trap method to determine the level of ·OH in rat renal tissue formed by the Fenton reaction and found that the magnitude of the increase in height of the DMPO-OH peak of rats that underwent LPS plus ischaemia–reperfusion was reduced by treatment with ECg, CuZnSOD and L-NIL (Table 2). These findings indicate that the effect of ECg on the highly reactive radical ·OH plays a crucial part in its protective action against $ONOO^-$-induced oxidative damage. Furthermore, the effects of ECg on $ONOO^-$ and ·OH were stronger than those of the other well-known free radical inhibitors tested, which can also be regarded as a mechanism distinct from the mechanisms of the others. In this study, uric acid exerted no ·OH-scavenging activity, although it blocked directly the formation of $ONOO^-$.

Although, physiologically, serum uric acid is a potent antioxidant capable of scavenging $ONOO^-$ and ·OH in man, excessive levels of uric acid are related to pathological conditions, such as renal and cardiovascular diseases (Hooper et al 1998; Kang et al 2002). The LPS plus ischaemia–reperfusion process resulted in significant elevation of the uric acid level, indicating that a pathological condition in the kidney had developed (Table 3). However, the administration of ECg reduced the uric acid level, while the other free radical inhibitors did not. This effect of ECg on excessive uric acid levels is also considered to be a property distinct from the other free radical scavengers.

The elucidation of the means whereby ECg improves renal dysfunction induced by the formation of $ONOO^-$ and ·OH will lend support to the usefulness of ECg as a therapeutic agent for $ONOO^-$-mediated oxidative conditions in pathogenesis. The parameters of renal function of serum urea nitrogen and Cr levels were elevated markedly by LPS plus ischaemia–reperfusion, while the administration of ECg reduced these levels significantly (Table 4), indicating amelioration of renal dysfunction by ECg. In addition, uric acid and L-NIL protected against renal dysfunction induced by this process, although their activity was relatively low compared with ECg.

Proteinuria, a hallmark of renal disease, is the indicator of protein loss and progression of renal failure. The presence of LMW proteins in the urine reveals tubular dysfunction and that of albumin and HMW proteins is evidence of glomerular damage, thus allowing preliminary diagnosis of the severity as well as the level of renal impairment (Lau & Woo 2002). Our results in rats showed that the LPS plus ischaemia–reperfusion process led to proteinuria, demonstrated by the SDS-PAGE pattern with an abundance of LMW and HMW protein bands (Figure 4). The administration of ECg and L-NIL reduced the intensity of the LMW and HMW protein bands more than the other radical inhibitors, which suggests that ECg would ameliorate proteinuria due to renal failure caused by $ONOO^-$-induced oxidative damage.

In the LPS plus ischaemia–reperfusion rat model, ECg, L-NIL and uric acid showed strong activity in protection against $ONOO^-$-induced oxidative damage, while CuZnSOD and ebselen exerted relatively low activity. In the light of the results of this study, we suggest that the activity of ECg is distinct from that of the other free radical inhibitors, especially L-NIL and uric acid. ECg scavenged $ONOO^-$ directly, but it did not scavenge its precursors O_2^- and NO, which is similar to the way that uric acid acts, but distinct from the mode of action of L-NIL. In addition, the inhibition of MPO activity by ECg, which was not observed with L-NIL, would contribute to effective inhibition of protein nitration and lipid peroxidation. ECg was also a stronger scavenger of the $ONOO^-$ decomposition product ·OH than any other free radical inhibitor tested. Furthermore, the improvement by ECg of the renal dysfunction caused by $ONOO^-$-related oxidative damage was marked and distinct from that by any of the other free radical inhibitors.

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