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Activity of (−)-Epigallocatechin 3-*O*-Gallate against Oxidative Stress in Rats with Adenine-Induced Renal Failure

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Methylguanidine (MG) is widely recognized as a strong uremic toxin. The hydroxyl radical (•OH) specifically plays an important role in the pathway of MG production from creatinine (Cr). In this study, we investigated whether oral administration of (−)-epigallocatechin 3-*O*-gallate (EGCg) suppresses MG production in rats with chronic renal failure after intraperitoneal Cr injection. MG production from Cr was significantly increased in rats with adenine-induced renal failure, which was more vulnerable to oxidative stress, compared with that in normal rats. However, oral administration of EGCg 30 min before and after Cr injection effectively inhibited MG production. Our findings suggest that EGCg, an excellent antioxidant from green tea, exerts protective activity in rats with chronic renal failure, resulting in suppression of Cr oxidation influenced by •OH.

KEYWORDS: (−)-Epigallocatechin 3-*O*-gallate; methylguanidine; creatinine; hydroxyl radical; chronic renal failure

INTRODUCTION

Green tea, prepared from the leaves of *Camellia sinensis* L., is a beverage that is popular worldwide. Polyphenols in green tea have been receiving much attention as potential compounds for the maintenance of human health due to their varied biological activity and low toxicity. In particular, the contribution of antioxidant activity to prevention of diseases caused by oxidative stress has been focused upon.

Our research group has been investigating the effects of green tea polyphenols against renal disease (*1*, *2*). We have demonstrated that green tea polyphenols exert a blood pressure lowering effect in rats with renal hypertension and that the kallikrein−prostaglandin system (i.e., a renal function- or blood pressure-regulating factor) is involved in these effects (*3*). We also found that green tea polyphenols suppressed the progression of renal failure in nephrectomized rats, assessed by measuring renal functional parameters and histopathological examination, and concurrently elevated endogenous radical-scavenging enzyme activities in the kidney (*2*, *4*). In addition, the increased levels of uremic toxins, such as methylguanidine (MG), in patients on dialysis and animal models of renal failure were also significantly reduced by continuous treatment with green tea polyphenols (*2*, *5*−*7*).

MG is a very strong uremic toxin that has been found to induce uremic symptoms such as anemia, ulcers of the gastrointestinal tract and decreased nerve conduction velocity (*8*). Under conditions of chronic renal failure, MG synthesis increases, and combined with renal dysfunction, results in MG accumulation in the body. MG is produced from the precursor creatinine (Cr) through the intermediates creatol, creatone A, and creatone B, as shown by our research group's studies using adenine-induced renal failure rats (*9*−*11*). We also found that the hydroxyl radical (•OH) is involved in the process of conversion of Cr to creatol, and this is the rate-determining step in MG synthesis from Cr (*12*−*14*). Therefore, it has been proposed that •OH participates as an important mediator in the pathway of Cr metabolism to MG, and the elimination of •OH may prevent or reverse uremic symptoms caused by MG accumulation in chronic renal failure. This raises the possibility that the ability of green tea polyphenols to ameliorate oxidative stress is involved in the MG-reducing mechanisms observed in our previous study.

To explore this possibility, we designed the present study to examine the effect of (−)-epigallocatechin 3-*O*-gallate (EGCg), which accounts for the largest fraction of the components of green tea polyphenols, on MG production from Cr.

MATERIALS AND METHODS

Animal Experiments. All experimental studies using animals were conducted in accordance with the "Recommendations on the Establishment of Animal Experimental Guidelines" approved by Toyama Medical and Pharmaceutical University. Male Wistar rats with a body weight of 150 g were obtained from Japan SLC, Inc. (Hamamatsu, Japan). The rats were kept in a wire-bottomed cage under a conventional lighting regimen with a dark night. The room temperature (about 23

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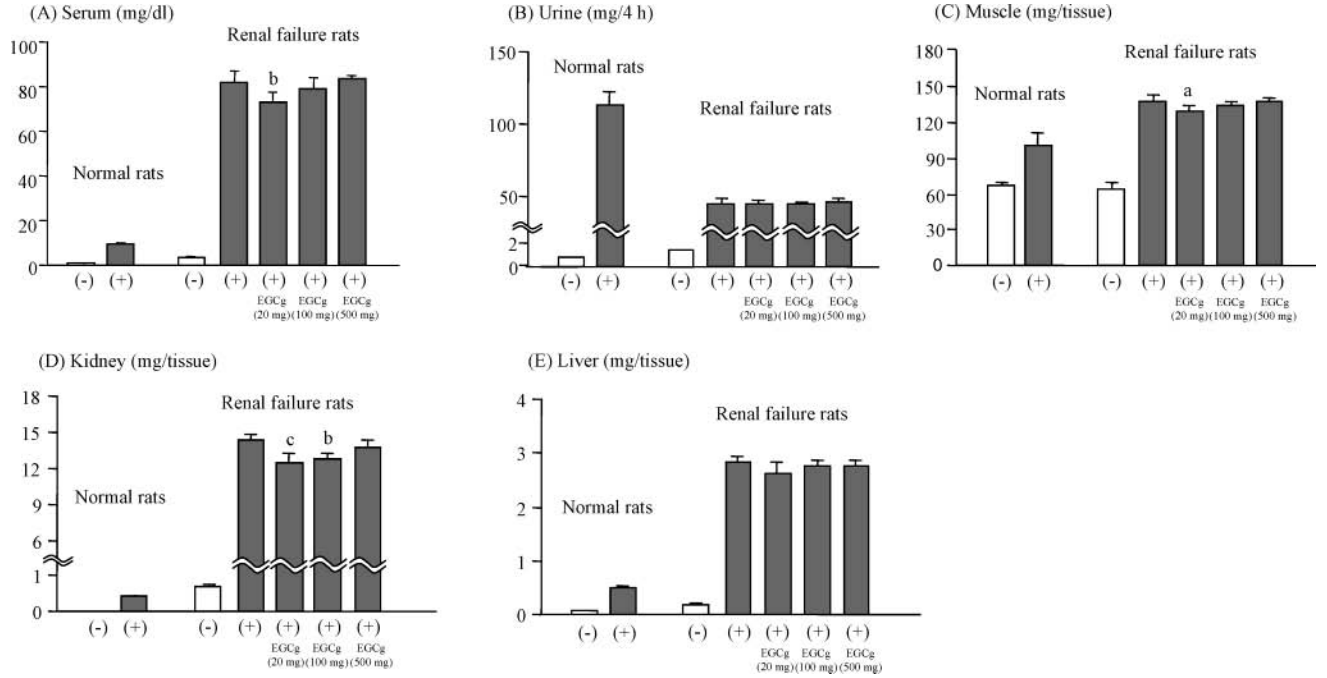

Figure 1. Cr levels in serum (**A**), urine (**B**), muscle (**C**), kidney (**D**), and liver (**E**). (–), without Cr loading; (+), with Cr loading. Statistical significance: (a) $p < 0.05$, (b) $p < 0.01$, (c) $p < 0.001$ vs renal failure control rats with Cr loading.

°C) and humidity (about 60%) were controlled automatically. Normal rats were fed on a basal 18% casein diet. Chronic renal failure was induced by feeding rats an 18% casein diet containing 0.75% adenine. As reported previously (*15*), the renal failure was aggravated by increasing the duration of adenine feeding. Feeding rats adenine produced metabolic abnormalities resembling chronic renal failure in humans, including accumulation of uremic toxins. During the experimental period, all rats were given pair-feeding to diet. After 25 days, the blood urea nitrogen levels were measured, and then the rats with chronic renal failure were divided into five groups, avoiding any intergroup differences in blood urea nitrogen levels. Normal rats were randomly divided into two groups. Four groups with chronic renal failure were given water or EGCg (20, 100, and 500 mg/kg body weight) orally via a stomach tube 30 min before and after Cr injection (100 mg/100 g body weight, intraperitoneally (i.p.)). One group of normal rats also received Cr injections (100 mg/100 g body weight, i.p.) and water was given orally 30 min before and after Cr injection. One group each of rats with chronic renal failure and normal rats was given water 30 min before and after physiological saline injection instead of Cr. The urine was collected for 4 h after Cr or saline injection. Following urine collection, blood samples were obtained by decapitation, and the serum was separated immediately by centrifugation. The liver, kidneys, and muscle tissue were subsequently extirpated from each rat, rinsed with cold physiological saline, and frozen at −80 °C until assayed.

Determination of Urea Nitrogen. Urea nitrogen levels were determined with the use of the commercial reagent BUN Kainos (Kainos Laboratories, Tokyo, Japan).

Determination of Cr and MG. Each tissue was homogenized with 10 volumes of ice-cold physiological saline solution at 0 °C. Each serum, tissue homogenate, and urine sample was deproteinized by adding trichloroacetic acid (final concentration 10%). The supernatant obtained after centrifugation at 3000 rpm for 10 min was filtered through a 0.2-μm membrane filter, and the filtrate was analyzed. Cr and MG concentrations were measured with the use of a Japan Spectroscopic liquid chromatograph employing a step-gradient system, according to the method of Higashidate et al. (*16*). A fluorescence spectrometer, model FP-210 (excitation 365 nm, emission 495 nm; Japan Spectroscopic Co., Tokyo, Japan) was used to detect Cr and MG on the column.

Statistics. The data presented are means ± SD of 7 determinations. Differences among groups were analyzed by Dunnett's test, and those at $p < 0.05$ were accepted as significant.

Table 1. Blood Urea Nitrogen Levels

group	blood urea nitrogen (mg/dL) Cr (–)[a]	Cr (+)[b]
normal rats	11.3 ± 0.8	16.8 ± 0.6
renal failure rats		
control	101.2 ± 4.6	110.5 ± 10.6
EGCg (20 mg/kg BW)	–	120.6 ± 12.3
EGCg (100 mg/kg BW)	–	121.4 ± 4.4
EGCg (500 mg/kg BW)	–	118.0 ± 6.4

[a] Cr (–), without Cr loading. [b] Cr (+), with Cr loading.

RESULTS

Blood Urea Nitrogen Levels. As shown in **Table 1**, the blood urea nitrogen levels of rats with renal failure were significantly higher, more than 100 mg/dL, than those of normal rats. There were no significant differences among the renal failure groups, showing that the blood urea nitrogen level was not changed by EGCg.

Cr Levels. Figure 1 shows the serum, urine, muscle, kidney, and liver Cr levels. The baseline serum Cr levels of the normal and renal failure groups were 1.16 mg/dL and 3.54 mg/dL, respectively. Due to Cr loading, the Cr levels increased to 9.66 mg/dL and 82.23 mg/dL, respectively, as shown in **Figure 1A**. Similarly, the muscle, kidney and liver Cr levels were also higher in rats with renal failure after Cr loading than those of normal rats (**Figure 1**, parts **C**−**E**). The amount of Cr excreted into the urine after Cr loading was greater, more than 100 mg/4 h, in normal rats than in those with renal failure, less than 50 mg/4 h, as shown in **Figure 1B**. These results showed that renal function deteriorated in adenine-fed rats. Oral administration of EGCg did not change the Cr levels, except the 20 mg- and/or 100 mg-treated groups showed changes in the serum, muscle and kidney levels.

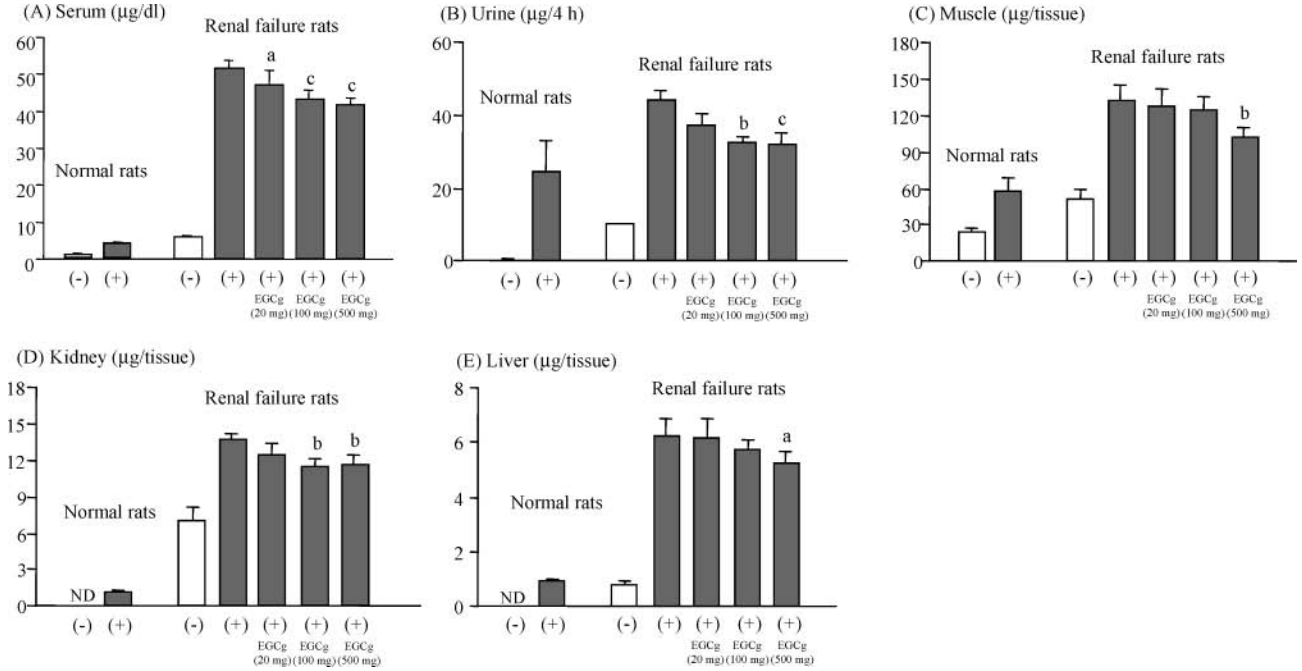

Figure 2. MG levels in serum (**A**), urine (**B**), muscle (**C**), kidney (**D**), and liver (**E**). (–), without Cr loading; (+), with Cr loading. Statistical significance: (a) $p < 0.05$, (b) $p < 0.01$, (c) $p < 0.001$ vs renal failure control rats with Cr loading.

MG Levels. The serum, urine, muscle, kidney, and liver MG levels are summarized in **Figure 2**. In normal rats, MG was not detected in the kidneys or liver, and the levels in serum, urine, and muscle were low. Compared with normal rats, rats with renal failure had higher serum, urine, muscle, kidney and liver MG levels (**Figure 2**). After Cr loading, MG levels increased in both normal and renal failure groups. However, MG production was higher in rats with renal failure than in normal rats. The serum MG level changed from 0.84 to 3.84 μg/dL in normal rats, whereas it changed from 5.66 to 51.38 μg/dL in rats with renal failure, as shown in **Figure 2**. Similar results were observed for urine, muscle, kidney and liver, showing that MG production from Cr was accelerated in rats with renal failure compared with normal rats. EGCg treatment at 20 mg/kg body weight significantly reduced serum MG levels. Further reductions were observed in the groups treated with 100 mg and 500 mg EGCg. Urinary excretion of MG was also reduced significantly in the 100 mg- and 500 mg-treated groups. Similar significant decreases occurred in muscle, kidney, and liver.

DISCUSSION

Clinical and experimental studies have resulted in considerable discussion of the link between renal disease and oxidative stress, which leads to excessive generation of oxygen-derived free radicals (*17*−*20*). These free radicals are highly reactive and injure lipids, proteins, and nucleic acids, resulting in structural and functional impairment. Increased levels of end products mediated by the reactions between biomolecules and free radicals, such as malondialdehyde, 3-nitrotyrosine, and 8-hydroxy-2′-deoxyguanosine, were observed with various pathological phenomena, such as acute renal failure, diabetic nephropathy, glomerular damage due to chronic renal failure, and hemodialysis (*21*−*24*). Inhibitors of free radicals and antioxidants have also been shown to protect against renal damage in a number of studies (*25*). These two lines of experimental systems have been extensively employed to evaluate the contribution of free radicals to pathological conditions, because it is difficult to establish the true entity of free radicals in the body due to their short half-lives.

To study the effects of free radical entities more directly, in vivo evaluation of free radical reactions in the body has been attempted in recent years. To this end, in a previous study, we used an L-band electron spin resonance (ESR) apparatus to analyze the free radical reactions in the bodies of rats with adenine-induced renal failure (*26*). We found that the elimination rate of 3-carbamoyl-2,2,5,5-tetramethyl pyrrolidine-N-oxyl was significantly lower in rats with adenine-induced renal failure than that in normal rats, resulting in significant prolongation of the radical half-life in rats with renal failure. In addition, using X-band ESR combined with spin-trapping with 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), we determined the radical species in the kidneys of rats given adenine and observed a marked increase in the size of the peak representing DMPO−OH (*27*, *28*). Based on this evidence, we concluded that rats with adenine-induced renal failure were in a state of augmented oxidation and that their bodies extensively generated free radicals, especially •OH. Under these conditions, considerable alterations in the metabolism of Cr, which accumulates in blood as renal failure progresses, are accelerated (i.e., Cr is oxidized to MG), suggesting that antioxidants that scavenge •OH may be useful therapeutic avenues for inhibition of MG production and further prevention of uremic symptoms.

Green tea polyphenols have been shown to act as metal chelators, preventing metal-catalyzed formation of radical species, antioxidant enzyme modulators, and scavengers of free radicals, including •OH, superoxide anion, nitric oxide, and peroxynitrite (*2*, *29*−*31*). These antioxidant activities are considered to be closely related to their protective effects against various diseases, including renal disease, arteriosclerosis, cancer, and inflammation caused by lipid peroxidation and excessive free radical production (*32*). The polyphenolic compounds of green tea mainly comprise EGCg (−)-epicatechin 3-*O*-gallate (ECg), (−)-epigallocatechin (EGC), and (−)-epicatechin (EC),

which are classified as the flavan-3-ol class of flavonoids. The structure−activity relationship of the antioxidative effect of green tea was examined by comparing the effects of seven kinds of polyphenols isolated from green tea (i.e., EGCg, (−)-gallocatechin 3-*O*-gallate, ECg, EGC, (−)-gallocatechin, EC and catechin). We found that structural specificity is involved in the manifestation of its antioxidative activity and that EGCg had the most effective structure for antioxidative activity (*29*, *31*, *33*). Moreover, EGCg accounts for the largest fraction of the components of green tea polyphenols. Thus, we investigated the effects of EGCg on the metabolism of Cr to MG in rats with adenine-induced renal failure who were subject to enhanced oxidative stress.

In the present study, both normal rats and rats with adenine-induced renal failure were subjected to intraperitoneal Cr loading. In normal rats, Cr was rapidly excreted into the urine after Cr loading, whereas in rats with renal failure, urinary Cr excretion was low, and high levels of Cr were present in the serum, muscle, kidneys and liver, suggesting the body was susceptible to oxidative alterations. After Cr loading, the MG levels in the serum, urine, muscle, liver, and kidneys of rats with renal failure were higher than those of normal rats, confirming that MG production from Cr was increased in rats with renal failure. Oral administration of EGCg dose-dependently reduced the serum MG levels, showing that EGCg effectively inhibited increased MG production in which oxidative reactions participate considerably. EGCg (20 mg/kg body weight) significantly reduced the urinary and kidney MG levels, which were reduced further and significantly in the 100-mg- and 500-mg-treated groups. In the muscle and liver, significant reduction was observed only in the high dose-treated group (500 mg).

We have already demonstrated that green tea polyphenols (daily dose, 400 mg) administered for 6 months to 50 patients on dialysis decreased the blood levels of MG (*6*) and that concomitant treatment with green tea polyphenols during 25-day adenine-feeding periods produced a dose-dependent decrease in the serum MG level (*7*). Furthermore, we reported that concomitant treatment with green tea polyphenols had protective effects against the increased serum Cr and urinary protein levels and the decreased creatinine clearance (Ccr) (*2*, *4*), indicating that green tea polyphenols can delay the deterioration of renal function. The deterioration of renal function influenced MG accumulation in the body through increases in the serum concentration of Cr, which is a precursor of MG, and reduced urinary excretion of MG. Thus, these findings suggest that the possible mechanisms by which green tea polyphenols decrease serum MG levels may involve reducing the serum Cr concentration and increasing MG excretion into urine due to improvement of the Ccr. In this study, to exclude any protective effects on the deterioration of renal function by concomitant treatment with green tea polyphenols, EGCg was administered 30 min before and after Cr injection. The blood urea nitrogen level, which is a renal function parameter, did not differ among the renal failure groups treated with or without EGCg. We found that oral administration of EGCg reduced MG levels even at the lowest dose (20 mg) and that further significant reductions were observed in the 100-mg- and 500-mg-treated groups. However, the high dose of EGCg (500 mg) did not change Cr levels in serum, urine, muscle, kidney, and liver. These results provide evidence that oral administration of EGCg inhibits MG production from Cr, and this is independent of its protective effects against renal dysfunction, suggesting its ability to scavenge •OH contributes to the former effect. Taking the evidence from previous and present studies into consideration, we propose that green tea polyphenols exert an MG-lowering effect in dialysis patients and rats with adenine-induced renal failure through, at least in part, two actions, improvement of renal dysfunction and inhibition of MG production from Cr due to their ability to scavenge •OH.

In the in vivo situation, to exert free radical-scavenging activity, absorption of EGCg into the body and its concentration in plasma and target tissues are important issues. However, limited information is available on the metabolism of EGCg after oral administration. Several studies showed that EGCg was detected in rat plasma after oral administration, indicating that EGCg is, at least in part, absorbed into the body (*34*, *35*). Benzie et al. (*36*) reported that although the amount of antioxidants absorbed was relatively small, some potentially polyphenolic antioxidants enter the systemic circulation and cause a significant increase in the plasma antioxidant status. These findings may explain the •OH-eliminating effects of EGCg. Although further investigations on the metabolism of EGCg in rats with renal failure, in particular on the form of EGCg after scavenging free radicals and EGCg excretion, are needed, the present study demonstrated that EGCg acted as an antioxidant on Cr oxidation in rats with chronic renal failure and thereby inhibited MG production.

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