Protective Effect of Green Tea Extract and Tea Polyphenols against the Cytotoxicity of 1,4-Naphthoquinone in Isolated Rat Hepatocytes

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The cytoprotective effect of green tea extract and its phenolic compounds against 1,4-naphthoquinone-induced hepatotoxicity was evaluated in primary cultured rat hepatocytes. After exposure to 1,4-naphthoquinone, lactate dehydrogenase (LDH) leakage and cell viability were both improved by the presence of the tea extract and tea polyphenols. This cytoprotective effect was related to the structure of tea polyphenols, the galloyl group of (−)-epigallocatechin-3-gallate and (−)-epicatechin-3-gallate being particularly effective. The production of liquid peroxidation by 1,4-naphthoquinone was not inhibited by the tea extract nor by tea polyphenol addition. After 2 h of incubation, the protein thiol concentration was reduced by 1,4-naphthoquinone, but this reduction was prevented by the tea extract and tea polyphenols. The reduction in protein thiol content of the cells closely paralleled the LDH leakage and loss of cell viability. These results suggest that the mechanism of protection by tea polyphenols against 1,4-naphthoquinone-induced toxicity to rat hepatocytes was due to the maintenance of protein thiol levels.

Key words: green tea extract; tea polyphenols; 1,4-naphthoquinone; protein-SH; hepatocytes

Tea is one of the most popular beverages consumed worldwide, and about 2.5 million metric tons are produced annually. Of this amount, about 20% is green tea, mainly consumed in Asian countries where tea is a major beverage.

Green tea contains polyphenols, which include flavonols, flavanols, flavonoids, and phenolic acids. Most green tea polyphenols are flavonols, which are commonly known as catechins. Some major green tea catechins are (−)-epigallocatechin-3-gallate (EGCG), (−)-epigallocatechin (EGC), (−)-epicatechin-3-gallate (ECG), (−)-epicatechin (EC), and (−)-catechin.11 These polyphenolic compounds in green tea have been reported to possess various physiological effects, such as antioxidant,22 antimitogenic,3,4 antitumoral,5 anti-inflammatory,6 abilities. Imai and Nakachi7 have recently reported that the inverse association between the consumption of green tea and various serum markers showed that green tea may act protectively against cardiovascular disease and disorders of the liver. However, these physiological effects of tea polyphenols have not been completely elucidated. We therefore examined the effect of tea polyphenols on vital functions, especially the liver function.

Quinones are a widely distributed class of compounds which occur naturally in many biological systems.8,9 Quinonoid compounds can exist in various redox states and participate in electron transport processes and photosynthesis. Quinones have also been detected as environmental pollutants in atmospheric particulate matter and cigarette smoke and are therefore of general toxicological relevance. The cytotoxicity of quinones has been shown in several studies,9—12 and provides a suitable experimental model for cytotoxicity studies.

Primary cultured rat hepatocytes represent a good in vitro experimental model which preserves its differentiated characteristics. In this study, we examined the effect of green tea extract and its phenolic compounds against 1,4-naphthoquinone-induced hepatotoxicity in primary cultured rat hepatocytes.

Materials and Methods

Materials. The green tea extract was prepared from a hot-water extract of green tea,21 the components of which are shown in the Table. (−)-Epicatechin, (−)-epigallocatechin, and (−)-epicatechin-gallate were obtained from Taiyo Kagaku Co. (Yokkaichi, Japan), and (−)-catechin, sodium NADH, and glutathione (reduced form) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). (−)-Epigallocatechin-gallate, collagenase (Type II), sodium pyruvate, 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB), 2,6-di-t-butyl-4-methylphenol (BHT), and 2-thiobarbituric acid (TBA) were from Wako Pure Chemicals (Osaka, Japan). The structures of these

Table Qualitative and Quantitative Analyses of Green Tea Extract

<table>
<thead>
<tr>
<th>Percentage in green tea extract (w/w)</th>
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<tr>
<td>Polyphenols</td>
</tr>
<tr>
<td>(−)-Catechin</td>
</tr>
<tr>
<td>(−)-Epicatechin</td>
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<tr>
<td>(−)-Gallocatechin</td>
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<tr>
<td>(−)-Epigallocatechin</td>
</tr>
<tr>
<td>(−)-Epicatechin gallate</td>
</tr>
<tr>
<td>(−)-Epigallocatechin gallate</td>
</tr>
<tr>
<td>Others</td>
</tr>
<tr>
<td>Caffeine</td>
</tr>
<tr>
<td>Sugars</td>
</tr>
<tr>
<td>Amino acids, peptides</td>
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<tr>
<td>Ash</td>
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five tea polyphenols are shown in Fig. 1. 1,4-Napthoquinone was obtained from Tokyo Kasei Co. (Tokyo, Japan), and Williams’ E medium was obtained from Life Technologies (Grand Island, NY, U.S.A.). Fetal bovine serum (FBS) was from JRH Biosciences (Lenexa, Australia).

Isolation and culture of hepatocytes. The protocol of the experiment was approved by the Animal Research Committee of Osaka City University, and care of the animals was in accordance with the standards of this Institution (Guide for Animal Experimentation, Osaka City University). Hepatocytes were isolated from male Sprague-Dawley rats weighing 200 to 250 g by collagenase perfusion. 1,4-Napthoquinone was added to dimethyl sulfoxide (DMSO) just before being added to the incubation medium. DMSO did not affect the results of our experiments. The green tea extract and tea polyphenols were dissolved in ethanol (final concentration of ethanol was 0.25%), before being added to the incubation medium. The control consisted of the medium, ethanol and DMSO.

Assessment of the viability of isolated hepatocytes. Lactate dehydrogenase (LDH) assay. Measuring the LDH activity in the suspension buffer as described previously 1,4-Napthoquinone was added to dimethyl sulfoxide (DMSO) just before being added to the incubation medium. DMSO did not affect the results of our experiments. The green tea extract and tea polyphenols were dissolved in ethanol (final concentration of ethanol was 0.25%), before being added to the incubation medium. The control consisted of the medium, ethanol and DMSO.

Cell viability. Cell viability was measured by the Neutral Red (NR) assay as described previously. 1,4-Napthoquinone was added to dimethyl sulfoxide (DMSO) just before being added to the incubation medium. DMSO did not affect the results of our experiments. The green tea extract and tea polyphenols were dissolved in ethanol (final concentration of ethanol was 0.25%), before being added to the incubation medium. The control consisted of the medium, ethanol and DMSO.

Effect of green tea extract on 1,4-napthoquinone-induced cytotoxicity

Isolated hepatocytes incubated with various concentrations of 1,4-napthoquinone showed dose-dependent cell damage as determined by released LDH (Fig. 2). 100 μM 1,4-napthoquinone produced an LDH leakage plateau between 2 to 4 hours of incubation. Hence, an exposure concentration of 100 μM 1,4-napthoquinone was used in subsequent experiments. The addition of the green tea extract to the cell incubation medium prevented LDH release and the loss of cell viability in a dose-dependent manner (Fig. 3).

Effect of tea polyphenols on cytotoxicity

To determine which tea component prevented cell damage by 1,4-napthoquinone, we examined the effect of five different tea polyphenol. ECG and ECG significantly reduced cytotoxicity in a dose-dependent manner, but catechin, EC and EGC had no effect on LDH release and cell viability (Figs. 4a and b).

The time-course for the effect of tea polyphenols (100 μM) also revealed that ECG and ECG had a significant effect.
Fig. 2. Cytotoxicity of 1,4-Naphthoquinone Measured as LDH Leakage in Primary Cultured Rat Hepatocytes.
Hepatocytes were exposed to various concentrations of 1,4-naphthoquinone for 2-4 hours. (a) control; (♦) 25 μM 1,4-naphthoquinone; (□) 50 μM 1,4-naphthoquinone; (■) 100 μM 1,4-naphthoquinone. Each value is the mean ± S.D. of three experiments. Values of LDH are expressed as μmol NADH converted to NAD per minute per liter of a sample, simply denoted as units per liter (U/liter). *p < 0.05, **p < 0.005, ***p < 0.0005 compared to control value.

Fig. 3. Effect of Various Concentrations of Green Tea Extract on 1,4-Naphthoquinone-induced Cytotoxicity in Primary Cultured Rat Hepatocytes.
Hepatocytes were incubated in Hank's buffer with or without the green tea extract for 4 hours. The effect of the green tea extract was examined on LDH leakage (□) and cell viability (■). All hepatocytes were exposed to 100 μM 1,4-naphthoquinone. Each value is the mean ± S.D. of three experiments. *p < 0.05, **p < 0.005, ***p < 0.0005 compared to hepatocytes exposed to 1,4-naphthoquinone.

within 2 to 4 hours (Fig. 5). Catechin had a protective effect at 2 hours, but had no effect thereafter, suggesting that the cytoprotective effect of catechin was relatively short.

Correlation of lipid peroxidation with cytotoxicity
Isolated hepatocytes incubated with 1,4-naphthoquinone (100 μM) underwent extensive lipid peroxidation in the incubation medium as measured by TBA-reactive substances. The addition of the green tea extract did not inhibit the formation of the peroxidative by-product, malondialdehyde (MDA) (Fig. 6). Furthermore, the addition of tea polyphenols, especially EGCg and ECg, reduced LDH leakage but did not inhibit the formation of the peroxidative by-product (Fig. 7). These results suggest that the cytoprotective effect of EGCg and ECg had no correlation with lipid peroxidation, which proceeded with the formation of lipid peroxidative by-products.

Cell toxicity and cellular thiol status
It has been observed that the toxicity of many xenobiotics, including various quinones, is associated with the depletion of protein thiols. Modification of protein thiol groups has been reported to be associated with the development of cytotoxicity. As shown in Figs. 8 and 9, the addition of 1,4-naphthoquinone to hepatocytes resulted in an extensive loss of cellular protein thiols. This loss began before a significant leakage of LDH was observed (Figs. 9a and b) and was inhibited by the addition of the green tea extract, EGCg and ECg. Furthermore, the inhibition occurred in a dose-dependent manner (data not shown for polyphenols). These results suggest that inhibition of the loss of protein thiol groups was related to the maintenance...
of cell viability by the green tea extract and tea polyphenols.

Discussion
This study has shown that green tea extract and tea polyphenols protected against 1,4-naphthoquinone-induced hepatotoxicity in primary cultured rat hepatocytes. After exposure to 1,4-naphthoquinone, LDH leakage and loss of cell viability were improved by the addition of the green tea extract and tea polyphenols, especially EGCg and ECg, and this was mediated by the maintenance of protein thiols.

Since the metabolism of many xenobiotics such as quinones in cells causes oxidative stress due to redox cycling and the formation of active oxygen species, the cytoprotective effects by tea polyphenols has been thought to be a consequence of the ability to remove reactive free radicals or to an antioxidative ability. However, in the present study, the addition of tea polyphenols, especially EGCg and ECg, did not inhibit the formation of peroxidative by-products in cells that had been incubated with 1,4-naphthoquinone. This suggests that the protective effect by green tea extract and tea polyphenols is not related to the prevention of lipid peroxidation.

O'Brien has reported that 1,4-naphthoquinone caused redox cycling and oxidative stress, which would result in the oxidation of critical thiol groups and is thought to be toxic via this mechanism. Free sulphhydryl groups in proteins play the role of highly reactive functional groups in biological systems and participate in several different reactions such as alkylation, arylation, oxidation, thiol-disulfide exchange, etc. Therefore, the modification of protein thiol groups can result in severe functional damage, including loss of enzyme activity, in biological systems. The inactivation of plasma membrane Ca\(^{2+}\)-ATPase activity can arise from the oxidation of protein thiol groups and induces...
Fig. 9. Effect of Tea Polyphenols on the Concentration of Protein Sulphydryl Groups (Protein-SH) (a) and Leakage of LDH (b) in Primary Cultured Rat Hepatocytes.

Hepatocytes were incubated with or without each tea polyphenol (100 μM) for up to 2 hours. (△) control; (△) 1,4-naphthoquinone (100 μM); (●) 1,4-naphthoquinone + catechin; (■) 1,4-naphthoquinone + EGCg; (□) 1,4-naphthoquinone + Ccg. The initial protein-SH value was 61.0 ± 8.1 μmol/5 × 10⁶ cells, and this is taken as 100%. Each value is a percentage of the initial value at 0 hour and is mean ± S.D. of three experiments. *p < 0.05 compared to hepatocytes exposed to 1,4-naphthoquinone.

high cytosolic Ca²⁺ concentration.²¹) Weis et al.²²) have recently shown that incubating hepatocytes with 3,5-dimethyl-acetaminophen in the presence of glucose/glucose oxidase and horseradish peroxidase caused a concentration-dependent loss of cell viability which was associated with decreased protein thiol levels. Restoration of the protein thiol levels arrested the cell killing. The results of the present study also show that the depletion of protein thiols by 1,4-naphthoquinone preceded leakage of LDH and was inhibited by the addition of the green tea extract and tea polyphenols. This inhibition was strongly correlated with the maintenance of cell viability. In conclusion, this study has demonstrated that tea polyphenols, especially the gallol group of EGCg and ECg, can protect against 1,4-naphthoquinone-induced cytotoxicity to primary cultured rat hepatocytes. The mechanism for this protection was not due to the ability to remove reactive free radicals or the antioxidative ability of polyphenols, but to the maintenance of the protein thiol level.

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References