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Antioxidative Action of Flavonoids, Quercetin and Catechin, Mediated by the Activation of Glutathione Peroxidase

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Antioxidative action of flavonoids have been attracted attention of many investigators and a good deal of studies on it were reported. While their interests were mostly centered to the direct scavenging action of flavonoids against free radicals and active oxygen species, we expected that the interaction of fiavonoids and intracellularly occurring antioxidative agents such as glutathione peroxidase (GSH-PO) could synergistically enhance their antioxidative activities. For this purpose, cultured rat hepatocytes (BL-9), which are highly expressing GSH-PO, were employed. One group of the cells were cultured with Se deficient media (Se(-) cells) to diminish the activity and the expression of GSH-PO protein and mRNA, and the other group was cultured with Se supplemented media (Se(+) cells). The oxidative cell damage was induced by the addition of H_2O_2 and two representative antioxidative flavonoids, quercetin and catechin, were added to the media to test their cytoprotective action. In Se(+) cells, the remarkable cytoprotective activity of those flavonoids were confirmed, whereas none of such activity was evidenced in Se(-) cells. It was proved that the intracellular antioxidative function of flavonoids requires the interaction with GSH-PO, at least in the cells expressing the enzyme. Interestingly, the flavonoid activated GSH-PO clearly, and its mechanism is discussed.

Key Words : Antioxidant, Flavonoids, Quercetin, Catechin, Glutathione Peroxidase

INTRODUCTION

Flavonoids are benzo-γ-pyrone derivatives which resemble coumarin and are ubiquitous in a wide variety of plants [1-4]. Multiple pharmacological effects of flavonoids have been reported, including vascular protection, anti-inflammatory, antitumor and anti-hypertension activities [5-10]. Flavonoids also posses free radical-scavenging abilities, and their anti-radical property is directed toward ·OH and O_2^-, which are highly reactive oxygen species implicated in the initiation of lipid peroxidation [11 - 16]. Furthermore, flavonoids have suppressive effects against cytotoxicity caused by those active oxygen species (H_2O_2 and O_2^- etc.) [17]. Only limited number of studies on the antioxidation action of flavonoids at the cellular level, however, has been reported [17-19] and little is known about the mechanisms of the cellular reactions. Most of those cytoprotective effects of flavonoids have been explained through the direct radical scavenge routes so far.

To prevent cell injuries induced by oxidative stresses, cells are equipped with enzymatic scavenging systems such as copper-zinc superoxide disumutase (Cu/Zn-SOD), manganese superoxide disumutase (Mn-SOD) and glutathione peroxidase (GSH-PO), and nonenzymatic antioxidant defenses [20]. We presumed that some flavonoids might display their antioxidative functions through the interaction with those antioxidative enzymes including GSH-PO. The only two reports, however, were found to have described that quercetin and its derivatives prevented oxidative cell damages by either increasing glutathione [21] which is indispensable for the peroxides reducing activity of GSH-PO, or protecting cells from glu-

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tathione depletion with the cooperation of ascorbic acids [22], but otherwise flavonoids were mostly proved to inhibit those antioxidative enzyme activity [23-26].

Among those antioxidative defenses, GSH-POs are known to directly reduce and eliminate intracellularly occurring hydrogen peroxide and lipid peroxides and to play the very important role in the protection against oxidative stress [27-32]. The antioxidative function of flavonoids may be synergistically promoted through the interaction with such GSH-PO. The present study was thus designed to investigate the cytoprotective action of flavonoids against hydroperoxide induced cell damages which may be promoted by the interaction between the flavonoids and GSH-PO. For this purpose, cultured rat hepatocyte BL-9, which are highly expressing GSH-PO, were employed for the experiment, dividing these cells into following two experimental groups: ① Cultured in selenium (Se) supplemented media (Se(+) cells; GSH-PO expressing). ② Cultured in Se depleted media (Se(-) cells; GSH-PO deficient).

As flavonoids, quercetin and catechin of which antioxidative action was proved to be most prominent [13, 16-17] were employed for the experiment.

MATERIALS AND METHODS

Materials. Nitrocellulose membranes (BA85) were obtained from Schleicher and Schuell (Dassel, Germany). Hydrogen peroxide (H_2O_2) was from Mitsubishi Gas Chemical Co. Ltd. (Tokyo, Japan). Quercetin, catechin, reduced glutathione (GSH), oxidized glutathione (GSSG), glutathione reductase and Williams E medium were from Sigma Chemical Co. (St. Louis, MO, USA). NADPH was from Oriental Yeast (Tokyo, Japan). 1-Chloro-2,4,-dinitrobenzen (CDNB) was from Wako Pure Chemical Co. Ltd. (Osaka, Japan). Fetal bovine serum was from Biosciences PTY. LTD. (N.S.W Australia). L-Glutamine, penicillin and streptomycin were from Immuno-Biological Laboratories (Gunma, Japan). Gentamycin was obtained from Schering-Plough (Osaka, Japan) .

Cell Culture. BL-9 is a normal rat liver derived hepatocyte cell line [33]. BL-9 were kindly obtained from Dr. M. Akao, Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University. BL-9 cells were cultured in Williams E medium containing 10% heat-inactivated fetal bovine serum (FBS), in a humidified 37℃ atmosphere consisting 5% CO_2 and 95% air. To attain a selenium deficient condition, the FBS concentration was gradually decreased from 10% to 1% in the medium [47]. Finally the selenium-free BL-9 cells (designated as Se(-) cells) were cultured in the medium containing 1% FBS, L-glutamine (2 mM), gentamycin (50 μg/ml), streptomycin (100 μg/ml), and penicillin (100U/ml) for 4 weeks. The selenium supplimented-BL-9 cells (Se(+) cells) were prepared by adding 100ng/ml of sodium selenium to the medium. Under normal culturing conditions (95% air/5% CO_2 at 37℃), the doubling time for Se(-) and Se(+) cells was found to be the same.

Cytotoxicity of H_2O_2 and protection by flavonoids. Quercetin and catechin were dissolved in dimethylsulfoxide (DMSO) which was maintained at constant concentration in additives (0.1%). The Se(-) and Se(+) BL-9 cells were seeded in 100-mm petri dishes (200 cells/dish) and cultured for 3 days in 15 ml of medium supplemented with 1% FBS. After changing the medium to FBS-free medium, quercetin and catechin were added to the medium within the range of 0−1 mM, and the cells were incubated for 4 hr at 37℃. The cells were then treated with 100 μM of H_2O_2 in flavonoid-free medium containing 1% FBS for 2 days. The cell viability was determined by trypan blue exclusion assay.

Enzyme Assays. After Se(-) and Se(+) cells were maintained in the medium containing l% FBS for 3 days, quercetin and catechin at different concentrations were added to the medium, and the cells were incubated for 4 hr at 37℃. The Se(-) and Se(+) cells were further cultured for 2 days flavonoid-free medium containing 1% FBS. Se(+) cells were collected in Eppendorf type tubes. The cells were suspended in 4 volumes of lysis buffer (0.01 M Tris-HCI pH 8.5, containing 1.5 mM $MgC1_2$, 0.14 M NaCl and 0.5% NP40) and centrifuged at 14,000 rpm for 15 min at 4℃. The supernatant was used as a sample for the measurement of antioxidant enzyme activities. Glutathione peroxidase (GSH-PO)

activity was determined according to the method of Beutler et al. [34] modified by Yoshimura et al. [35], with cumene hydroperoxide (0.23 mM) as substrate. One unit of enzyme activity is defined as 1 μM NADPH oxidized/min. at 37℃. Glutathione reductase (GSSG-RD) activity was assayed according to the method of Horn [36], by means of measurement of the reduction of GSSG to GSH by NADPH at 340nm. Glutathione-S-transeferase (GSH-S-T) activity was determined according to the method of Habing [37], by means of measurement of the formation of the conjugate of glutathione and CDNB. The enzyme activities of GSH-PO, GSSG-RD and GSH-S-T were expressed as units per gram tissue weight.

Immunoblot Analysis. Cell lysates were solubilized in 2% SDS containing 10% 2-mercaptoethanol, electrophoresed in a SDS-12.5% polyacrylamide gels and then electrotransferred to nitrocellulose membranes [38-39]. The membranes were blocked with 3% BSA in 0.01M phosphate-buffered saline (PBS) for 1hr at 37℃. After three washes with PBS containing 0.05% Tween 20 (T-PBS), membranes were incubated with 1 μg/ml of rabbit anti-rat liver cytosolic GSH-PO antibody, with PBS containing 0.1% BSA and 0.1% gelatin, for 1hr at room temperature. After five washes with T-PBS, the membranes were incubated with horseradish peroxidase-conjugated donkey anti-rabbit Ig antibody (Amersham Japan, Tokyo), 1:400 dilution with T-PBS, for 1hr at room temperature. After five washes with T-PBS, the horseradish peroxidase-labels on the nitrocellulose membranes were developed with 3,3'-diaminobenzidine and hydrogen peroxide as substrate [40].

RNA preparation and Northern-blot Analysis. Total RNA was isolated from cells by the guanidine isothiocyanate-CsCl method [41]. Ten micrograms of total RNA was electrophoresed on 1% agarose gels containing 18% formaldehyde and blotted onto nitrocellulose filters in 10×SSC (1×=0.15 M NaCl, 0.015 M sodium citrate), as described by Maniatis et al. [42]. A 0.6 kb Sac II-Sal I fragment of cytosolic GSH-PO cDNA [43] was labeled with ^{32}P with a random priming kit (Takara Biomedicals, Japan). The cDNA probe for chicken β-actin was used as an internal positive control (housekeeping gene mRNA). The filters were prehybridized at 42℃ for 4hr in a mixture comprising 50% formamide, 0.1% bovine serum albumin, 0.1% Ficoll 400, 0.1% polyvinylpyrrolidone, 5×SSC, 0.1% sodium lauroylsarcosine, 0.05M sodium phosphate (pH 7.0) and 0.1mg/ml denatured salmon sperm DNA. Hybridization was performed at 42℃ for 18hr with heat-denatured ^{32}P-labeled DNA probes in the same solution as described above. After three washes with 2×SSC containing 0.1% SDS at room temperature, bands were autoradiographed with Kodack XRP-5 film for 24hr at room temperature. The signals of GSH-PO and β-actin were quantified using an IBAS image analyzer (Carl Zeiss, Germany).

RESULTS

Cytotoxicity of H_2O_2 on Se (-) and Se(+) BL-9.

While BL-9 cells were grown in selenium-free medium containing 1% FBS for 4 weeks (Se(-) cells), cells expressed far lower expression less than 10% of GSH-PO activity than Se-supplemented counter parts (Se(+) cells). Cells growth and morphological characteristics of Se(-) cells were similar to Se(+) cells (data not shown). As shown Fig. 1, the viability of both Se(-) and Se(+) cells significantly decreased until the H_2O_2 concentration reached 120 μM. However, Se(-) cells were far more sensitive to H_2O_2 (LD_{50} = 22 μM) than Se(+) cells (LD_{50} = 67 μM), as indicated in Fig. 1.

Cytoprotective effects of flavonoids against H_2O_2 under the condition of Se(+) and Se(-).

The protective effects of flavonoids against H_2O_2-induced cytotoxicity under the condition of Se(+) or Se(-) were studied (Fig. 2). Under the Se(+) condition, treatment of quercetin and catechin prior to H_2O_2 exposure resulted in a significant increase of the cell survival compared to the flavonoids-free controls (Fig. 2A). On the other hand, in Se (-) cells, the cytoprotective effects of flavonoids against H_2O_2 exposure were entirely unobtainable (Fig. 2B).

Effect of flavonoids on antioxidative enzyme activities.

It is known that selenium is an essential trace element for the biosynthesis of GSH-PO [27, 32, 43] which catalyzes the reduction

of a variety of hydroperoxides, including H_2O_2 [25-28]. We therefore examine the effect of quercetin and catechin on GSH-PO activities in Se(+) cells (Fig. 3). On treating Se(+) cells with both 5,20 μM quercetin for 4 hr, the activities of GSH-PO significantly increased to 254% ($P<0.01$) at 5 μM, 261% ($P<0.02$) at 20 μM of quercetin concentration. In catechin-treated Se(+) cells, GSH-PO activities also markedly increased to 269% ($P<0.001$) at 500 μM, 259% ($P<0.009$) at 1mM. In contrast, no significant changes were observed for glutathione-S-transeferase (GSH-S-T) and glutathione reductase (GSSG-RD) activities with the flavonoids treatment given to the Se(+) cells (Table 1).

Effect of quercetin and catechin on cGSH-PO protein expression.

As shown in Fig. 4 (lane 1), cGSH-PO protein was not detected in Se(-) cells with the immunoblot analysis. However, the synthesis of cGSH-PO was clearly recovered by the adding sodium selenite to the medium (Fig. 4, lane 2). A single immunoreactive band of 21 kDa were detected in Se(+) cells. The staining intensities of the bands were significantly increased in Se(+) cells treated with either quercetin or catechin (Fig. 4).

Induction of cGSH-PO mRNA expression by quercetin,and catechin.

To confirm the immunoblot results, we analyzed RNA from Se(+) cells treated with

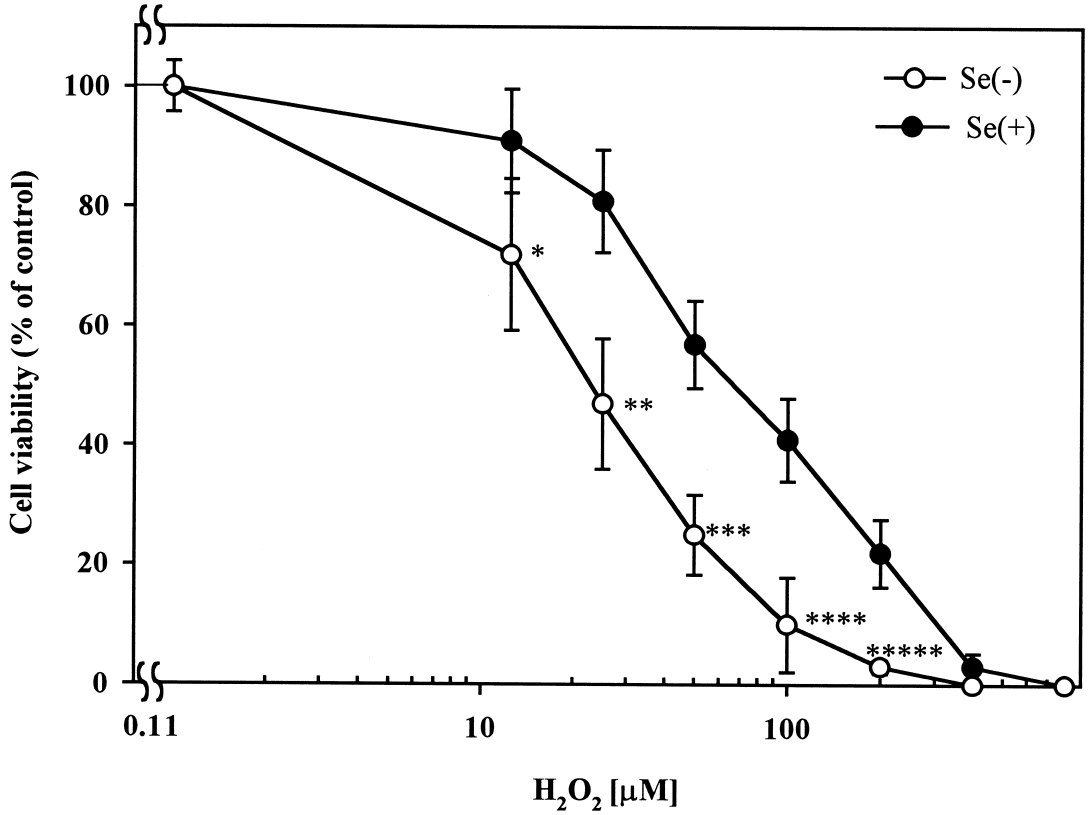

Fig. 1 Effects of selenium supplement on H_2O_2- induced cytotoxicity. To attain a selenium deficient condition, the FBS concentration was gradually decreased from 10% to 1% in the medium. Finally the selenium-free BL-9 cells (designated as Se(-) cells) were cultured in the medium containing 1 % FBS for 4 weeks. The selenium supplemented-BL-9 cells (Se(+) cells) were prepared by adding 100ng/ml of sodium selenium to the medium. Se(+) and Se(-) cells were treated with H_2O_2 for 48hr in a medium containing 1 % FBS. Cell viability was assessed by trypan blue exclusion assay. (●) Se(+); (○) Se(-). Confidence levels were determined using Student's *t* - test; *, $P<0.005$, **, $P< 0.0005$, ***, $P< 0.000005$, ****, $P< 0.0001$,*****, $P< 0.00003$.

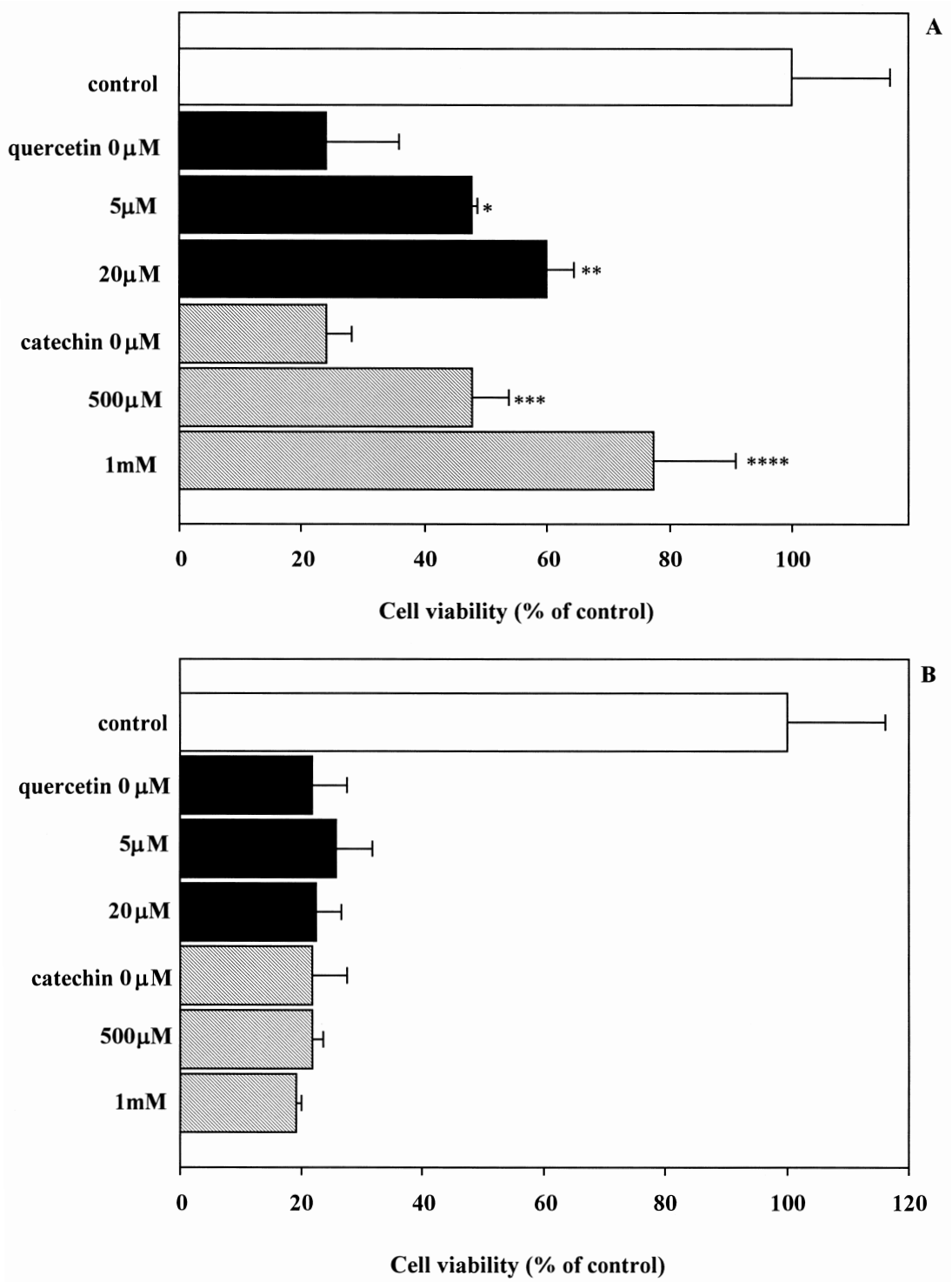

Fig. 2 Effect of quercetin and catechin on H_2O_2- induced cytotoxicity in Se(+) (A) and Se(-) cells (B). After Se(+) and Se(-) cells were treated for 4hr with quercetin or catechin at different concentrations in FBS-free medium, the cells were exposed for 48hr with 100 μM of H_2O_2 in a medium containing 1% FBS as described in Materials and Methods. The cell viability was determined by trypan blue exclusion assay. Columns and bars represent means ± SD (n=5). *, P< 0.0004, **, P< 0.001, *** P< 0.0007, ****, P< 0.0003 relative to flavonoids-free control.

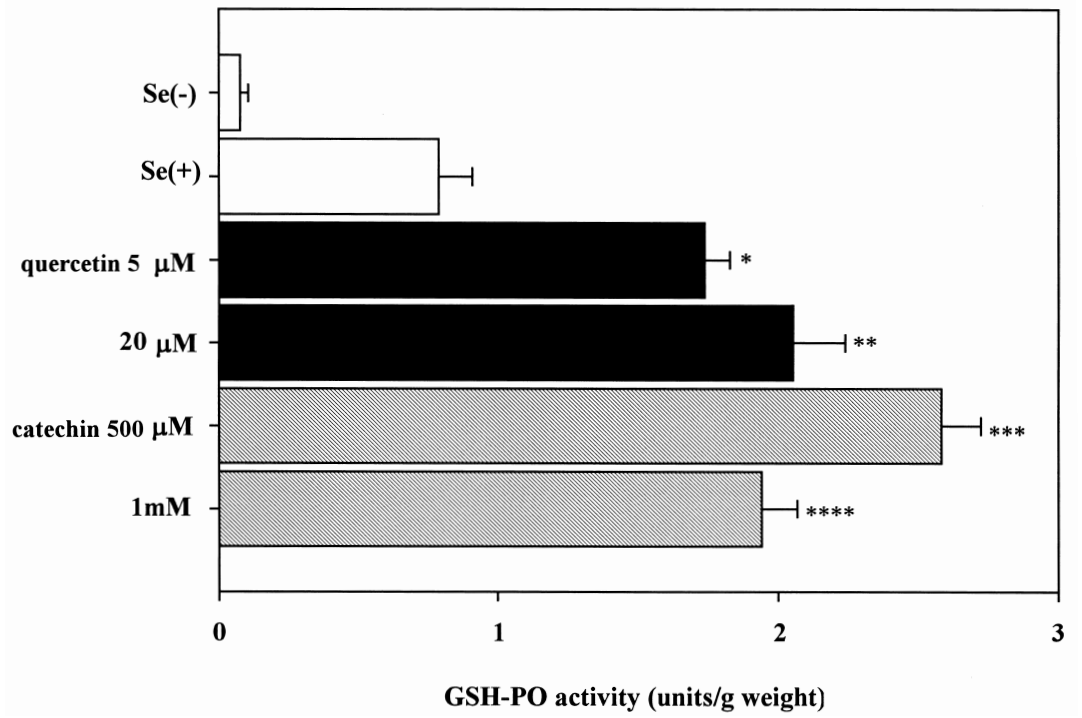

Fig. 3 GSH-PO activity increased by the treatment of either quercetin or catechin in Se(+) cells. After cells were treated with quercetin or catechin at different concentrations in FBS-free medium for 4hr, cells were further cultured in a medium containing 1% FBS for 48hr and assayed for cGSH-PO activity as described. Columns and bars represent means ± SD (n=7). Confidence levels were determined using Student's *t* - test; *, P< 0.01, **, P< 0.02, ***, P< 0.001, ****, P< 0.009.

either quercetin or catechin for cGSH-PO mRNA (Fig. 5). The rat cGSH-PO probe detected a single mRNA transcript. Densitometry of resulting autoradiograms indicated that treatment of quercetin and catechin elicited cGSH-PO mRNA increase of 98% at 5 μM, 15% at 20 μM of quercetin and 55% at 1mM of catechin (Table 2).

DISCUSSION

It is well documented fact that some flavonoids, mostly quercetin and its derivatives and catechin, posses scavenging (or quenching) abilities against free radicals and active oxygen species [11-16]. However, studies on cytoprotective actions of those flavonoids against cell damages induced by free radicals and/or oxidative stress as including lipid peroxidation are rather rare [17-19], and the mechanism of those cytoprotective actions of flavonoids have not been clearly understood. The mechanism was mostly ascribed to the direct scavenging (or quenching) action of flavonoids against free radicals and active oxygen species, and some investigators added the iron-chelating activity of flavonoids to this [11-16]. On the other hand, in aerobic organism using oxygen for energy production, active oxygen species including O_2^-, ·OH, H_2O_2 and subsequent by occurring lipid peroxides, which are all eminently cytotoxic, are inevitably formed in cells as by-products of metabolism. Cells of those aerobic organisms, therefore, are generating enzymes and non-enzymatic defenses scavenging those cytotoxic oxidants [13]. Among them, various types (cytosolic [27, 43], phospholipid-[44], plasma-[45] and intestinal [46]) of glutathione peroxidase (GSH-POs) are known to reduce H_2O_2 and lipid peroxides most effectively. In this context, we expected that the cytoprotective action of flavonoids against active oxygen species and lipid peroxides might be synergistically enhanced if the flavonoids can effectively interact with GSH-POs to pro-

mote their expression and/or their activities. To confirm the availability of this interaction, we employed proliferating cultured rat hepatocyte (BL-9) [33] in which prominent expression of the cytosolic GSH-PO (cGSH-PO) is evident. In order to obtain cGSH-PO deficient cells, BL-9 was cultured with Se depleted culture medium prepared following to the method of Geiger et al [47] (Se(-) BL-9). 100ng/ml of sodium selenite was added to the above medium to prepare Se(+) BL-9 cells. The effects of flavonoids (quercetin and catechin) on the cell viability after H_2O_2 exposure, on the activities of antioxidative enzymes and on cGSH-PO expressions (both protein, by immunoblot, and mRNA, by Northern blot) were compared between these Se(-) and Se(+) BL-9 cells.

Cytotoxicity of H_2O_2 on Se(-) and Se(+) BL-9 cells.

Se(-) cells were far more sensitive to H_2O_2 exposure than Se(+) cells. This may be simply due to the action of cGSH-PO which was far more pronouncedly expressed and activated in Se(+) cells. Although cGSH-PO is not only Se dependent protein in rat hepatocytes but other isozymes of GSH-PO, thioredoxin reductase [48] and iodothyronine 5 '-deiodinase [49] are also Se-dependent ones, cGSH-PO exists in overwhelmingly greater amount in the hepatocytes and the latter two enzymes do not react with H_2O_2 directly.

Cytoprotective effect of flavonoids against H_2O_2 induced cytotoxicity under the condition of Se(+) and Se(-).

Both quercetin and catechin exhibited definitely increased cell viability in dose dependent manner in Se(+) cells, but apparently no change in cell viability was elicited in Se(-) cells by the addition of both flavonoids (Fig. 2). These results would indicate that Se is indispensable to effect the antioxidative (anti-H_2O_2) functions of the flavonoids, in other words the anti-H_2O_2 functions of the flavonoids are totally dependent on cGSH-PO (Se dependent protein) activity which may be enhanced by the flavonoids under the condition of Se(+). Incidentally, cGSH-PO activity was definitely enhanced by the addition of the flavonoids added to the culture media as shown in Fig. 3. The direct interaction between Se itself

Table 1 Effect of treatment with either quercetin or catechin on the activities of various antioxidant enzymes in Se(+) cells.
After cells treated with each flavonoid in FBS-free medium for 4hr, they were cultured in medium for 48hr as described in Materials and Methods.
Values are means ± SD, n=5. Significant differences were determined by Student's *t* - test; *, P< 0.01, **, P< 0.02, ***, P< 0.001, ****, P< 0.009.

	GSH-PO activity [units/g・weight]	GSH-S-T activity [units/g・weight]	GSSG-RD activity [units/g・weight]
Se(−)	0.080 ± 0.028	6.874 ± 0.983	4.556 ± 0.235
Se(+)	0.791 ± 0.118	6.091 ± 0.435	4.996 ± 0.037
+ quercetin 5 μM	1.741 ± 0.086*	6.163 ± 2.012	3.481 ± 1.262
+ quercetin 20 μM	2.055 ± 0.183**	4.580 ± 0.949	4.293 ± 0.546
+ catechin 500 μM	2.586 ± 0.142***	4.507 ± 1.680	4.218 ± 0.563
+ catechin 1mM	1.942 ± 0.125****	4.514 ± 1.217	4.149 ± 0.824

Table 2 The changes in the relative amounts of cGSH-PO mRNA in quercetin or catechin treated Se(+) cells.

	GSH-PO mRNA/β-actin mRNA	Relative level (%)
Se(+)	1.16	100
+ quercetin 5 μM	2.29	198
+ quercetin 20 μM	1.33	115
+ catechin 500 μM	1.14	98
+ catechin 1mM	1.79	155

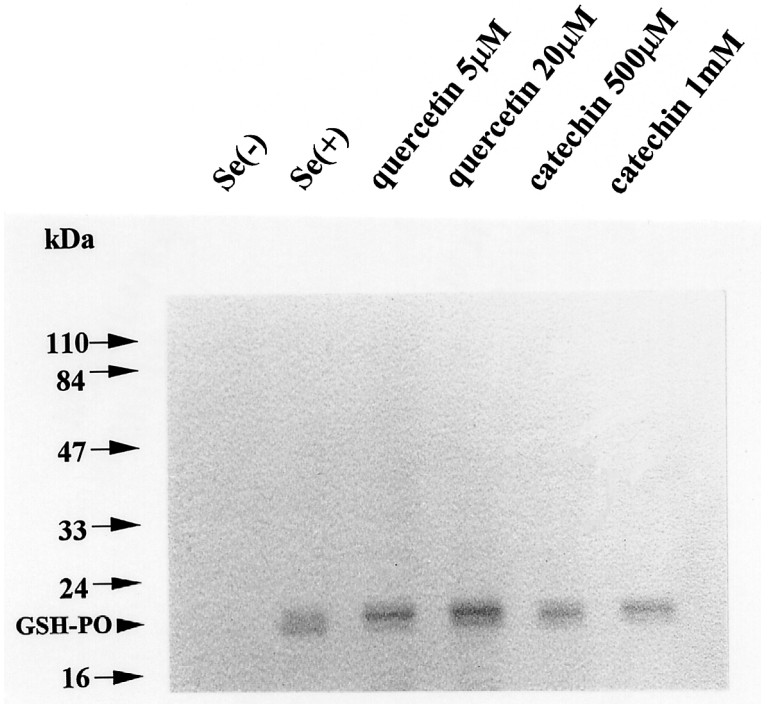

Fig. 4 Effect of treatment with quercetin and catechin on the expression of cGSH-PO proteins in Se(+) cells. Immunoblot analysis was performed on Se(+) cells treated with 5,20 μM quercetin or with 500 μM, 1mM catechin. Samples were electrophoresed on SDS-12.5% polyacrylamide gel, transferred to nitrocellulose membrane, and treated with anti-rat liver cGSH-PO antibodies, as described.

and the flavonoids should also be taken into account regarding the cytoprotective mechanism against H_2O_2- induced cell damage, but it is unlikely because the anti-H_2O_2-activity of cGSH-PO is far more pronounced than that of Se itself.

Effect of flavonoids on antioxidative enzyme activities.

Among the antioxidative enzymes here dealt with (GSH-PO, GSH-S-T, GSSG-RD), only GSH-PO was meaningfully activated by Se supplementation (Table 1) and also by the addition of the flavonoids under the condition of Se(+) (Fig. 3 and Table 1). So far as this BL-9 cells, highly GSH-PO expressing cells, is concerned, it appears that cytoprotective abilities of the flavonoids are largely or may be totally dependent on H_2O_2 and lipid peroxides scavenging activity of GSH-PO.

In these cytoprotective activities of the flavonoids (the recovery of the cell viability and the activation of GSH-PO), quercetin worked at much lower concentrations (5〜20 μM) than those of catechin (500 μM〜1mM). Both flavonoids are usually detected in plasma of non-supplemented human at the levels of approximately 2 μM [50-51]. It was also reported that when rats were fed with flavonoids quercetin was most stably present in their plasma [52]. Taking these evidences into account, the cytoprotective ability of quercetin is more powerful and consistent than that of catechin.

Induction of cGSH-PO protein and mRNA by the flavonoids.

Expression of both cGSH-PO protein and mRNA was markedly diminished with Se deficiency and prominently recovered by the supplementation of Se. In Se(+) cells, the addition of both flavonoids clearly increased the expression of both GSH-PO protein and

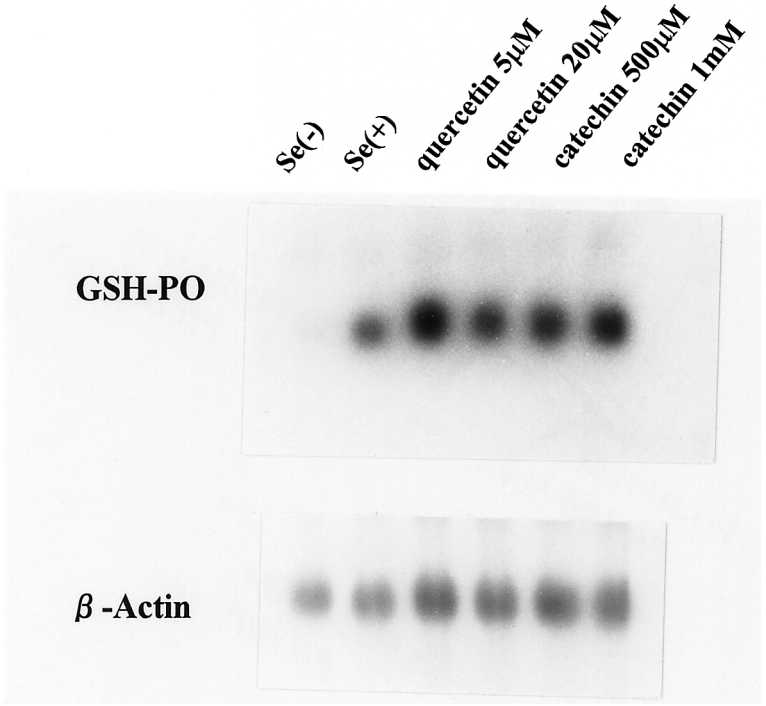

Fig. 5 Induction of cGSH-PO mRNA by the treatment of quercetin or catechin. Ten-micrograms of total RNA were fractionated on formalin-agarose gels, transferred to nitrocellulose membranes and then hybridized with ^{32}P-labeled rat cGSH-PO cDNA. The same filter was rehybridized with chicken β-actin cDNA.

mRNA (Figs. 4 and 5). R. A. Sunde's group and our group reported for the first time and at the same time [53, 43] that Se was transcriptionally regulating cGSH-PO synthesis. Recently, however, the majority of GSH-PO investigators insist that Se is working on stabilizing cytoplasmic mRNA by preventing nonsense codon-mediated decay of the mRNA [54-56]. The exact mechanism of this phenomenon remains unexplained. The evidence that the flavonoids enhanced the cytoplasmic mRNA in Se(+) cells would provide us with precious clues to solve the mystery concerning the role of Se in GSH-PO expression. The flavonoids could be preventing the decay of the mRNA through its active oxygen species scavenging ability under the cooperation of Se.

In conclusion, we have investigated the mechanism of the cytoprotective action of flavonoids, quercetin and catechin, against H_2O_2 cytotoxicity induced in proliferating cultured rat hepatocytes BL-9, which are highly cGSH-PO expressing cells, and we found that the cytoprotective action was very intimately related to the activation of the cGSH-PO through the fact that the changes were totally dependent on the sufficiency of Se in those cells. The cytoprotective actions of the flavonoids should also be tested on cells with low expression of GSH-PO to determine the exact mechanism of the antioxidative action of the flavonoids.

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