Effects of Quercetin and Catechin on Hepatic Glutathione-S Transferase (GST), NAD(P)H Quinone Oxidoreductase 1 (NQO1), and Antioxidant Enzyme Activity Levels in Rats

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Cell culture data indicate that quercetin and catechin may affect the activity of phase II and antioxidant enzymes. However, little is known about the impact of dietary flavonoids in vivo. Therefore, the present study aimed to investigate the in vivo effects of the flavonoids quercetin and catechin on mRNA and activity levels of phase II enzymes glutathione-S transferase (GST) and NAD(P)H quinone oxidoreductase-1 (NQO1) in rat liver. Furthermore, the activity of the hepatic antioxidant enzymes catalase (CAT), glutathione peroxidase (GPx), and superoxide dismutase (SOD) was determined. Feeding male Wistar rats (3 × 6 animals) over 3 wk with semisynthetic diets enriched with quercetin and catechin (2 g/kg diet) did not affect liver enzyme activity of CAT, GPx, and SOD as well lipid peroxidation and glutathione levels. Dietary quercetin significantly decreased activity of hepatic GST (24%), whereas dietary catechin significantly decreased NQO1 activity (26%) compared to controls. Changes in GST and NQO1 activity were partly reflected on mRNA levels. Current data indicate that dietary flavonoids have little effects on liver oxidant/antioxidant status but do significantly affect the phase II enzymes GST and NQO1 in rat liver. This in turn may affect the ability of the organism to detoxify endogenous and exogenous xenobiotics.

INTRODUCTION

The hepatic detoxification of xenobiotics is partly mediated by the phase II enzymes glutathione-S transferase (GST) and NAD(P)H quinone oxidoreductase 1 (NQO1) (1,2). GST catalyzes the conjugation of endogenous and xenobiotic electrophiles with glutathione (3). The major cytosolic GST sub-classes in humans and rats are GSTA, GSTM, and GSTP (3,4). GST subunits exhibit species- and tissue-specific expression patterns and display partly overlapping substrate specificities (1). NQO1 reduces compounds with a quinone structure due to two-electron transfer (5). The NQO1 enzyme thereby prevents the generation of toxic semiquinone radicals and reactive oxygen species. The resulting compounds are less reactive hydroquinones (6). NQO1 is predominantly (>90%) localized in the cytosol and mainly expressed in liver, kidney, and the gastrointestinal tract (5). Furthermore, reactive oxygen species such as hydrogen peroxide and superoxide anion free radicals are detoxified by the antioxidant enzymes catalase (CAT), glutathione peroxidase (GPx), and superoxide dismutase (SOD) (7).

On the one hand, the induction of phase II and antioxidant enzymes by dietary constituents, such as flavonoids, may affect the capacity of organisms to detoxify xenobiotics and free radicals (2,8,9). On the other hand, an inhibition of GST and NQO1 may be a strategy in cancer therapy in order to combat the drug resistance of cancer cells against chemotherapeutics (10,11).

Although there is evidence from in vitro experiments and cell culture studies that quercetin and catechin may affect phase II (12–14) and antioxidant enzymes (15–17), little is known on their activity in vivo (18–20). Therefore, the present study aimed to investigate the in vivo effects of the flavonol quercetin and the flavanol catechin on mRNA and activity levels of hepatic GST and NQO1 in rats. Additionally the liver redox system was characterized by measurement of activity of the hepatic antioxidant enzymes CAT, GPx, and SOD. Since GST uses glutathione (GSH) for conjugation reactions, liver tissue levels of GSH were determined.
MATERIALS AND METHODS

Chemicals and Reagents

(+)-Catechin hydrate, 1-chloro-2,4-dinitrobenzene (CDNB), 5,5′-Dithiobis[2-nitrobenzoic acid] (DTNB), 1,1,3,3-tetraethoxypropane (TEP), menadione (2-methyl-1,4-naphthoquinone), and trichloroacetic acid (TCA), were purchased from Sigma-Aldrich (Schnelldorf, Germany); and quercetin dihydrate, β-nicotinamide-adenine-dinucleotide-phosphate-tetrasodium salt (NADPH) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were obtained from Roth (Karlsruhe, Germany).

Animals and Diets

Eighteen male Wistar Unilever rats (HsdCpb:WU, Harlan & Winkelmann, Borchen, Germany) were housed pair wise (2 animals per cage) in Macrolon cages with spruce and fir wood bedding in a controlled environment (21 ± 2°C and 55 ± 5% relative humidity, 12-h light-dark cycle). The animals were fed either a flavonoid-free, semisynthetic diet (ssniff special diets GmbH, Soest, Germany) or a diet enriched with quercetin or catechin at concentrations of 2 g/kg diet. The composition of the basal semisynthetic diet was cornstarch, 472 g; glucose, 110 g; cellulose, 50 g; casein, 240 g; coconut oil concentrate, 38 g; corn oil, 20 g; mineral and trace element premix, 60 g; and vitamin premix, 10 g/kg diet. The experimental diets were prepared weekly and stored at 4°C. The rats had free access to feed and water throughout the experiment.

The rats were randomly divided into 3 groups of 6 animals each with initial body weights of 89 ± 0.9 g and fed the experimental diets for 22 days. Body weight and food intake were recorded weekly. At the end of the experiment, the rats were food deprived for 12 h, anaesthetized with carbon dioxide, and decapitated. Liver tissue was quickly excised, rinsed with 0.9% sodium chloride solution, frozen in liquid nitrogen, and stored at −80°C until analyzed.

RNA Isolation and Real-Time qRT-PCR

Total RNA was isolated from rat liver tissue using RNeasy Mini kit (Qiagen, Hilden, Germany). RNA was quantified photometrically and RNA quality was controlled by gel electrophoresis. Real-time PCR was performed as one step procedure with QuantiTect SYBR Green RT-PCR kit (Qiagen). Transcription levels of target genes were related to transcription of the housekeeping gene β-actin. The primers were designed with a standard program (primer3, NCBI Spidey and Blast) and purchased from MWG (Ebersberg, Germany). Primer specifications are given in Table 1.

Liver Tissue Preparation

A ratio of 1:10 homogenates of liver were prepared with ice cold phosphate buffered saline (PBS) buffer (pH 7.4) and centrifuged at 3,800 g at 4°C for 10 min (Beckman CS-15R, Rotor S4180, Krefeld, Germany). The supernatant was stored at −80°C until further use. To prepare the cytosolic fraction, 1 g of liver tissue and 4 × vol/wt of ice cold PBS buffer (pH 7.4) was homogenized and centrifuged at 10,000 g at 4°C for 20 min (Eppendorf 5804 R, Rotor F34-6-38, Wesseling-Berzdorf, Germany). The supernatant was transferred into a new tube and centrifuged at 100,000 g at 4°C for 1 h (Kontron Instruments Centrikon T-1065 Ultra centrifuge, Rotor TST 28.38, Neufahrn, Germany). The resulting supernatant is equivalent to the cytosolic fraction.

### TABLE 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene ID</th>
<th>Sequence (5'-3')</th>
<th>AT</th>
<th>Product Size</th>
</tr>
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<tbody>
<tr>
<td>Gsta2</td>
<td>24422</td>
<td>F GGAGAGAGCCCTGTATTGACA R TTCAAAAGCCCAGGCAAGTACC</td>
<td>58°C</td>
<td>151bp</td>
</tr>
<tr>
<td>Gstp1</td>
<td>24426</td>
<td>F CTTTTGAGACCCTGCTGTCC R GAGCCACATAGGCAGAGACG</td>
<td>59°C</td>
<td>162bp</td>
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<tr>
<td>Gstm2</td>
<td>24424</td>
<td>F TTTCCTGTTCCTTGGATAT R TTGCTCTGGGTGATCTTGTG</td>
<td>57°C</td>
<td>172bp</td>
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<tr>
<td>NQO1</td>
<td>24314</td>
<td>F CGCAGAGAGACATCATCCA R CGCCAGAGATGACTCAACAG</td>
<td>57°C</td>
<td>190bp</td>
</tr>
<tr>
<td>Gclc</td>
<td>25283</td>
<td>F CTGGGGGATGGATTTCTGCTAT R AGATCTCGTGCTGTGATGTC</td>
<td>57°C</td>
<td>176bp</td>
</tr>
<tr>
<td>Gclm</td>
<td>29739</td>
<td>F TTGTGTGATGCCACAGAGATT R GCTTTTCACGATGACCAGGT</td>
<td>57°C</td>
<td>189bp</td>
</tr>
<tr>
<td>Actb</td>
<td>81822</td>
<td>F GGTTTTGGAAGGTTCTCACA R TGTCATCAACTGGGAGCAGATA</td>
<td>56°C</td>
<td>165bp</td>
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</table>

*Abbreviations are as follows: qRT-PCR, quantitated reverse transcription polymerase chain reaction; AT, annealing temperature; F, forward primer; R, reverse primer; GST, glutathione S-transferase; α, class alpha; p, class pi; m, class mu; NQO1, NAD(P)H quinone oxidoreductase 1; Gclc, glutamate-cysteine ligase, catalytic subunit; Gclm, glutamate cysteine ligase, modifier subunit; Actb, beta-actin.*
Enzyme Activity Measurements

Total cytosolic GST activity was measured according to the method of Habig et al. (21) using CDNB as substrate at 340 nm. A standard curve was generated with equine liver GST (Sigma) and used for calculation of enzyme activity. NQO1 activity was measured in the cytosolic fraction based on the method of Prochaska and Santamaria (22). NQO1 catalyzes the reduction of menadione to menadiol by NADPH utilization. Simultaneously, MTT is reduced by menadiol, producing a blue dye that can be quantified at 610 nm spectrophotometry. NQO1 activity was calculated by use of a standard curve with human recombinant diaphorase (Sigma). Antioxidant enzyme activities were measured in whole cell liver homogenates according to the method of Marklund and Marklund (23) for SOD, Lawrence and Burk (24) for GPx, and Johansson and Borg (25) for CAT. Enzyme activities are expressed as units per milligram protein.

Protein concentration was determined with a commercial kit (Pierce BCA Protein Assay, IL, USA) using bovine serum albumin as a standard.

Glutathione Concentration

Total concentration of GSH was measured according to the method of Dringen and Hamprecht (26). GSH reacts spontaneously with DTNB whereby free 2-nitro-5-thiobenzoate (TNB) molecules are generated. TNB is a chromogenic thiol compound that can be spectrophotometrically quantitated at 405 nm.

Lipid Peroxidation

Lipid peroxidation was fluorometrically assayed as thiobarbituric acid reactive substances (TBA-RS) in liver homogenates after protein precipitation with TCA and extraction in 1-butanol. The samples were measured with and without ferrous provocation. Excitation and emission wavelengths were 520 nm and 560 nm, respectively. Calibration curve was prepared with TEP as an external standard (27).

Quercetin Analysis

Aliquots (300 µl) of each plasma sample were spiked with rhamnetin (internal standard, 50 mg/l methanol) and treated with β-glucuronidase (Roth) and sulfatase (crude enzyme extract from Helix pomatia, Sigma) for cleavage of all ester bonds of glucuronides/sulfates. HPLC analysis was performed according to the method described by Hollman et al. (28) with minor modifications (29). Calibration curves for quercetin andisorhamnetin (Roth) were obtained by the addition of these flavonols from methanolic stock solutions to flavonol-free plasma, with rhamnetin serving as an internal standard.

Catechin Analysis

The plasma samples (200 µl) were acidified (to pH 5.0) with 0.58 mol/l acetic acid containing 0.11 mol/l ascorbic acid and analyzed both with and without hydrolysis. Sulfatase from Helix pomatia with secondary β-glucuronidase activity (Sigma) was added to the samples for digestion of conjugated catechins. Catechin was extracted by addition of cold methanol with 0.2 mol/l HCl, phases were separated by centrifugation (9,000 g, 10 min, 4°C), the supernatant was collected and the residue was extracted again twice, each with cold methanol/0.2 mol/l HCl under the same conditions as used in the first extraction. The supernatants were combined and vacuum evaporated. The residue was dissolved in 80% methanol and measured by HPLC with post column derivatization (30) and additional by LC/MS/MS. The 4000 Q TRAP MS-MS Modul from Applied Biosystem (Darmstadt, Germany) was used to analyze the samples in full scan and multiple reaction monitoring mode. The recovery of (+)catechin in plasma without hydrolysis and after incubation with the enzyme was 68% and 65%, respectively. The limit of detection for catechin was 20 ng/ml.

Statistical Analysis

Statistical analysis was performed using the statistical software SPSS (version 13.1). To assess effects of various treatments, a 1-way ANOVA followed by a Dunnett T (two-sided) post hoc test was used. Differences were considered significant if $P < 0.05$. Results are expressed as mean with SEM.

RESULTS

Body Weight Gain and Food Intake

Feeding growing rats with the quercetin and catechin supplemented diets did not significantly affect their body weight and food intake over the 3 wk experimental period (Table 2).

<table>
<thead>
<tr>
<th>Week</th>
<th>Body Weight (g)</th>
<th>Estimated Food Intake (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Quercetin</td>
</tr>
<tr>
<td>0</td>
<td>89 ± 1.6</td>
<td>89 ± 1.7</td>
</tr>
<tr>
<td>1</td>
<td>139 ± 2.8</td>
<td>143 ± 1.9</td>
</tr>
<tr>
<td>2</td>
<td>188 ± 3.9</td>
<td>197 ± 2.4</td>
</tr>
<tr>
<td>3</td>
<td>237 ± 4.9</td>
<td>249 ± 3.8</td>
</tr>
</tbody>
</table>
Plasma Quercetin and Catechin Levels

Feeding the quercetin-supplemented diet significantly enhanced the plasma levels of quercetin and its methylated metabolite isorhamnetin. The plasma concentrations in the quercetin-supplemented group were 1.90 μmol/l for quercetin and 13.0 ± 1.17 μmol/l for isorhamnetin, respectively. The plasma concentrations in the control group were < 0.03 μmol/l for quercetin and for isorhamnetin, respectively. Following catechin supplementation, no catechin in plasma could be detected after 12 h starvation of the animals.

Liver Antioxidant Enzyme Activity, Glutathione, and Lipid Peroxidation Levels

There was no statistical significant effect of dietary quercetin and catechin on liver activity of the antioxidant enzymes CAT, GPx, and SOD (Table 3). Furthermore, hepatic glutathione as well as liver lipid peroxidation levels, as measured by TBA-RS in the absence and presence of iron, remained unchanged in response to the dietary treatments. The mRNA levels of both subunits of γ-GCS, which is the rate limiting enzyme in glutathione synthesis (31), were similar between control rats and rats fed the diets supplemented with quercetin and catechin (Table 3).

Liver Phase II Enzyme Activity and mRNA Levels

Feeding the quercetin-supplemented diet significantly lowered cytosolic GST activity by 24% in rat liver (Table 4). However, the dietary supplementation of catechin had no effect on hepatic GST levels. Feeding the catechin-supplemented diet signifi-
Following dietary quercetin supplementation, plasma levels of isorhamnetin (methylated quercetin metabolite) were higher than of quercetin, indicating that most of quercetin in rats is metabolised by methylation in the gut and in the liver (36,37). However, plasma catechin levels following catechin supplementation were under the limit of detection of the LC/MS/MS. In this context, it needs to be taking into account that catechin is rapidly eliminated from the circulation with a half life of 3 to 4 h (38,39). In the present rat study, animals were starved for 12 h, which may explain that no catechin could be detected in plasma. Furthermore, the elimination of catechins is expected to be nearly complete after an overnight fast (40).

In accordance with recent in vitro studies using recombinant (13) and purified (12,41) GST, quercetin inhibited hepatic GST activity also in the present rat study. However, quercetin supplementation only resulted in a moderate decrease in GST mRNA levels, indicating that GST inhibition due to quercetin, as observed in the present study, may be only partly regulated on the posttranscriptional level. The inhibition of GST by quercetin may have different implications: Quercetin supplemented at very high doses in humans may retard the metabolism of different drugs that are conjugated with glutathione in order to make them more hydrophilic. Thus, the capacity of the liver to detoxify xenobiotics can possibly be impaired by high dose quercetin. Multidrug resistance in cancer patients is frequently associated with enhanced chemotherapeutics efflux or enhanced metabolism via GST. Thus, the use of GST inhibitors such as quercetin may offer a therapeutic strategy to modulate anticancer drug resistance against drugs such as doxorubicin (42) and chlorambucil (43), which are substrates of GST. Since quercetin but not catechin inhibited GST activity, and both molecules do differ only in one residue and the double bond in the C ring, our data indicate that the presence of the 4-oxo group and the double bond between C atom 2 and C atom 3 may be an important structural requirement for the GST inhibitory activity of quercetin as compared to catechin. In this context, Van Zanden and coworkers (44) further specified the inhibitory mechanism of GSTP1 by quercetin. It was suggested that quinone-type oxidation products of quercetin likely act as specific inhibitors of GSTP1 by binding to cysteine 47. It needs be taken into account that the substrate used for assaying total GST activity in our rat study was 1-chloro-2,4-dinitrobenzene (CDNB), which is recognized as a general substrate for all GST subunits. However, the individual GST subunits exhibit different substrate affinity to CDNB, whereas the subunits of class Alpha have the highest specific activity to CDNB (21,45). Thus, changes in the activity of GST subunits by quercetin not belonging to class Alpha may have been underestimated in this study. Interestingly, in a study by Mitchell and coworkers (46), an isoenzyme specific change of hepatic GST due to flavone was recently observed in mice.

Under the conditions investigated, dietary catechin decreased hepatic NQO1 activity. It is well known that NQO1 is generally considered to be a detoxification enzyme that has been shown to prevent the formation of reactive oxygen species (6). NQO1 exhibits antioxidant activity by maintaining ubiquinones, α-tocopherolhydroquinone, and α-tocopherol in their reduced forms (47) and regulates intracellular redox state by controlling the NAD(P)H:NAD(P) ratio (48). However, in this study, the inhibition of NQO1 (which may lead to enhanced cellular levels of reactive oxygen species) by dietary catechin was not accompanied by a change in liver oxidant/antioxidant status as determined by hepatic TBA-RS (although a relative unspecific biomarker of lipid peroxidation) and liver glutathione levels.

It is well known that NQO1 is overexpressed in several types of cancer (49,50). Thus, NOQ1 is important target for cancer therapeutics (51). It was shown in cultured cells that inhibition of NQO1 with dicoumarol increased intracellular O2 and inhibited the in vitro malignant phenotype of pancreatic cancer (52). Moreover, genetic deletion of NQO1 potentiates apoptosis in transformed cells (53).

To the best of our knowledge, an inhibition of NQO1 by dietary catechin, as observed in the present study, has yet not been reported. Further studies are warranted to test the hypothesis whether there is a synergistic effect between dicoumarol (a known inhibitor of NQO1) and catechins on induced cell death in cancer cells. Furthermore, potential anticancer properties of catechin in relation to its NQO1 inhibitory activity should be investigated in cancer-relevant rodent models.

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REFERENCES


