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Phytochemicals Inhibit Catechol-O-Methyltransferase Activity in Cytosolic Fractions from Healthy Human Mammary Tissues: Implications for Catechol Estrogen-Induced DNA Damage

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Phytochemicals are natural dietary constituents of fruits and vegetables. Some of these phytochemicals are known to affect estrogen-metabolizing enzymes. In breast tissue, estradiol can be metabolized to the catechol estrogens 2- and 4-hydroxyestradiol (2-OHE_2 and 4-OHE_2). Catechol estrogens are suspected carcinogens potentially involved in the etiology of breast cancer. Catechol-O-methyltransferase (COMT) converts the catechol estrogens to their inactive methoxy derivatives (2-$MeOE_2$ and 4-$MeOE_2$). In this study we investigated the effects of several phytochemicals on COMT activity in cytosolic fractions of seven healthy human mammary tissues from reduction mammoplasty. Large interindividual variations were observed in the constitutive levels of COMT activity. However, in all cytosol samples the catalytic efficiency of COMT was greater for 2-$MeOE_2$ formation than for 4-$MeOE_2$ formation. The known COMT inhibitor Ro 41-0960 and several phytochemicals with a catechol structure (quercetin, catechin, and (-)-epicatechin) concentration-dependently inhibited COMT activity, while phytochemicals without a catechol structure (genistein, chrysin, and flavone) showed no effect up to 30 μM. Distinct interindividual variations were observed in sensitivity toward COMT inhibition among the various tissue samples, as was shown by the range in IC_{50} values for Ro 41-0960 (5–42 nM). The toxicological relevance of COMT inhibition and the effect of reduced inactivation of catechol estrogens was studied by determining the amount of catechol estrogen-induced DNA damage in MCF-7 cells using the comet assay. Catechol estrogens alone caused no increase of DNA damage compared with control treated cells. However, both Ro 41-0960 and quercetin caused a decrease of methoxy estradiol formation and an increase of catechol estrogen-induced DNA damage in MCF-7 cells. This suggests that phytochemicals with a catechol structure have the potential to reduce COMT activity in mammary tissues and may consequently reduce the inactivation of potentially mutagenic estradiol metabolites and increase the chance of DNA damage.

Key Words: **phytochemicals; catechol estrogens; COMT; DNA damage.**

During the last decades an increase has been observed in the occurrence of malignant neoplasms of the female breast in industrialized countries. Presently, it is one of the major causes of death among women in Western countries. In the etiology of breast cancer, estrogens play a key role in tumor development. The role of estrogen in carcinogenesis has been suggested to be dual; it may act by stimulating cell transformation and cell proliferation, and it may act as a tumor initiator through its metabolites by inducing damage to cellular macromolecules such as DNA (Cavalieri *et al*., 1997; Clemons and Goss, 2001; Lemon *et al*., 1992; Liehr, 1997; Yager, 2000). The tumor-initiating action of estrogens is believed to be a result of hydroxylation of the main estrogen in premenopausal women, 17β-estradiol (E_2), to the catechol estrogens 2- and 4-hydroxyestradiol (Badawi *et al*., 2001; Martucci and Fishman, 1993; Ziegler *et al*., 1997). Unless inactivated, catechol estrogens can undergo oxidation to reactive quinones (Cavalieri *et al*., 1997). Quinones of 2-hydroxyestradiol (2-OHE_2) can form stable DNA adducts that remain in the DNA unless repaired, but quinones of 4-hydroxyestradiol (4-OHE_2) can form depurinating DNA adducts, a potential tumor-initiating event in human cancers (Cao *et al*., 1998; Cavalieri *et al*., 1997; Liehr, 1997; Yager, 2000). Catechol-O-methyltransferase (COMT) plays an important role in the inactivation of catechol estrogens (Ball and Knuppen, 1980; Weisz *et al*., 1998). COMT is a phase II enzyme involved in the inactivation of many endogenous catechol substrates by transferring a methyl group from S-adenosyl-L-methionine (SAM) to the substrate and thus converting them into their methoxy derivatives (reviewed by Männistö and Kaakkola, 1999).

Low COMT activity has been associated with increased breast cancer risk (reviewed by Yue *et al*., 2003). There are several ways in which COMT activity might be altered. Lachman *et al*. have described a low-activity form of COMT resulting from a genetic polymorphism (Lachman *et al*., 1996). A single nucleotide substitution in codon 108 causes an amino acid transition (Val → Met) which results in a high- (Val/Val) or low-activity (Met/Met) form of the COMT enzyme with a three-to-four-fold difference in activity. COMT activity can also be inhibited by substrate competition for the enzyme. There are many naturally

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occurring substrates for COMT in the body, but some exogenous compounds have also been identified as substrates for the enzyme. For example, certain catechol metabolites of PCBs have been shown to inhibit COMT activity (Garner *et al*., 2000). In addition, many dietary catechols, such as phytochemicals, can be a substrate for COMT. Phytochemicals are a diverse group of chemicals which can be found in fruits and vegetables. This group of biologically active compounds occurs in high concentrations in our diet, and the daily intake can comprise a few hundreds of milligrams per day (Hollman and Katan, 1999). As a result, submicromolar plasma levels can be reached (Hollman and Katan, 1999; Rein *et al*., 2000; Warden *et al*., 2001). Phytochemicals have been shown to possess antioxidant, anticancer, and antiviral properties. Because of these properties, they are generally regarded as safe, and many phytochemicals are sold in high concentrations as dietary supplements with recommended intake levels that exceed normal daily intake up to a 100-fold. However, in addition to the beneficial properties, phytochemicals may also affect various enzyme activities. For example, Zhu and Liehr described the effect of quercetin, a phytochemical found in many food items, on COMT activity in male Syrian hamsters (Zhu and Liehr, 1996). In hamsters fed with quercetin, a decreased COMT activity was found that resulted in increased catechol estrogen concentrations in the kidneys and subsequent enhancement of estradiol-induced tumorigenesis.

In the present study, we investigated COMT activity in healthy mammary tissues, where COMT plays an important role in the inactivation potentially genotoxic catechol estrogens. We studied the constitutive rates of O-methylation of catechol estrogens and the effects of phytochemicals on this activity in healthy human mammary tissue cytosol. We hypothesized that phytochemicals with a catechol structure, like quercetin, catechin, and (-)-epicatechin (chemical structures in Fig. 1), make a suitable substrate for the COMT enzyme and thus potentially inhibit the formation of methoxy estrogens. We also investigated the effects of several phytochemicals without a catechol structure (genistein, chrysin, and flavone) on COMT activity. Ro 41-0960, a known selective COMT inhibitor, was used as a standard positive control. COMT inhibition results in decreased inactivation of catechol estrogens, which in turn may lead to increased DNA damage. Therefore, we studied the implications of decreased COMT activity caused by phytochemicals on catechol estrogen-induced DNA damage by performing the alkaline comet assay using the malignant human mammary tumor cell line MCF-7.

MATERIALS AND METHODS

Chemicals and reagents. S-adenosyl-L-methionine (SAM), dithiothreitol (DTT), 3,(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Ro 41-0960 (2′-fluoro-3,4-dihydroxy-5-nitrobenzophenone), quercetin, catechin, (-)-epicatechin, chrysin, genistein, and flavone were obtained from Sigma (St. Louis, MO). Estrogen standards were obtained from Steraloid Inc. (Newport, RI), and N,O-bis(trimethylsilyl)trifluoroacetamide/trimethylchlorosilane (BSTFA/TMCS) was purchased from Supelco (Bellefonte, PA).

Sample preparation. Tissues from reduction mammoplasty ($n = 7$) were obtained from the Antonius Hospital (Nieuwegein, the Netherlands). The study was approved (number TME/Z-02.09) by the medical ethical committee of the hospital. All women gave permission for the removed tissue to be used by an informed consent. Tissues were diagnosed as histologically normal breast tissue by a pathologist. Upon arrival in our laboratory, fresh tissues were snap frozen in liquid nitrogen and stored at −70°C until use. Before preparation of cytosolic fractions, the tissues were thawed at 4°C and kept on ice. Adipose tissue was removed with a surgical knife, and the remaining parenchyma was cut into small pieces. The tissue pieces were weighed, and 3 ml cold phosphate buffer (50 mM, pH 7.6 containing 0.1 mM EDTA) was added per mg tissue. This mixture was homogenized with a Potter-Elvehjem Teflon-glass homogenizer. Cytosolic fractions were prepared through ultracentrifugation (Beckman L7-55). Homogenates were first centrifuged at 10,000 × *g* for 15 min at 4°C to remove the cell debris and remaining adipose tissues. Subsequently, the supernatant was centrifuged at 100,000 × *g* for 75 min at 4°C to separate the cytosolic (supernatant) from the microsomal (pellet) fractions. Aliquots of the cytosolic fractions were stored at −70°C until analysis. Protein contents of the fractions were determined by the method of Lowry *et al*. (1951) using bovine serum albumin (BSA) as protein standard.

COMT activity. In order to study the O-methylation activity, cytosolic protein (300 µg) was incubated with 50 mM phosphate buffer (pH 7.6), 5 mM $MgCl_2$, 150 µM SAM, 1 mM DTT, and various concentrations of a phytochemical or the solvent vehicle (0.1% v/v MeOH) to a final volume of 492.5 µl. Reaction mixtures were incubated at 37°C for 5 min before the reaction was started by adding 2-OHE_2 and 4-OHE_2 (3.75 µM each). After 30 min, the reaction was stopped by putting the reaction tubes on ice. The metabolite extraction procedure was adapted from Spink *et al*. and performed as described previously (Spink *et al*., 1990; van Duursen *et al*., 2003). Briefly, the internal standard (20 µl equilin, 10 µM) was added, and 2- and 4-$MeOE_2$ were extracted with dichloromethane. Trimethylsilyl derivatives of the estrogens were prepared and analyzed by GC/MS. Peak areas were determined at m/z 446 and 340 for 2- and 4-$MeOE_2$ and equilin, respectively. Peak identification and quantification was performed with the corresponding standards.

Cell lines and cell culture. MCF-7 cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and cultured in Dulbecco's Modified Eagle's Medium supplemented with 0.01 mg/ml insulin, 5% fetal calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were cultured in a humidified atmosphere with 5% CO_2 at 37°C.

Cell viability. The cell viability was determined by measuring the capacity of the cells to reduce MTT (3,(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to the blue-colored formazan (Denizot and Lang, 1986). Cell cultures of MCF-7 were exposed to the catechol estrogens (7.5 µM 2- and 4-OHE_2), Ro 41-0960 (10 µM), or quercetin (30 µM) for 6 h. Then, serum-free medium containing 1 mg MTT/ml was added for 1 h. The medium containing MTT was then removed, and the cells were washed twice with warm PBS. Formazan was extracted by adding 1 ml isopropanol at room temperature. Formazan formation was measured spectrophotometrically at an absorbance wavelength of 560 nm, and cell viability was calculated using solvent vehicle-treated cells (ethanol, methanol, and DMSO, total of 0.17% v/v) as 100% viable control cells.

Alkaline single-cell gel electrophoresis (comet) assay. The effect of COMT inhibition on DNA damage caused by catechol estrogens was determined using the single-cell gel electrophoresis (comet) assay as described by Singh *et al*. with some modifications (Singh *et al*., 1988). For this assay, 5×10^5 MCF-7 cells were plated onto 12-well plates and placed in a humidified atmosphere with 5% CO_2 at 37°C. The next day, cells were exposed for 5 h to serum-free medium containing the solvent vehicles (ethanol, methanol, and DMSO, total of 0.17% v/v) and catechol estrogens (7.5 µM 2-OHE_2 and 4-OHE_2), Ro 41-0960 (10 µM),

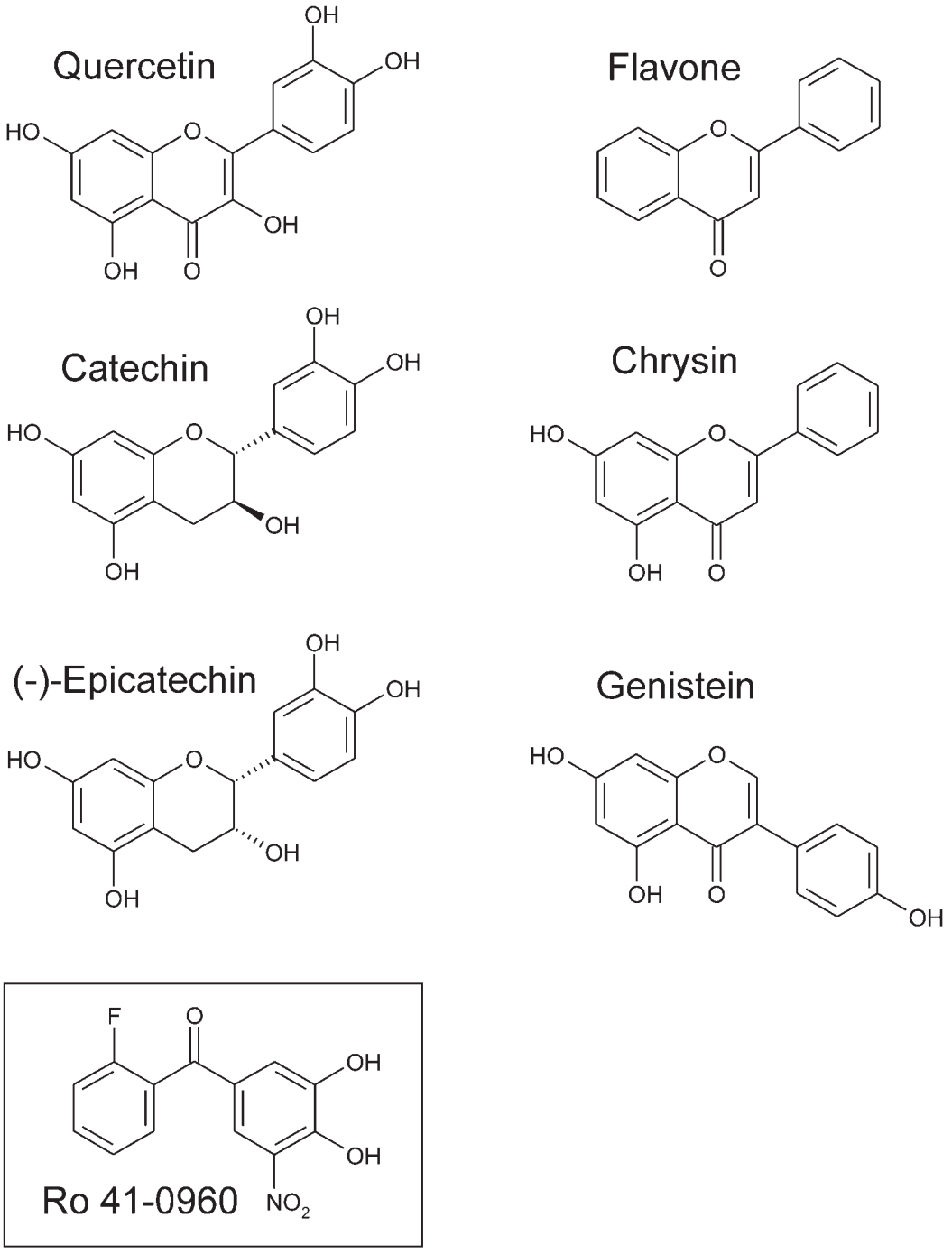

FIG. 1. Chemical structures of the compounds used in this study; phytochemicals with a catechol structure (quercetin, catechin, and (-)-epicatechin), without a catechol structure (flavone, chrysin, and genistein) and the known COMT inhibitor Ro 41-0960.

and quercetin (10 μM or 30 μM), alone or in combination. Then, media were removed and analyzed for methoxy estradiol concentrations as described above. The cells were washed with PBS, and 100 μl trypsin was added. As soon as the cells detached, 1 ml of warm medium containing 5% FBS was added, and the cells were suspended and transferred to a 1.5-ml Eppendorf cup. The cell suspension was briefly centrifuged, and 1000 μl of the supernatant was removed. The remaining cells were gently resuspended, and a 10 μl aliquot was added to 90 μl warm 0.5% low-melting agarose. This mixture was spread onto a frosted slide covered with 1.5% normal melting agarose and placed on a ice-cold glass plate to solidify. Then, the slides were placed in freshly prepared cold lysis solution (2.5 M NaCl, 0.1 M Na_2EDTA, 10 mM Tris containing 1% Triton-X, and 10% DMSO, pH 10) for 1 h at 4°C. After this, the slides were kept in the dark to prevent DNA damage by exposure to direct light. Subsequently, the slides were placed in a horizontal slide holder in an electrophoresis unit containing cold electrophoresis

solution (0.3 M NaOH, 0.001 M EDTA) for 25 min and then electrophoresed for 25 min at 25 V, 290–310 mA. Then, the slides were washed three times with a sterile neutralization buffer (0.4 M Tris/HCl, pH 7.5) and dehydrated for 10 min in 100% ethanol. The slides were kept in a dark box at 4°C until analysis. Prior to analysis, the slides were stained with ethidium bromide (20 μg/ml). Analysis was performed under a fluorescence microscope using a 20× objective and a filter of 450–490 nm equipped with a digital camera. Of each treatment 175–200 cells (four slides per treatment, 40–50 cells per slide) were analyzed and the tail moment (comet tail length × % tail DNA) was determined using the PC image-analysis program Casp described by Konca *et al*. (2003).

Data analysis. Enzyme kinetic parameters (V_{max} and K_m values in pmol/min/mg protein and μM, respectively) were calculated with Prism 3.0 (GraphPad Software, San Diego, CA). Statistical significance of difference of the mean was determined by the Student's *t*-test. Variance and differences among the means were determined by a one-way ANOVA with a Tukey-Kramer Multiple Comparisons test using GaphPad InStat 3.06 (GraphPad Software Inc., San Diego, CA).

RESULTS

Constitutive COMT activity

Enzyme kinetics were studied in samples 1 and 4 by determining the $MeOE_2$ formation at various equimolar concentrations of 2- and 4-OHE_2. The concentration of SAM, the methyl donor for O-methylation, in the incubation mixture was 150 μM, which was a saturating concentration (data not shown). After 30 min, $MeOE_2$ concentrations were determined. In both samples the catalytic efficiency of COMT was higher for 2-$MeOE_2$ formation than for 4-$MeOE_2$ formation. In sample 1, catalytic efficiencies (V_{max}/K_m) were 3.0/5.0 = 0.6 and 1.3/9.5 = 0.1 for 2- and 4-$MeOE_2$ formation, respectively. In sample 4, catalytic efficiencies were 16.2/9.4 = 1.7 and 4.2/19.7 = 0.2 for 2- and 4-$MeOE_2$ formation, respectively. In both samples, the rate of methoxy formation was linear up to a concentration of 25 μM for at least 45 min (data not shown). An incubation of 30 min with 7.5 μM catechol estrogens was chosen to study the constitutive COMT activity and the effects of phytochemicals.

The constitutive rates of methylation of catechol estrogens in the various tissue samples are shown in Table 1. The mean 2-$MeOE_2$ formation of seven tissues was 8.12 ± 1.32 pmol/min/mg protein with a range of activity of 2.14–19.03 pmol/min/mg protein. For 4-$MeOE_2$, the mean metabolite formation and range of activity were 1.83 ± 0.29 and 0.37–3.81 pmol/min/mg protein, respectively. ANOVA analysis showed significant differences in 2- and 4-$MeOE_2$ formation between the tissue samples ($p = 0.0004$ and $p = 0.0044$, respectively). Further analysis showed that cytosol from tissue sample 3 had substantially higher rates of methoxy estradiol formation compared with other tissue samples. However, despite the variation in rates of methylation, the ratio of 4- $MeOE_2$/2-$MeOE_2$ formation at 7.5 μM 2- and 4-OHE_2 was not significantly different statistically between the tissues (ANOVA analysis, $p = 0.125$). The average 4-/2-$MeOE_2$ ratio in seven tissue samples was 0.26 ± 0.02.

COMT Inhibition by Ro 41-0960

Because substantial interindividual variation in methylation rates was observed, a concentration-response curve was made with Ro 41-0960, a known COMT inhibitor, for every tissue sample. IC_{50} values for every tissue sample are listed in Table 1. Large variations between the tissue samples in responsiveness to Ro 41-0960 were observed. Sample 4 was the least responsive to Ro 41-0960, with an IC_{50} value for COMT inhibition of 42.1 nM, and this IC_{50} value was significantly different from samples 3 and 7. Although COMT has a higher constitutive activity with 2-OHE_2 than with 4-OHE_2 as substrate, inhibition of methylation occurred in the same order of magnitude for both 2- and 4-$MeOE_2$ formation (Fig. 2).

TABLE 1
2- and 4-$MeOE_2$ Formation, 4-$MeOE_2$/2-$MeOE_2$ Ratio, and IC_{50} Values for Ro 41-0960 Inhibition in Mammary Tissue

	2-$MeOE_2$ formation (pmol/min/mg protein)	4-$MeOE_2$ formation (pmol/min/mg protein)	4-$MeOE_2$/2-$MeOE_2$	IC_{50} (nM) for Ro 41-0960
Sample 1	5.38 ± 1.45[a]	1.13 ± 0.14	0.21 ± 0.06[b]	33.6 ± 5.8[c]
Sample 2	2.17 ± 0.31	0.37 ± 0.05	0.17 ± 0.03	11.4 ± 5.0
Sample 3	19.03 ± 4.81[d]	3.81 ± 1.21[e]	0.20 ± 0.08	5.1 ± 2.7
Sample 4	5.38 ± 2.35	1.16 ± 0.24	0.22 ± 0.10	42.1 ± 14.5[f]
Sample 5	2.14 ± 0.47	0.67 ± 0.11	0.30 ± 0.02	27.5 ± 9.8
Sample 6	9.10 ± 1.34	2.31 ± 0.30	0.27 ± 0.03	8.6 ± 1.5
Sample 7	9.36 ± 1.30	2.53 ± 0.41	0.27 ± 0.04	5.8 ± 2.0

Note: After incubation of cytosol with an equimolar concentration of 7.5 μM 2- and 4-OHE_2.
[a]Mean ± standard error of the mean of 4 to 7 determinations.
[b]4-$MeOE_2$ formation (pmol/min/mg protein) divided by 2-$MeOE_2$ formation (pmol/min/mg protein).
[c]Mean IC_{50} value (nM) of two duplicate determinations ± SEM.
[d]Significantly different from sample 1 and 4 ($p < 0.05$) and sample 2 and 5 ($p < 0.01$).
[e]Significantly different from sample 2 and 5 ($p < 0.05$).
[f]Significantly different from sample 3 and 7 ($p < 0.05$).

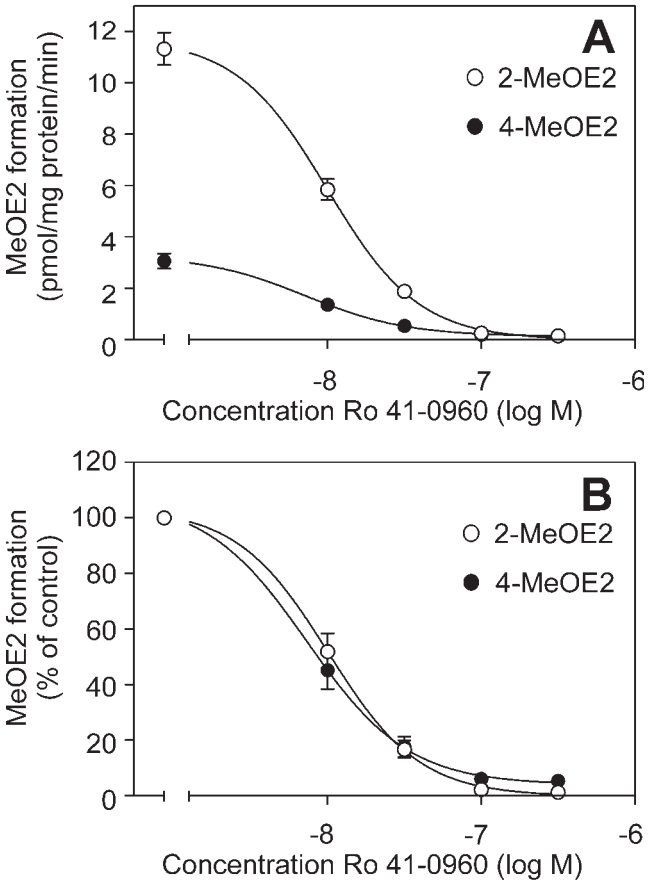

FIG. 2. Inhibition of COMT by Ro 41-0960 in tissue sample 6. COMT activity is represented (A) as absolute $MeOE_2$ formation (pmol/mg protein/min) and (B) as percentage of control activity. Cytosolic fractions from human mammary tissue sample 6 were incubated with an equimolar concentration of 7.5 μM 2- and 4-OHE_2. After 30 min, methoxy estradiols were extracted and analyzed by GC/MS. Data are represented as mean and range of two determinations.

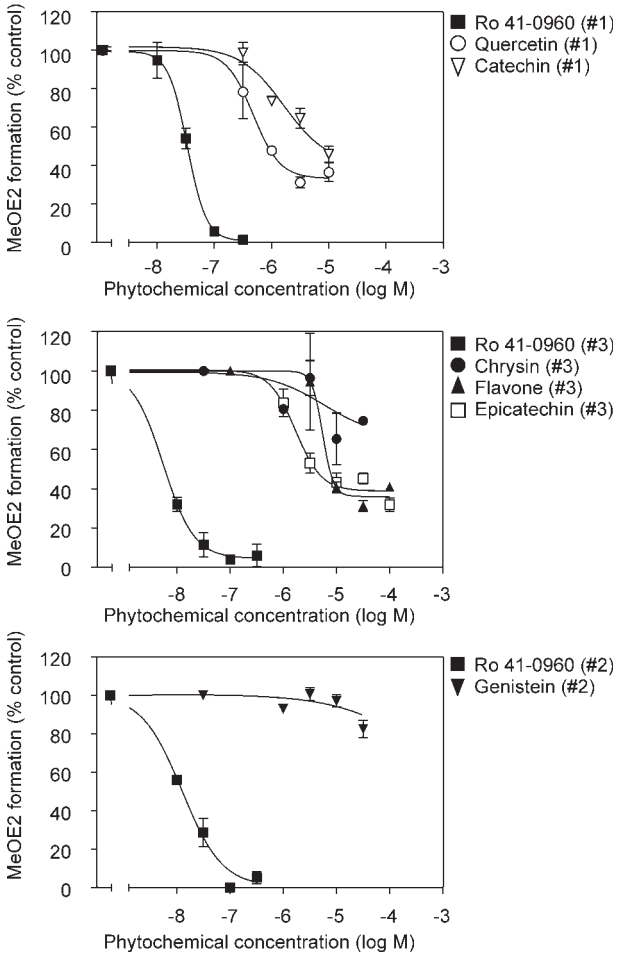

FIG. 3. Effect of various phytochemicals on COMT activity in cytosolic fractions from human mammary tissues. Cytosolic fractions were incubated with an equimolar concentration of 7.5 μM 2- and 4-OHE_2. After 30 min, methoxy estradiols were extracted and analyzed by GC/MS. Data are represented as means and range of two duplicate determinations. The numbers in the legend refer to the tissue sample numbers. Open and closed symbols are phytochemicals with and without a catechol structure, respectively.

Effects of Phytochemicals on COMT Activity

The *O*-methylation of 7.5 μM 2- and 4-OHE_2 in cytosolic fractions of human mammary tissues was concentration-dependently inhibited by quercetin, catechin, and epicatechin, as was expected based on their catechol structure (Fig. 3). While Ro 41-0960 fully inhibited COMT activity at the highest concentration tested (0.3 μM) in all tissue samples, the phytochemicals decreased COMT activity to about 30–40% of the control activity at the highest concentrations tested (10–100 μM). IC_{50} values for COMT inhibition were 0.48 μM (tissue #1), 1.64 (tissue #1) and 1.96 μM (tissue #3) for quercetin, catechin, and epicatechin, respectively (Table 2). Phytochemicals without catechol structure were less potent COMT inhibitors. Genistein and chrysin appeared to reduce COMT activity slightly at the highest concentration tested (30 μM). Flavone, on the other hand, decreased COMT activity in tissue sample 3 to 40% of the control levels at the highest concentration tested (100 μM) with an IC_{50} value of 5.5 μM. ANOVA analysis showed that only the differences in IC_{50} values for quercetin and flavone were statistically significant ($p < 0.05$). However, in tissue sample 5, which was less responsive to COMT inhibition by Ro 41-0960, no inhibitory effect on COMT activity could be detected (data not shown).

Because of the large variations among the tissue samples in responsiveness to COMT inhibition, the potencies of the phytochemicals to inhibit COMT activity were calculated relative to the inhibitory potency of Ro 41-0960 in the same tissue sample (Table 2). For quercetin, catechin, epicatechin, and flavone the relative potencies were 0.07 (tissue #1), 0.021 (tissue #1), 0.0026 (tissue #3), and 0.0009 (tissue #3), respectively.

Catechol Estrogen-Induced DNA Damage in MCF-7 Cells

To investigate the implications of possible COMT inhibition by quercetin in whole cells, the effects of quercetin on catechol

TABLE 2
IC_{50} Values and Relative Potencies (RP) for COMT Inhibition by Phytochemicals in Human Mammary Tissue Cytosol

	QUE	CAT	GEN	EPI	CHR	FLA
Tissue sample number	1	1	2	3	3	3
IC_{50} (μM) phytochemical[a]	0.48 ± 0.19*	1.64 ± 0.84	NA	1.96 ± 0.44	NA	5.49 ± 1.92
IC_{50} (nM) Ro 41-0960	33.6 ± 5.8	33.6 ± 5.8	11.4 ± 5.0	5.1 ± 2.7	5.1 ± 2.7	5.1 ± 2.7
RP (Ro 41-0960 = 1)[b]	0.070	0.021	NA	0.0026	NA	0.0009

Note: Quercetin (QUE), catechin (CAT), epicatechin (EPI), chrysin (CHR), genistein (GEN) and flavone (FLA).
[a]Mean IC_{50} value of two duplicate determinations ± SEM.
[b]RPs are calculated by dividing the IC_{50} value for COMT inhibition by Ro 41-0960 by the IC_{50} value of the phytochemical in cytosol from the corresponding tissue sample.
*Significantly different from IC50 value for flavone.

estrogen-induced DNA damage were studied. MCF-7 cells were exposed to catechol estrogens with or without Ro 41-0960 and quercetin. Then the amount of DNA damage was determined using the comet assay, and methoxy estrogen levels were determined in the culture medium by GC/MS analysis.

An equimolar concentration of catechol estrogens (7.5 μM), catechol estrogens together with quercetin (30 μM) or Ro 41-0960 (10 μM), or a combination of the two COMT inhibitors, did not cause cytotoxicity in MCF-7 cells, as determined by the MTT test (data not shown). Incubation of MCF-7 cells with catechol estrogens, Ro 41-0960, or quercetin alone did not cause a significant increase of DNA damage compared with the solvent vehicle-treated cells (Fig. 4). The extent of background DNA damage was significantly increased (by about 200%) in catechol estrogen-exposed cells when COMT was inhibited by 10 μM Ro 41-0960 ($p < 0.05$). When quercetin was added to the cells together with Ro 41-0960, catechol estrogen-induced DNA damage increased even further, in an apparent concentration-dependent manner, compared with Ro 41-0960 treated cells. A concentration-dependent increase of catechol estrogen-induced DNA damage was also seen after incubation with catechol estrogens and quercetin alone. Catechol estrogen-induced DNA damage levels increased 76 and 160% when cells were co-incubated with 10 and 30 μM quercetin, respectively, compared with vehicle-treated control cells.

To study whether the increase of catechol estrogen-induced DNA damage could be attributed to decreased COMT activity, methoxy estrogen levels were determined in the culture media of the MCF-7 cells used for the comet assay. Constitutive methoxy estradiol formation in MCF-7 cells was 148.1 ± 5.4 and 85.5 ± 4.9 pmol/h/10^6 cells for 2-$MeOE_2$ and 4-$MeOE_2$, respectively (Fig. 5). Incubation with 10 μM Ro 41-0960 inhibited methoxy estradiol formation by about 98% compared with control cells. Incubation with both Ro 41-0960 and quercetin did not significantly change methoxy estradiol formation compared with Ro 41-0960 alone (data not shown). In culture media of MCF-7 cells incubated with catechol estrogens and quercetin, a concentration-dependent decrease in methoxy estradiol formation compared with control cells was seen. Incubation

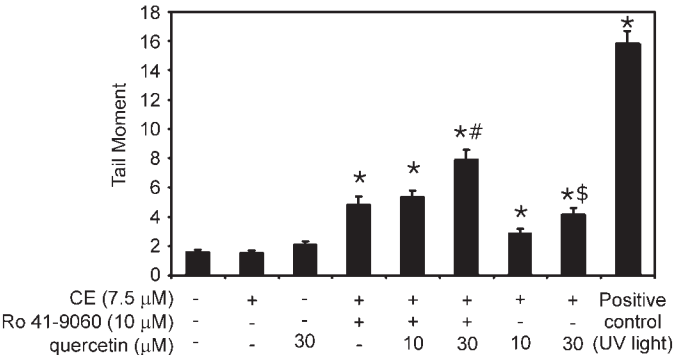

FIG. 4. Catechol estrogen-induced DNA damage in MCF-7 cells and the effect of COMT inhibition. MCF-7 cells were incubated with 7.5 μM catechol estrogens (CE), 10 μM Ro 41-0960, and/or quercetin (10 or 30 μM). Exposure of the cells to UV light (1 min) was used as a positive control. The experiment were performed twice in duplicate, and 40–50 comets per slide were analyzed. Data are represented as mean Tail Moment (comet tail length × % of total DNA in the comet tail) of four slides ± SEM. * Significantly different from vehicle control-treated and CE-treated cells ($p < 0.05$). # Significantly different from CE + Ro 41-0960 and CE + Ro 41-0960 + quercetin (10 μM)-treated cells ($p < 0.01$). $ Significantly different from CE + quercetin (10 μM)-treated cells ($p < 0.01$).

with 10 μM quercetin resulted in a 46% and 18% decrease in 2- and 4-MeOE2 formation, respectively. For 30 μM the decrease was 82% and 73% for 2- and 4-MeOE2 formation, respectively.

These data indicate that the increase in catechol estrogen-induced DNA damage in MCF-7 cells shown in the comet assay was due to COMT inhibition resulting in a decreased inactivation of the catechol estrogens, as shown by decreased methoxy estradiol formation.

DISCUSSION

Constitutive COMT Activity and Inhibition by Ro 41-0960

In this study we compared constitutive COMT activities in seven healthy human mammary tissue samples. In line with other studies, we found a higher catalytic activity of COMT

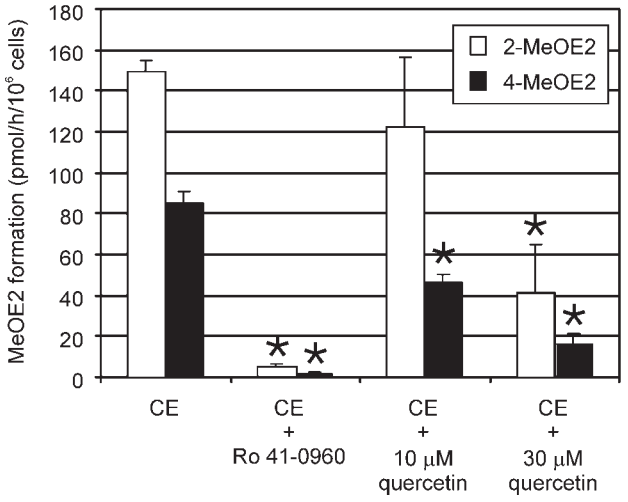

FIG. 5. Methoxy estradiol formation (pmol/h/10^6 cells) in MCF-7 cells after incubation with 7.5 µM catechol estrogens (CE) or co-incubation with CE and Ro 41-0960 (10 µM) or quercetin (10 or 30 µM). Data are represented as means ± SD (n = 3). * Significantly lower than CE-treated cells.

for 2-$MeOE_2$ formation than for 4-$MeOE_2$ formation (Goodman *et al*., 2002; Männistö and Kaakkola, 1999). As expected for human tissues, there was a large interindividual variation in constitutive COMT activity. One other study found the rates of 2-$MeOE_2$ formation by COMT in healthy human breast tissues (n = 12) to be 6.65 pmol 2-$MeOE_2$/min/mg protein (range of 0.8–14.6 pmol 2-$MeOE_2$/min/mg protein) (Hoffman *et al*., 1979). These values are in good agreement with the rates of methoxy formation that we found in our study, with a mean 2-$MeOE_2$ formation of 8.3 ± 2.0 pmol/min/mg protein and a range of activity of 2.1–19.0 pmol/min/mg protein. In addition to a large variation in constitutive activity, a large variation in interindividual responsiveness to the selective inhibitor Ro 41-0960 was observed. Generally, in samples with a high constitutive COMT activity, the activity was most readily inhibited by Ro 41-0960. This might be caused by difference in cytosolic expression of COMT. Although we corrected for protein content of the cytosolic fraction, we did not determine the expression level of the COMT enzyme. Another possibility for the large variation in activity might be a genetic conformational difference between the COMT enzymes. It has been reported that a genetic polymorphism of COMT (Val108Met) can result in a high- and a low-activity isoform of the enzyme (Lachman *et al*., 1996). However, several studies have described reduced activity in the low-activity isoform of COMT that is mainly caused by a change in thermostability of the enzyme and not as much by a change in catalytic activity of the enzyme (Goodman *et al*., 2002; Lachman *et al*., 1996; Männistö and Kaakkola, 1999).

Effect of Phytochemicals on COMT Activity

In this study we showed that phytochemicals with a catechol structure are capable of inhibiting COMT activity in cytosolic fractions of healthy human mammary tissues. Zhu and Liehr reported that quercetin acted as inhibitor of COMT activity in hamster kidney cytosol, with IC_{50} values of 8 µM and 2 µM at substrate concentrations of 10 µM 2-OHE_2 and 4-OHE_2, respectively (Zhu and Liehr, 1996). We found in this study lower IC_{50} values for COMT inhibition with quercetin (0.5 µM for both 2-OHE_2 and 4-OHE_2). This is probably due to differences in study design; we added both 2- and 4-OHE_2 together to the cytosol, and we used human COMT. Zhu and Liehr concluded that quercetin acted as a noncompetitive inhibitor of COMT activity by competing for SAM. Quercetin and other phytochemicals containing a catechol structure have shown to be a substrate for COMT and, thus, compete for cofactors necessary for *O*-methylation of the substrate, such as SAM (Zhu *et al*., 2000). Upon *O*-methylation, a methyl group from SAM is transferred to the catechol substrate resulting in S-adenosyl-L-homocysteine (SAH). An increasing concentration of SAH was shown to (non-competitively) inhibit the association of the methyl donor SAM with COMT. This might also explain why phytochemicals reduced COMT activity to 60% of the control activity, while Ro 41-0960 fully inhibited methylation. Ro 41-0960 is a poor substrate for COMT, but it binds tightly to the catalytic site of the enzyme, thus inhibiting methylation of other substrates without depletion of cofactors (Backstop *et al*., 1989; Ding *et al*., 1996).

The tested phytochemicals with a catechol structure, quercetin, catechin, and (-)-epicatechin, all reduced COMT activity, but large differences in inhibitory potency were found. This might be a result of structural differences of these phytochemicals, but interindividual variations among the tissue samples in COMT activity and responsiveness toward inhibition might also play a role. Interindividual variation between the tissue samples was especially apparent with flavone, which showed COMT inhibition in sample 3, but not in sample 5. In an attempt to correct for interindividual variations in sensitivity toward COMT inhibition, we calculated the potency of a phytochemical relative to the potency of Ro 41-0960 to inhibit COMT activity in the same tissue sample. The relative potencies (RPs) varied less than a 100-fold with the RPs of the three phytochemicals with a catechol structure (e.g. quercetin, catechin, and epicatechin) being higher than the RP of flavone, the phytochemical without a catechol structure. However, we did not study the potencies of all phytochemicals in all the tissue samples. As a result, it is not clear whether the RPs represent the differences between individuals or differences between the potencies of the phytochemicals. Therefore, the calculated inhibition potencies of the phytochemicals, both absolute and relative to Ro 41-0960, should be considered with care.

Catechol Estrogen-Induced DNA Damage in MCF-7 Cells

Although COMT activity was inhibited by the phytochemicals in cytosol from healthy mammary tissues, the question was raised if this inhibition is relevant in a more complex system such as whole cells. We showed that incubation with Ro 41-0960 or quercetin caused a significant increase in DNA damage by

catechol estrogens compared with catechol estrogens alone. Chen *et al*. also showed by the comet assay a low potency of another catechol estrogen, 4-hydroxyestrone (4-OHE_1), to induce DNA damage in MCF-7 cells (Chen *et al*., 2000). They mainly attributed this low potency to the fact that 4-OHE_1 does not auto-oxidize and requires oxidative enzymes to generate the highly reactive quinone. However, our study suggests that the inactivation of catechol estrogens plays an important role in the potential of these compounds to cause DNA damage. We showed that a decrease in inactivation of the potentially genotoxic catechol estrogens by COMT inhibition caused a significant increase in catechol estrogen-induced DNA damage. Our data concur with the results described by Lavigne *et al*. (2001). They found a clear association between catechol estrogen levels and 8-oxo-dG levels in MCF-7 cells after treatment with estradiol and the COMT inhibitor Ro 41-0960. These data show that catechol estrogens have the potential to induce DNA damage, but that this is strongly dependent on the cellular capacity for inactivation by COMT.

Implications for Breast Cancer Development

Phytochemicals are often studied in relation with hormone-dependent cancers such as breast cancer. The low breast cancer incidence in Asian countries is often attributed to the soy-rich diet, which contains high concentrations of isoflavones like genistein. On the other hand, some studies describe a deleterious effect of certain phytochemicals in women with breast cancer (Lesperance *et al*., 2002; Tagliaferri, 2001). Our study shows that phytochemicals with a catechol structure have the potential to reduce COMT activity in cytosol of healthy mammary tissues at concentrations which are well within the range of plasma levels that are reached by regular daily intake. We found for quercetin an IC_{50} value of 0.5 μM for COMT inhibition. Hollman and Katan found plasma levels up to 0.74 μM quercetin after consumption of a meal rich in plant products (Hollman and Katan, 1999a). It is not unlikely that higher levels can be reached, since quercetin plasma levels return to basal levels after about 20 h, so repeated consumption of high levels of quercetin can result in accumulation in the blood. Furthermore, the COMT-inhibiting properties of quercetin also resulted in decreased inactivation of potentially genotoxic catechol estrogens and an increase in catechol estrogen-induced DNA damage. Yet, it is difficult to predict the effects of excessive phytochemical intake in individuals, as large variations in COMT activity and responses to COMT inhibition between the various breast tissue samples were found. Nevertheless, this study shows that adverse effects of high levels of certain phytochemicals are not unlikely. Therefore, high intake of phytochemicals, for example through dietary supplements, should be considered with care.

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