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Quercetin and resveratrol potently reduce estrogen sulfotransferase activity in normal human mammary epithelial cells

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Abstract

Estrogen sulfotransferase (EST) is the sole sulfotransferase expressed in normal human breast epithelial cells and has an important function in determining free estrogen hormone levels in these cells. In the present study we examined the inhibitory effect of the dietary polyphenols quercetin and resveratrol on EST activity, i.e. 17β-estradiol (E2) sulfation. Both the compounds potently inhibited recombinant human EST in a competitive fashion with K_i values of about 1 μM. In fact, both polyphenols could serve as substrates for EST. In order to extend the studies to more physiologically relevant conditions, we examined whether inhibition of EST also occurred in the intact cultured human mammary epithelial (HME) cells. The mean baseline EST activity (E2 sulfate formation) in the HME cells was 4.4 pmol/h per mg protein. The IC_{50} for resveratrol was very similar to that for recombinant EST, i.e. about 1 μM. Surprisingly, quercetin was 10 times more potent in the HME cells with an IC_{50} of about 0.1 μM, a concentration that should be possible to achieve from the normal dietary content of this flavonoid.

Keywords: hEST; Estrogen sulfotransferase; Human mammary epithelial cells; Quercetin; Resveratrol; Polyphenols

1. Introduction

Estrogen hormones are important for the growth and development of normal human breast tissue. The presence of estrogen, however, is also a requirement for the growth and development of most breast cancers. Although the major proportion of active estrogen in premenopausal women is produced by the ovaries, and thus requires transport to the breast tissue, an increasing proportion is synthesized in peripheral tissues, including the breast [1,2], in postmenopausal women. Biological processes that affect the intracellular availability of estrogen hormones to their receptors will, therefore, influence the progression of breast cancer. This has led to the development of multiple strategies to decrease the cellular exposure and response to estrogen hormone. Two therapeutically accepted approaches to accomplish this are inhibition of estrogen action by antiestrogens, which interact with estrogen receptors [3], and inhibition of estrogen production by inhibitors of aromatase, the enzyme responsible for estrogen synthesis [4].

The intracellular levels of estrogen can also be affected by conjugating enzymes, e.g. UDP-glucuronosyl transferase [5], and in particular estrogen sulfotransferase (EST), an enzyme highly specific for 17β-estradiol (E2) and estrone [6,7]. Interestingly, EST has been demonstrated to be highly expressed in the normal human mammary epithelial cells, but with very low or no expression in breast cancer cells [6,7]. A high EST expression, which can be stimulated by progesterone [8], may result in diminished estrogen hormone levels and a protective effect [6–8]. The resulting estrogen sulfates can, however, be hydrolyzed by estrogen sulfatase [2,9]. A recent study indicated that dietary flavonoids, including quercetin, may inhibit estrogen sulfatase, suggesting a protective effect of these dietary polyphenols [10].

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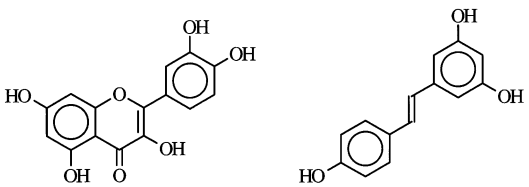

Fig. 1. Chemical structures of the dietary polyphenols quercetin and resveratrol.

Previous studies have demonstrated that flavonoids can be potent inhibitors of a human sulfotransferase, P-PST [11,12], which can sulfonate high concentrations of estrogen hormones [13]. In the present study we demonstrate very potent inhibition of EST by the polyphenols quercetin and resveratrol (Fig. 1), using both recombinant EST, as well as cultured normal human mammary epithelial (HME) cells.

2. Materials and methods

2.1. Materials

E2, quercetin, quercitrin, and resveratrol were purchased from Sigma (St. Louis, MO). [^{3}H]Estradiol ([^{3}H]E2) (48 Ci/mmol) and [^{35}S]-labeled 3′-phosphoadenosine-5′-phosphosulfate (PAPS) (1.0–1.5 Ci/mmol) were purchased from DuPont-New England Nuclear (Wilmington, DE). Ultrapure PAPS was purchased from Dr S.S. Singer (University of Dayton, Dayton, OH). Quercetin 4′-monoglucoside was purified from red onions as previously described [14].

2.2. Isolation of recombinant EST

EST cDNA subcloned into the pKK233-2 bacterial vector and expressed in *Escherichia coli* XL1-Blue strain was donated by Dr C.N. Falany (University of Alabama at Birmingham). Recombinant human EST was isolated and purified from EST/pKK233-2 XL1-Blue cultures after induction with 1 mM isopropyl-β-D-thiogalactopyranoside for 5 h at 37°C, as previously described [15] with several modifications [16].

2.3. In vitro incubations

E2 sulfation was assayed as previously described [15] using a chloroform extraction procedure (pH 8) [17]. The reaction mixtures containing 0.01 μl human recombinant EST, 20 nM [^{3}H]E2 and 25 μM PAPS in Tris–HCl buffer, pH 7.4, with 8 mM dithiothreitol and 0.0625% BSA in a final volume of 200 μl, were incubated at 37°C for 30 min. Reactions were performed in the presence and absence of the inhibitors quercetin, quercetin 4′-monoglucoside, quercitrin, and resveratrol (0.1–100 μM). The reactions were terminated by adding 250 μl 0.25 M Tris–HCl buffer, pH 8.7, and 3 ml chloroform. After mixing and centrifugation, aliquots of the aqueous phase were subjected to liquid scintillation counting. To determine the mode of EST inhibition by quercetin and resveratrol, a range of [^{3}H]E2 concentrations (5–50 nM), as well as inhibitor concentrations (0.5–2 μM) were used.

2.4. Culturing of human mammary epithelial (HME) cells

Primary human mammary epithelial (HME) cells, at passage 7, were obtained from Clonetics (San Diego, CA). These cells were derived from a 22-year-old healthy woman who had undergone reduction mammoplasty. Cell cultures were maintained as recommended by Clonetics [18]. Serum-free mammary epithelial growth medium was supplemented with the following (final concentrations) bovine pituitary extract (52 μg/ml), human recombinant epidermal growth factor (10 ng/ml), insulin (5 μg/ml), hydrocortisone (0.5 μg/ml), gentamicin (50 μg/ml), and amphotericin B (50 ng/ml). Experiments were conducted with cells at passage 9–11.

2.5. In situ HME cell incubations

For studies of E2 sulfation in intact HME cells, cells were seeded in 6-well plates and allowed to grow to confluency (8–10 days) [18]. The cells were then incubated for 1 h at 37°C with 20 nM [^{3}H]E2 in 1 ml of a HEPES-buffered balanced salt solution (HBSS) of the following composition: 10 mM glucose, 20 mM HEPES, 1.2 mM Na_2HPO_4, 1.2 mM $MgSO_4$, 145 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, and NaOH to a pH of 7.4. Incubations were performed in the presence and absence of the inhibitors quercetin and resveratrol (0–10 μM). 100 μl aliquots of the HBSS incubation buffer were sampled at various times and E2 sulfate formation was assayed as previously described [15] using the alkaline-chloroform extraction procedure [17].

2.6. Sulfation of quercetin and resveratrol

EST-catalyzed sulfation of quercetin and resveratrol was determined using the previously described ion-pair extraction method [19]. The typical reaction mixture contained 0.1–10 μM of the polyphenol substrate, 1 μM [^{35}S]PAPS and 0.1 μl of recombinant EST in 33 mM Tris–HCl buffer, pH 7.4, with 8 mM dithiothreitol and 0.0625% BSA in a total volume of 100 μl. The samples were incubated for 30 min at 37°C and the reactions were terminated by the addition of 10 μl 2.5% acetic acid, 20 μl of 0.1 M tetrabutylammonium hydrogen sulfate and 500 μl ethyl acetate. After mixing and

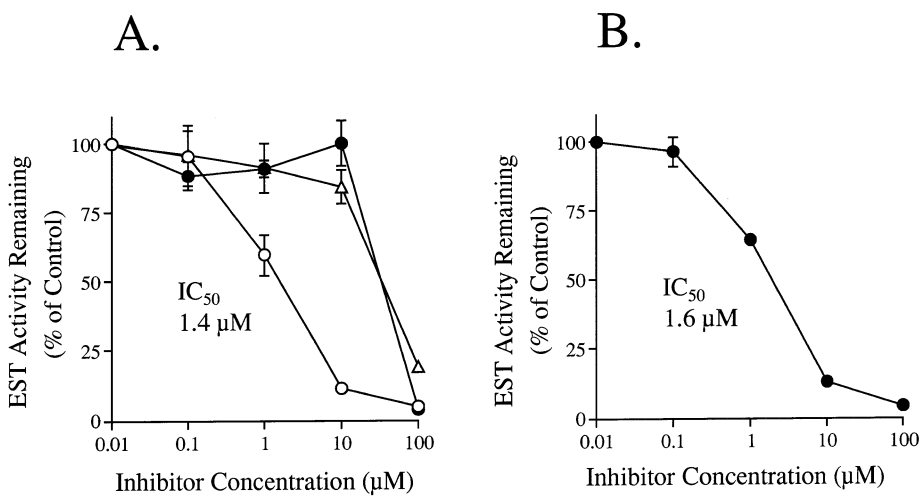

Fig. 2. Inhibition of E2 sulfation by (A) quercetin (○), quercetin 4′-monoglucoside (●) and quercitrin (△) and (B) resveratrol, using recombinant human EST. Experiments are mean ± S.E.M. ($N = 3$). For some points the error bars are smaller than the symbols.

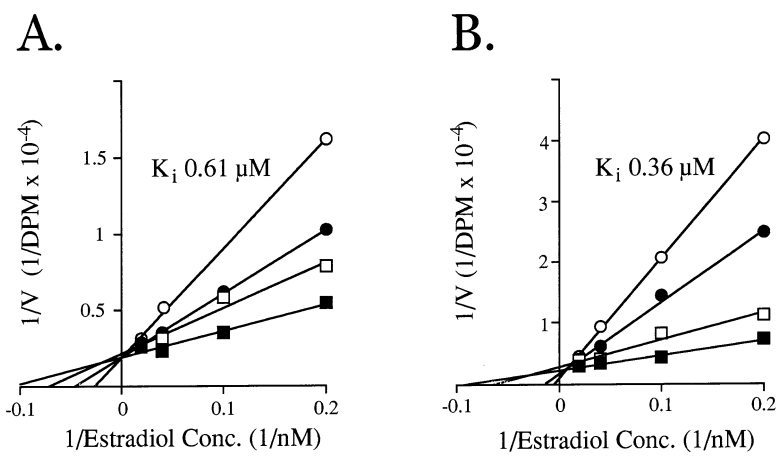

Fig. 3. Inhibition of E2 sulfation by (A) quercetin and (B) resveratrol, using recombinant EST. In these double reciprocal plots of velocity versus substrate concentration the inhibitor concentrations used were 0 (■), 0.5 (□), 1 (●) and 2 (○) μM. The E2 concentrations used were 5–50 nM. The data represent mean values of two experiments.

centrifugation, 400 μl of the ethyl acetate extract was subjected to liquid scintillation counting.

2.7. Data analysis

The K_m values were derived with UltraFit (Biosoft, Cambridge, UK) from V versus S plots, using the Michaelis-Menten equation [20]. The IC_{50} values for the concentration-activity curves from individual experiments were derived with UltraFit, using an equation for double exponential decay with offset. The mode of quercetin and resveratrol inhibition of E2 sulfation by recombinant EST and its K_i values were derived graphically from Lineweaver–Burk plots (1/V vs. 1/S) and replots of the slope versus inhibitor concentration [21].

3. Results

The recombinant human EST was isolated as described [15] and characterized catalytically with its main natural substrate E2. The formation of E2 sulfate had an apparent K_m value of 21 nM. Saturating concentrations of the co-substrate PAPS were reached at 10 μM.

The sulfation of E2 by recombinant EST was inhibited potently by quercetin, yielding an IC_{50} value of 1.4 μM. The naturally occurring glycosides of quercetin, i.e. quercetin 4′-glucoside and quercitrin (quercetin 3-rhamnoside) were considerably less potent, with estimated IC_{50} values of about 30 μM, Fig. 2A. Resveratrol had a potency very similar to that of quercetin with an IC_{50} value of 1.6 μM, Fig. 2B.

To determine the mode of inhibition, varying concentrations of quercetin and resveratrol were used (0.5–2 μM) together with varying concentrations of the substrate E2 (5–50 nM). As shown in Fig. 3A and B, both quercetin and resveratrol appeared to be competitive inhibitors of E2 sulfation. The K_i values, although somewhat lower than the IC_{50} values in Fig. 2, were very similar for quercetin and resveratrol, 0.58 and 0.36 μM, respectively.

The finding that the inhibition was competitive suggested that both quercetin and resveratrol are substrates for the human EST. This was tested using an ion-pair extraction method [19], particularly suitable for labile sulfate conjugates. The data shown in Fig. 4A and B clearly demonstrated that both quercetin and resveratrol are sulfated, with apparent K_m values of 0.32 and 0.53 μM, respectively, i.e. very similar to their K_i values for inhibition of E2.

To determine the potential physiological significance of EST inhibition by quercetin and resveratrol, experiments were carried out in cultured intact human mammary epithelial (HME) cells. These cells have previously been shown to express high EST activity [6,18]. The mean baseline EST activity of the HME cells in these experiments was 4.4 pmol of E2 sulfate formed per hr and mg protein, using 20 nM E2. Surprisingly, the quercetin inhibition of E2 sulfation in the intact HME cells was more potent than by recombinant EST, yielding an IC_{50} value as low as 0.13 μM, Fig. 5A. Complete inhibition occurred at 1 μM quercetin. For resveratrol, the inhibition was similar to that observed with recombinant EST, resulting in an IC_{50} value of 1.3 μM, Fig. 5B.

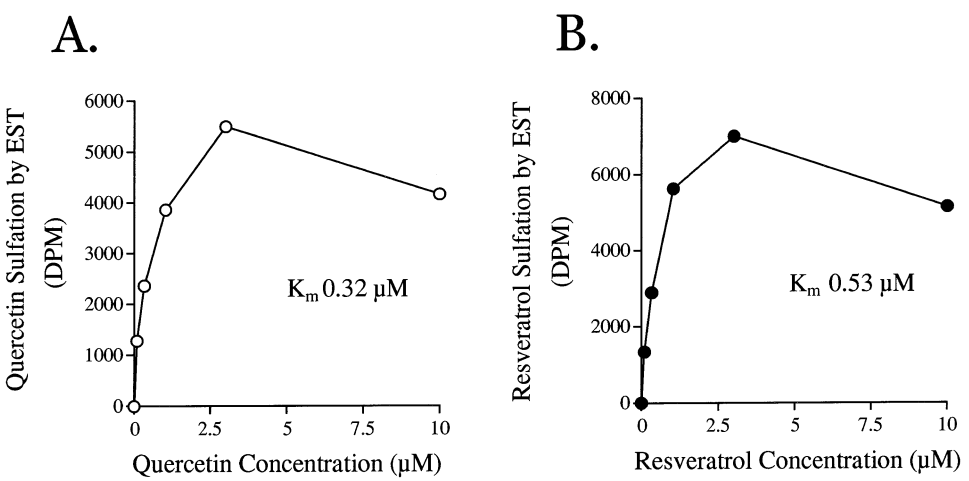

Fig. 4. EST-catalyzed sulfation of (A) quercetin and (B) resveratrol. The data represent mean values of two determinations.

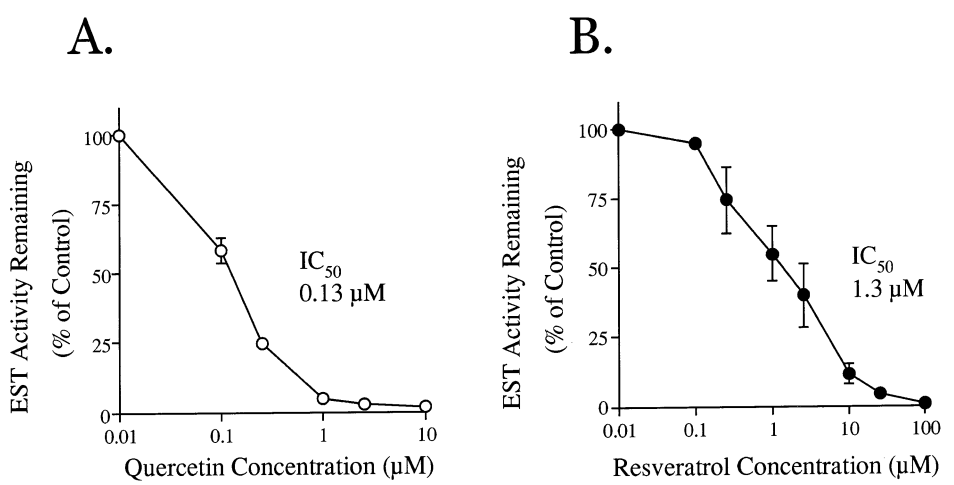

Fig. 5. Inhibition of E2 sulfation by (A) quercetin and (B) resveratrol in the cultured intact HME cells. The cells were incubated for 3 h with [^{3}H]E2 in the absence and presence of varying concentrations of inhibitor. [^{3}H]E2 sulfate was then measured in the medium. The data shown represent mean ± S.E.M. of three experiments. For some points the error bars are smaller than the symbols.

4. Discussion

Quercetin and resveratrol demonstrated very similar inhibition of the catalytic activity of the recombinant human EST with an IC_{50} value of about 1 μM, whereas two of the naturally occurring glycosides of quercetin were about 30 times less potent. Interestingly, the inhibition by both quercetin and resveratrol appeared to be simply a competitive type of interaction. Therefore, as expected, both of these dietary components were substrates for EST, with K_m values similar to their K_i values for inhibition of E2 sulfation. This finding is interesting in that P-PST, the most ubiquitous of the human STs, responsible for the sulfation of most foreign phenolic compounds [22] and highly related to EST [15], does not appear to use quercetin as a substrate [12].

Quercetin has previously been shown to also inhibit the catalytic activity of P-PST with an IC_{50} value as low as 0.1 μM [11,12]. This inhibition was noncompetitive in nature. In the intact human hepatoma cell line Hep G2, which has P-PST expression similar to the human liver [23], the potency of quercetin inhibition of P-PST decreased about 25-fold, yielding an IC_{50} value of 2.5 μM [12]. This was speculated to be due to a combination of factors, including poor plasma membrane penetrability as well as extensive metabolism of quercetin. This was thought to be consistent with the generally held view that flavonoids have a low cellular availability.

The very potent inhibitory effect of quercetin on the sulfation of estradiol by EST in the intact HME cells is therefore most surprising. Thus, quercetin is 25 times more potent inhibiting the EST activity in these cells than inhibiting the P-PST activity in Hep G2 cells, even though quercetin is 10 times less potent inhibiting recombinant EST than P-PST. The mechanism for this potent inhibition is unclear. It could involve (1) a mechanism concentrating quercetin inside the breast cell, (2) bioactivation to a more potent form, e.g. by *O*-methylation [24,25], (3) inhibition of synthesis of the cofactor PAPS, or perhaps most likely, (4) inhibition of some signaling pathway important for the regulation of EST expression. The IC_{50} of 0.1 μM corresponds to a quercetin concentration of about 30 ng/ml, which is five to ten times lower than concentrations in plasma reported in humans after consuming common foodstuffs rich in quercetin, such as onions and apples [26]. Even when taking into account the high plasma binding of quercetin [27], this potent effect on EST function in the breast cell may be relevant in humans.

Even though inhibition of EST by quercetin, resulting in elevated estrogen hormone levels in the normal breast cell, may be a potentially harmful effect, other considerations may be of importance. In a recent study, it was demonstrated, also in the HME cells, that EST could catalyze the bioactivation of the cooked-food mutagen and procarcinogen *N*-hydroxy-2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (N-OH-PhIP) and its subsequent binding to genomic DNA [18]. This potential breast cancer initiating reaction was highly correlated to EST expression. Thus, inhibiting EST with quercetin and other dietary flavonoids could serve as a protective mechanism in breast cancer initiation. Although inhibition of EST may increase circulating levels of estradiol, quercetin has been shown to compete with a similar affinity as E2 for type II estrogen binding sites [28]. The interaction of quercetin at these sites serves to inhibit the estradiol induction of cellular proliferation [28–31]. Therefore, as quercetin may increase mean estradiol concentration by inhibiting EST, this potentially harmful effect may be countered by the opposing, growth inhibitory, action of quercetin at the type II estrogen binding sites.

Acknowledgements

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