Archives of Biochemistry and Biophysics 559 (2014) 62–67

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

journal homepage: www.elsevier.com/locate/yabbi

Glucuronidation does not suppress the estrogenic activity of quercetin in yeast and human breast cancer cell model systems

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ARTICLE INFO

Article history:
Received 4 December 2013
and in revised form 3 March 2014
Available online 18 March 2014

Keywords:
Flavonols
Quercetin
Quercetin metabolites
Phytoestrogen

ABSTRACT

Several plant-derived molecules, referred to as phytoestrogens, are thought to mimic the actions of endogenous estrogens. Among these, quercetin, one of the most widespread flavonoids in the plant kingdom, has been reported as estrogenic in some occasions. However, quercetin occurs in substantial amounts as glycosides such as quercetin-3-*O*-glucoside (isoquercitrin) and quercetin-3-*O*-rutinoside (rutin) in dietary sources. It is now well established that quercetin undergoes substantial phase II metabolism after ingestion by humans, with plasma metabolites after a normal dietary intake rarely exceeding nmol/L concentrations. Therefore, attributing phytoestrogenic activity to flavonoids without taking into account the fact that it is their phase II metabolites that enter the circulatory system, will almost certainly lead to misleading conclusions. With the aim of clarifying the above issue, the goal of the present study was to determine if plant-associated quercetin glycosides and human phase II quercetin metabolites, actually found in human biological fluids after intake of quercetin containing foods, are capable of interacting with the estrogen receptors (ER). To this end, we used a yeast-based two-hybrid system and an estrogen response element-luciferase reporter assay in an ER-positive human cell line (MCF-7) to probe the ER interaction capacities of quercetin and its derivatives. Our results show that quercetin-3-*O*-glucuronide, one of the main human phase II metabolites produced after intake of dietary quercetin, displays ERα- and ERβ-dependent estrogenic activity, the functional consequences of which might be related to the protective activity of diets rich in quercetin glycosides.

Introduction

Epidemiological evidence shows that women have lower cardiovascular risk than men, an advantage progressively disappearing after menopause, when their estrogen levels decline. Estrogens, especially 17-β-estradiol (E2), protect arteries from damage that can lead to heart disease. In addition to the regulation of several other physiological functions, E2 is also implicated in the development or progression of numerous diseases and this activity is often mediated through its receptors. E2 interacts with two different nuclear receptors, estrogen receptor alpha (ERα)[1] and beta (ERβ) [1].

Several plant-derived molecules, referred to as phytoestrogens, are thought to mimic the actions of endogenous estrogens, an effect that is mainly based on their *in vitro* ability to act as agonists and/or antagonists of the ERs. Even if they do act as endocrine disruptors (EDs), as reported for several pesticides such as polychlorobiphenyls, phthalates and bisphenol A [2], phytoestrogens appear to have many beneficial effects on human health, including preventive or therapeutic actions in carcinogenesis, atherosclerosis, and osteoporosis [3]. The best-known and studied phytoestrogens

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[1] *Abbreviations used:* ERα, estrogen receptor alpha; ERβ, estrogen receptor beta; EDs, endocrine disruptors; DMSO, dimethyl sulfoxide; LC, liquid chromatography; LBD, ligand-binding domain; Q, quercetin; Q3GA, quercetin-3-*O*-β-D-glucuronide; IQ, isoquercitrin (Quercetin-3-*O*-β-glucoside); R, rutin (quercetin-3-*O*-rutinoside); Q3′S, quercetin-3′-*O*-sulfate; E2, estradiol; FBS, fetal bovine serum; PBS, phosphate-buffered saline.

http://dx.doi.org/10.1016/j.abb.2014.03.003
0003-9861/

are isoflavones and lignans. However in the last decade, many other compounds, nearly all sharing a polyphenolic structure, have been shown to be endowed with phytoestrogen-like activity. One example is the flavonol quercetin, a potent ROS scavenger [4–6] capable of activating the p38/MAPK pathway, thus promoting pro-apoptotic caspase-3 activation and poly(ADP-ribose) polymerase cleavage in the presence of ERα [7]. Quercetin has also been shown to counteract the deleterious effects of estrogen deficiency on bone-stimulating osteoblastic activity [8].

Quercetin, however, is present only occasionally and often at very low concentrations *in planta*, where it is found widely, and in some instances in substantial amounts, as glycosides such as quercetin-3-*O*-glucoside (isoquercitrin) and quercetin-3-*O*-rutinoside (rutin). It is now well established that flavonols, and flavonoids in general, undergo substantial phase II metabolism after ingestion by humans, with plasma metabolites after a normal dietary intake rarely exceeding nmol/L concentrations [9]. The two major phase II metabolites of quercetin glucosides produced after consumption of onions by humans are quercetin-3′-*O*-sulfate and quercetin-3-*O*-glucuronide [10]. They appear in the circulatory system with a combined peak plasma concentration of ~1 μM, approximately 1 h after ingestion. Their levels rapidly decline thereafter, with an half life of 1.7–2.3 h, as they undergo rapid turnover and are removed from the bloodstream via renal excretion [10]. The aglycone quercetin and the parent glycosides are rarely, if ever, detected in human plasma or urine [10,11].

Moreover, it has recently been reported that resveratrol and its main human circulating metabolite resveratrol-3-*O*-sulfate elicit different effects on MCF-7 cells, the former showing super-estrogenic activity and the latter acting as an antiestrogen [12]. Thus, attributing phytoestrogenic activity to polyphenolic compounds ignoring the fact that it is their phase II metabolites that enter the circulatory system, will almost certainly lead to misleading conclusions. Aim of the current study was to evaluate the estrogenic activity of the main quercetin derivatives present *in planta*, along with that of its two major phase II metabolites found in human plasma.

Material and methods

Chemicals

17-β-Estradiol (E2), quercetin, rutin and isoquercitrin were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Quercetin-3-*O*-glucuronide was purchased from Extrasynthese (Genay Cedex, FRANCE). Quercetin-3′-*O*-sulfate was synthesized according to a previously described procedure [13]. The compounds were dissolved in dimethyl sulfoxide (DMSO) to obtain stock solutions at concentrations ranging from 1 to 10 mM. All liquid chromatography (LC) solvents were purchased from Carlo Erba Reagents (Milan, ITALY).

Yeast two-hybrid assay (Y2H)

Saccharomyces cerevisiae strains co-transformed with expression vectors for the hERα (or hERβ) ligand-binding domain (LBD) and the hTif2 coactivator receptor-interacting domain (pGBT9-GAL4DBD-ER-LBD and pGAD424-Gal4AD-TIF2, respectively) were produced and handled as described previously [12]. Estrogen ligand-dependent interaction between the LBD and the transcriptional coactivator hTif2 [14] recruits the basal transcriptional machinery to the promoter of the 'reporter gene' (β-galactosidase), the expression of which was used to assay phytoestrogenic activity. Briefly, overnight yeast cultures were diluted in glucose-supplemented (0.1%) selective medium (SD) lacking tryptophan and leucine to bring OD600 to 0.1, and aliquots of the resulting cell suspension (200 μl) were transferred to 96-well microtiter plates together with 2 μl of the compounds to be tested dissolved in DMSO (100× stock solutions). DMSO alone (2 μl) served as a negative control for all the experiments, which were carried out in triplicate. Plates were incubated at 30 °C for 18 h without shaking. Estrogenic activity was determined by measuring the β-galactosidase-dependent conversion of the chromophoric substrate chlorophenol red β-D-galactopyranoside (Sigma-Aldrich Co., St. Louis, MO, USA), followed by monitoring absorbance at 570 nm [12,14].

Anti-estrogenic activity was determined by incubating yeast transformants with a fixed suboptimal concentration of E2 (250 pM) in the presence of increasing concentrations of individual test compounds.

Determination of estrogenic activity in mammalian cells

The B17 clone of the human breast cancer cell line MCF-7, stably transfected with a plasmid containing the luciferase gene under the control of an estrogen responsive promoter [15], was grown at 37 °C in a humidified atmosphere (99% humidity, 5% CO_2) in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS, Euroclone, U.K.), 1 mM sodium pyruvate and antibiotics (50 U/mL penicillin G, 50 μg/mL streptomycin sulfate). Cells were split twice a week by seeding 2×10^6 cells in 100 mm diameter Petri (Corning) dishes [12].

For the *in vitro* analysis of agonistic or antagonistic activity, 1×10^5 cells/well were seeded in a 24 well plate in phenol red-free RPMI 1640 medium (Invitrogen) supplemented with 10% dextran-coated charcoal stripped-FBS, non-essential amino acids (1%), 1 mM sodium pyruvate, 2 mM L-glutamine and antibiotics (50 U/mL penicillin G, 50 μg/mL streptomycin sulfate) and maintained at 37 °C in a humidified incubator for 24 h. The culture medium was replaced with phenol red-free RPMI 1640 supplemented with 1% stripped-FBS for a minimum of 2 h before adding E2, Q or Q3GA (agonistic assay) or a combination of E2 and increasing concentrations of the tested flavonols as specified in the text. After 24 h, cells were rinsed once with PBS (phosphate-buffered saline) before preparing the extracts for total protein quantification and for determination of luciferase activity. Luciferase assays were performed with a commercial kit (Luciferase assay system, Promega) based on quantification of photon emission produced by luciferin/luciferase reaction. Light emission, measured with a GlomaxTM 96 microplate luminometer (Promega), is expressed as relative light units over 10 s/μg total protein (RLU/μg total protein). Protein concentration was determined with the Coomassie dye method (Bio-Rad) using bovine serum albumin as standard; each experiment was carried out in duplicate.

UHPLC-Linear ion trap mass spectrometry (LITMS) analyses of cell lysates

Yeast cells (100 ml) treated for 18 h with the various test compounds were harvested by centrifugation at 8000 rpm for 15 min at 4 °C and cytosolic fractions were processed as described previously [12].

MCF-7 cells treated for 24 h with quercetin-3-*O*-glucuronide were rinsed once with PBS, resuspended in lysis buffer (25 mM Tris–phosphate, pH 7.8, 2 mM dithiothreitol, 2 mM 1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid, 10% glycerol and 1% Triton X-100) and incubated at 4 °C for 15 min, before LITMS analysis.

Yeast and mammalian cell lysates were analyzed using an Accela UHPLC 1250 system equipped with a linear ion trap-mass spectrometer (LTQ XL, Thermo Fisher Scientific Inc., San Jose, CA) fitted with a heated electrospray ionization probe (H-ESI-II; Thermo Fisher Scientific Inc., San Jose, CA). Extract fractionation was