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The flavonoid quercetin inhibits thyroid-restricted genes expression and thyroid function

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ABSTRACT

Quercetin is the most abundant flavonoid present in a broad range of fruit and vegetables. Furthermore, quercetin is available as dietary supplements that are based on its antioxidant, antiproliferative and anti-inflammatory properties. However, concerns have been raised about the potential toxic effects of excessive intake of quercetin, and several studies have demonstrated that flavonoids, included quercetin, can interfere with thyroid function. In a previous report, we showed that quercetin inhibits thyroid-cell growth and iodide uptake. The latter effect was associated with down-regulation of sodium/iodide symporter gene expression. In the present study, we have evaluated the effects of quercetin on the expression of other thyroid-restricted genes, and we show that quercetin decreases the expression of the thyrotropin receptor, thyroid peroxidase and thyroglobulin genes. We further investigated the inhibitory effects of quercetin on thyroid function *in vivo* through evaluation of radioiodine uptake in the Sprague–Dawley rat, which was significantly decreased after 14 days of quercetin treatment. These data confirm that quercetin can act as a thyroid disruptor, and they suggest that caution is needed in its supplemental and therapeutic use.

1. Introduction

Quercetin (3,3′,4′,5,7-pentahydroxyflavone) is the most abundant dietary flavonoid in fruit and vegetables (Russo et al., 2012). According to the literature, the daily intake of quercetin varies widely. In the western world, the average daily intake of quercetin has been estimated to be between 20 mg and 40 mg, although it can increase up to 500 mg/day in individuals who ingest large quantities of apples, onions and tomatoes (Manach et al., 2005, 2004; Russo et al., 2012). Furthermore, quercetin is available as a dietary supplement, and ingestion of 1 g/day or even more has been reported (Manach et al., 2005; Russo et al., 2012).

Several studies have shown that quercetin and other flavonoids have many therapeutically relevant properties, such as induction of apoptosis in tumor cells, and antiviral, antioxidant, anti-inflammatory and antiproliferative activities (Arts and Hollman, 2005; Boots et al., 2008; Gupta et al., 2010; Pandey et al., 2012; Russo et al., 2012).

As well as these positive health effects of flavonoids, there have been some cautionary reports in the literature that raise concerns about potential side effects of excessive flavonoid intake (Egert and Rimbach, 2011; Mennen et al., 2005; National Toxicology Program, 2013). With respect to the thyroid, several reports have shown anti-thyroid and goitrogenic effects of flavonoids, which differ in terms of the mechanisms and potencies among the individual flavonoids (Chandra and De, 2013; de Souza dos Santos et al., 2011). The data in the literature concerning the anti-thyroid effects of quercetin show that it can inhibit iodide organification and has a thiourea-like action, inhibiting thyroid peroxidase (TPO) enzyme activity (de Souza dos Santos et al., 2011; Divi and Doerge, 1996). Furthermore, quercetin interferes with thyroid hormone metabolism, particularly through inhibition of type I 5′-deiodinase activity (de Souza dos Santos et al., 2011; Ferreira et al., 2002). In our previous report, we showed that treatment of the FRTL-5 rat thyroid cell line with quercetin inhibits cell growth, iodide uptake, and expression of the sodium/iodide symporter (NIS) gene

Abbreviations: 5H, five-hormone; 6H, six-hormone; ip, intraperitoneal; MHC, major histocompatibility complex; NIS, sodium/iodide symporter; PI3K, phosphatidylinositol 3-kinase; SD, standard deviation; TG, thyroglobulin; TPO, thyroid peroxidase; TSHR, thyrotropin receptor.

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(Giuliani et al., 2008). These data are of particular interest, as quercetin is the most abundant dietary flavonoid, and shows high bioavailability (de Souza dos Santos et al., 2011; Manach et al., 2005, 2004; Russo et al., 2012; Williamson and Manach, 2005). Moreover, quercetin is used in the treatment and prevention of several inflammatory diseases in human (Askari et al., 2013; Cherniack, 2011; Kanzaki et al., 2012; Russo et al., 2012).

In the present study, we further investigated the effects of quercetin on thyroid gene expression using FRTL-5 thyroid cells, which is a nontransformed thyroid cell line in continuous culture (Ambesi-Impiombato, 1986; Giuliani et al., 2006; Kohn and Valente, 1989). We show that quercetin down-regulates the expression of other thyroid-restricted genes; namely, for the thyrotropin receptor (TSHR), TPO and thyroglobulin (TG). Furthermore, we investigated the *in vivo* effects of quercetin on thyroid radioiodine uptake in the Sprague–Dawley rat. Treatment with quercetin significantly decreased thyroid radioiodine uptake, in comparison with the control vehicle, confirming an anti-thyroid effect *in vivo*.

These data indicate a potential role for quercetin as a thyroid disruptor, and suggest caution with the ingestion of large amounts of quercetin. Further studies are required to confirm these data in human and to evaluate the potential use of quercetin in hyperthyroidism.

2. Materials and methods

2.1. Materials

Quercetin was from Sigma–Aldrich Co. (St. Louis, MO, USA). Heat-treated, mycoplasma-free calf serum was from Life Technologies Europe (Monza, Italy). [α-^{32}P]-dCTP and [^{125}I]-NaI were from Perkin Elmer Italia (Monza, Italy). The source of all of the other materials was Sigma–Aldrich, unless otherwise specified.

2.2. Cell culture

The F1 subclone of FRTL-5 rat thyroid cells (American Type Culture Collection, CRL-8305) was a gift from Interthyr Research Foundation (Woodinville, WA, USA). These FRTL-5 cells were grown in a six-hormone (6H) medium consisting of Coon's modified Ham's F-12 medium supplemented with 5% calf serum, 2 mM glutamine, 1 mM nonessential amino acids, and the 6H mixture: bovine TSH (1×10^{-10} M), insulin (10 μg/ml), cortisol (0.4 ng/ml), transferrin (5 μg/ml), glycyl-L-histidyl-L-lysine acetate (10 ng/ml), and somatostatin (10 ng/ml) (6H5% medium). The cells were diploid, between the 5th and 25th passage, and had all of the functional properties described previously (Ambesi-Impiombato, 1986; Giuliani et al., 2008, 2006; Kohn et al., 1995; Kohn and Valente, 1989). Fresh 6H medium was added every 2–3 days, and the cells were passaged every 7 days. In individual experiments, the cells were shifted to a five-hormone (5H) medium: again with 5% calf serum (5H5% medium), as described above, but without TSH.

In all of the experiments with quercetin, the medium was changed every 24 h, adding fresh medium with quercetin. Quercetin was taken from an absolute ethanol stock solution, with control cells treated with the same amount of vehicle. The final ethanol concentration was thus identical in control and treated samples, and did not exceed 0.5% (vol/vol).

2.3. RNA isolation and Northern analysis

The FRTL-5 cells were grown to 60% confluency in 6H5% medium, and then maintained in 5H5% medium for 6 days, as indicated above. In individual experiments, the cells kept in 5H5% medium were shifted to 6H5% medium for 24 h before treatment with control vehicle or quercetin for the indicated times. RNA was prepared using a RNeasy Mini kits (Qiagen Inc., Valencia, CA, USA). Twenty μg of the different RNA samples were run on denaturing agarose gels, capillary blotted on Nytran membranes (Schleicher & Schuell-Whatman, Florrham Park, NJ, USA), UV cross-linked, and hybridized using QuickHyb Hybridization Solution (Stratagene, La Jolla, CA, USA), following the manufacturer protocol. The probes were labeled with [α-^{32}P]-dCTP using Ladderman Labeling kits (Takara Mirus Bio, Madison, WI, USA). The major histocompatibility complex (MHC) class I probe was a 1.0 kb HpaI fragment of the MHC class I pH 7 clone that spanned the entire cDNA insert (Cosman et al., 1982). The NIS, TSHR, TPO, TG, and β-actin probes were as described previously (Isozaki et al., 1989; Giuliani et al., 2008; Saji et al., 1992a,b). Quantitation was performed using a BAS 1500 bioimaging analyzer (Fuji Medical Systems USA Inc., Stamford, CT, USA).

2.4. Preparation of whole-cell extracts and Western blotting

To prepare whole-cell lysates, the cells were collected, washed with ice-cold phosphate-buffered saline, and resuspended in ice-cold RIPA lysis buffer (Sigma–Aldrich). The cells were incubated on ice for 15 min before being vortexed. After centrifugation to remove cellular debris, the cell lysates were subjected to 10% SDS–PAGE, and the separated proteins were transferred to nitrocellulose membranes by electrophoretic blotting. After the transfer, the membranes were incubated according to the manufacturer instructions with the following primary antibodies: mouse monoclonal anti-NIS (NBP1-70342, Novus Biologicals Europe, Cambridge, UK), mouse monoclonal anti-TG (ab80783, Abcam, Cambridge, UK), mouse monoclonal anti-TPO (ab76935, Abcam), mouse monoclonal anti-TSHR (ab6044, Abcam), and rabbit polyclonal anti-actin (ab1801, Abcam). Membranes were subsequently washed and incubated with horseradish peroxidase-conjugated anti-mouse (ab6789, Abcam) or anti-rabbit (sc-2004, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) secondary antibodies, following the manufacturer instructions. Protein was detected using ECL plus (GE Healthcare Italia, Milan, Italy). Quantitation was performed using a Molecular Dynamics STORM 860 Imager (GE Healthcare).

2.5. Animals and radioiodine uptake

All of the experiments performed on animals were carried out in accordance with EU Directive 2010/63/EU for animal experiments and were approved by the Interuniversity Animal Research Ethics Committee (CEISA) of the Chieti–Pescara and Teramo Universities (Italy). Twelve male adult Sprague–Dawley rats (8 weeks old; 250 g) from our breeding colony were housed in a temperature-controlled room under standard conditions of light and dark cycles, with food pellets and water *ad libitum*. The rats were treated by daily intraperitoneal (i.p.) injections of the control vehicle or of quercetin 50 mg/kg, suspended by sonication in phosphate-buffered saline containing 20% polyethylene glycol 400 and 2% Tween-80, over 14 days. On the last day of treatment, each rat received 185 kBq of [^{125}I]-NaI diluted in sterile saline, by i.p. injection. After 24 h, the rats were killed by carbon dioxide narcosis, the thyroid glands were removed, and the radioiodine uptake was evaluated using a gamma-counter (Packard Cobra II auto-gamma, Perkin Elmer). The data were evaluated as % [^{125}I] uptake/mg thyroid gland.

2.6. TSH and thyroid hormones determination

Plasma TSH, total T3 and total T4 were measured by ELISA using a commercial kit (Sunred biological technology, Shangai, China). The inter- and intra-assay coefficients of variation were less than 9%. All the procedures were carried out following the manufacturer instructions.

2.7. Other assays

Protein concentrations were determined using BCA protein assays kits (Pierce Biotechnology Inc., Rockford, IL, USA), with crystalline bovine serum albumin used as the standard.

2.8. Statistical analysis

The data are expressed as means ± standard deviation (S.D.). The significance between experimental values was determined by two-way analysis of variance. Differences were considered significant when $P < 0.05$.

3. Results

3.1. Quercetin decreases RNA expression of the NIS, TSHR, TPO and TG thyroid-restricted genes in FRTL-5 cells

The FRTL-5 cells were grown in 6H5% medium until 60% confluent, and then switched to 5H5% medium (i.e., without TSH) for 6 days, to become quiescent; they were then cultured again in 6H5% medium for 24 h, and treated with quercetin at the doses and times indicated. As shown in Fig. 1 and Table 1, quercetin decreased RNA expression of the thyroid-restricted NIS, TSHR, TPO and TG genes. These effects were concentration and time dependent (Table 1). After 48 h of treatment, 5 μM quercetin significantly decreased NIS, TSHR and TPO gene expression (16 ± 5%, 56 ± 8%, and 33 ± 5% of control, respectively), with a significant decrease in TG gene expression observed at 10 μM quercetin (38 ± 3% of control; Table 1). These quercetin effects were maximal for all of the thyroid-restricted genes at 10 μM, and no further significant decreases were observed at 20 μM or 40 μM (Table 1). Time-course