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Resveratrol has anti-thyroid effects both *in vitro* and *in vivo*

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ABSTRACT

Resveratrol is a natural polyphenol with antioxidant, anti-inflammatory, and antiproliferative properties. We have shown previously that resveratrol decreases sodium/iodide symporter expression and iodide uptake in thyrocytes, both *in vitro* and *in vivo*. In the present study, we further investigated the effects of resveratrol, with evaluation of the expression of additional thyroid-specific genes in the FRTL-5 rat thyroid cell line: thyroglobulin, thyroid peroxidase, TSH receptor, Nkx2-1, Foxe1 and Pax8. We observed decreased expression of these genes in FRTL-5 cells treated with 10 μM resveratrol. The effects of resveratrol was further evaluated *in vivo* using Sprague-Dawley rats treated with resveratrol 25 mg/kg body weight intraperitoneally, for 60 days. No clinical signs of hypothyroidism were seen, although the treated rats showed significant increase in thyroid size. Serum TSH and thyroid hormone levels were in the normal range, with significantly higher TSH seen in resveratrol-treated rats, compared with control rats. Histological and immunohistochemical analyses confirmed increased proliferative activity in the thyroid from resveratrol-treated rats. These data suggest that resveratrol acts as a thyroid disruptor and a goitrogen, which indicates the need for caution as a supplement and for therapeutic uses.

1. Introduction

Resveratrol (3,4',5-trihydroxystilbene) is a natural polyphenol that is present in grapes, berries, peanuts, and other plants, and it is known to have many therapeutically relevant properties, such as antioxidant, anti-inflammatory, and antiproliferative activities (Britton et al., 2015; Diaz-Gerevini et al., 2016; Han et al., 2015; Park and Pezzuto, 2015). For these reasons, there is great interest in the potential use of resveratrol in several chronic human diseases, such as cancers and diabetes, and in neurodegenerative and cardiovascular disorders. Indeed, resveratrol is available as a dietary supplement, and its use is being investigated for various disorders in several clinical trials, both completed and ongoing (Heebøll et al., 2016; Novelle et al., 2015; Park and Pezzuto, 2015; Thazhath et al., 2016; Turner et al., 2015).

With respect to the thyroid, little data are available on the effects of resveratrol on thyroid growth and function (Duntas, 2011). Most studies have focused on the effects of resveratrol on thyroid cancer cells, where it has been shown to have antiproliferative actions (Duntas, 2011; Hosseinimehr and Hosseini, 2014; Kang et al., 2011). Furthermore, resveratrol induces redifferentiation of anaplastic thyroid cancer cell lines, and up-regulation of expression of several thyroid-specific genes (Yu et al., 2013). Recently, these antiproliferative and redifferentiation properties of resveratrol were observed even in thyroid cancer stem-like cell clones, where treatment with resveratrol resulted in decreased cell proliferation and increased apoptosis, which were associated with up-regulation of the thyroid differentiation markers sodium/iodide symporter (NIS) and TTF-1 (Hardin et al., 2016). Therefore, a potential role for resveratrol in treatment of undifferentiated thyroid cancer has been proposed. This concept is further strengthened by the observation that resveratrol enhances the toxic effects of radioiodine on thyroid cancer cells (Hosseinimehr and Hosseini, 2014).

Despite these data on the effects of resveratrol on thyroid cancer cells, little information is available for its effects on normal thyroid

Abbreviation: 5H, five-hormone; 6H, six-hormone; HPLC, high-performance liquid chromatography; ip, intraperitoneal; NIS, sodium/iodide symporter; PCNA, proliferating cell nuclear antigen; SD, standard deviation; TG, thyroglobulin; TPO, thyroid peroxidase; TSHR, thyrotropin receptor.

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cells. In a previous study, we showed that resveratrol can inhibit expression of the NIS gene and iodide uptake in nonneoplastic thyroid cells, both *in vitro* and *in vivo* (Giuliani et al., 2014b). Given these data, we wanted to further evaluate the effects of resveratrol through examination of the expression of additional thyroid-restricted genes. Indeed, in the present study, we show that resveratrol down-regulates expression of the thyroid stimulating hormone receptor (TSHR), thyroid peroxidase (TPO), and thyroglobulin (TG) genes in the FRTL-5 rat thyroid cell line, as a function of time. A similar effect was observed on the expression of the transcription factors Nkx2-1 (known also as TTF-1), Foxe1 (known also as TTF-2) and Pax-8, which are involved in regulation of the aforementioned thyroid-restricted genes. We also show that long-term treatment (i.e., 60 days) with resveratrol in Sprague-Dawley rats has goitrogenic effects. These data confirm the role of resveratrol as an anti-thyroid agent, and thus we suggest caution with ingestion of large amounts of resveratrol. Further studies are required to confirm these data in human.

2. Materials and methods

2.1. Materials

Resveratrol was from Sigma-Aldrich Co (St. Louis, MO, USA). Resveratrol 3-*O*-β-D-glucuronide was from Spectra2000 Srl (Rome, Italy). Acetonitrile was from Delchimica Scientific Glassware Srl (Naples, Italy). Heat-treated, mycoplasma-free calf serum was from Life Technologies Europe (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 the Interthyr Research Foundation (Marietta, OH, USA). These FRTL-5 cells were grown in the six-hormone (6H) medium of Coon's modified Ham's F-12 supplemented with 5% calf serum, 2 mM glutamine, 1 mM nonessential amino acids, and the 6H mixture (6H5% medium): 1 mU/mL bovine TSH, 10 μg/mL insulin, 0.4 ng/mL cortisol, 5 μg/mL transferrin, 10 ng/mL glycyl-L-histidyl-L-lysine acetate, and 10 ng/mL somatostatin. These cells were diploid, between the 5th and 25th passage, and had all of the functional properties described previously (Ambesi Impiombato, 1986; Giuliani et al., 2014b, 2010, 2006; Kohn et al., 1995; Kohn and Valente, 1989; Lin et al., 2010). Fresh 6H5% medium was added to the cells every 2–3 days, and they were passaged every 7 days. In individual experiments, the cells were transferred to a five-hormone (5H) medium (i.e., without TSH), again with 5% calf serum (5H5% medium), as described in Section 3.1.

The treatments were performed with 10 μM resveratrol, which was chosen based on our previous studies (Giuliani et al., 2014b). In all of the experiments with resveratrol, the medium was changed every 24 h, with addition of fresh medium with resveratrol. Resveratrol was used from a stock solution in absolute ethanol, with control cells treated with the same amount of vehicle. The final ethanol concentration was thus identical in the control and treated samples, and did not exceed 0.1% (v/v).

2.3. Real-time quantitative PCR analysis

Total RNA was prepared using RNeasy minikits (QIAGEN Inc, Valencia, CA, USA). The yield and quality of the RNA were determined by spectrophotometry and denaturing agarose gel electrophoresis. Total RNA was reverse transcribed using high-capacity cDNA reverse transcription kits (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer instructions. Real-time quantitative PCR (RT-qPCR) was performed using TaqMan Gene Expression assay kits (Applied Biosystems) and an ABI Prism 7900 Sequence Detection System (Applied Biosystems), following the manufacturer instructions. Taq-Man Gene Expression Assays (Applied Biosystems) were used, for the rat genes: *Tg* (assay ID: Rn00578496_m1), *Tpo* (assay ID: Rn00571159_m1), *Tshr* (assay ID: Rn00563612_m1), *Nkx2-1* (assay ID: Rn01512482_m1), *Foxe1* (assay ID: Rn00594363_s1), *Pax8* (assay ID: Rn00579743_m1), and *Actb* (assay ID: Rn00667869_m1). RT-qPCR analysis was carried out in triplicate and repeated at least three times. The relative mRNA expression levels were normalized with respect to β-actin using the ΔΔCt method.

2.4. Whole-cell extracts and western blotting

To prepare whole-cell lysates, FRTL-5 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 cell debris, cell lysates were subjected to 10% SDS-PAGE, and the separated proteins were transferred to nitrocellulose membranes by electrophoretic blotting. After this transfer, the membranes were incubated according to the manufacturer instructions, with the following primary antibodies: 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). The 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. The proteins were detected using ECL plus (GE Healthcare Italia, Milan, Italy). Quantitation was performed using a STORM 860 Imager (Molecular Dynamics, GE Healthcare).

2.5. Animals

All of the experiments performed on animals were carried out in accordance with European Union Directive 2010/63/EU for animal experiments, and were approved by the Interuniversity Animal Research Ethics Committee of the Chieti–Pescara and Teramo Universities (CEISA, Italy; prot.12/2013/CEISA/COMM/PROG.47). Twelve male adult Sprague-Dawley rats from our breeding colony (age, 8 weeks; weight, 250 g) were housed in a temperature-controlled room with standard light and dark cycles, and food pellets and water *ad libitum*. The rats were treated daily for 60 days by intraperitoneal (ip) injection of the control vehicle or of resveratrol 25 mg/kg body weight, resuspended by sonication in phosphate-buffered saline containing 20% polyethylene glycol 400 and 2% Tween-80. After 60 days of treatment, the rats were sacrificed using carbon dioxide narcosis, and their thyroid glands were removed, weighted, and fixed in 10% neutral buffered formalin for histological examination.

2.6. TSH and thyroid hormones determination

Serum TSH was measured by ELISA using commercial kits from ALPCO Diagnostics (Salem NH, USA), and total T_3 and T_4 were measured by ELISA using commercial kits from Genway Biotech, Inc. (San Diego CA, USA).

2.7. Morphological analysis

Tissue samples were fixed in 10% neutral buffered formalin and