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Xanthohumol stimulates iodide uptake in rat thyroid-derived FRTL-5 cells

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Sodium-iodide-symporter (NIS), an integral plasma membrane glycoprotein, mediates the sodium-dependent active uptake of iodide (I^-) into the thyroid gland, which is a fundamental step in thyroid hormone synthesis. In this work, we analyzed the influence of xanthohumol (XN), a prenylated chalcone from hops (*Humulus lupulus* L.), on the I^- uptake in a cell culture model of normal, nontransformed rat thyrocytes (FRTL-5). Acute treatment with nanomolar concentrations of XN does not influence I^- uptake, but after 2 and 3-days of XN stimulation an increase in I^- uptake was observed; I^- uptake was maximally increased by 50% compared to control after 3-days of XN stimulation at 1 nM. A clear time-dependent stimulation was observed which showed no marked concentration relationship, however. To investigate whether expression of NIS mRNA is also increased, we grew FRTL-5 cells for 3-days in a medium containing increasing concentrations of XN (0.1 nM – 1 μM). Northern blot analysis showed no difference in NIS mRNA transcript levels between control cells and those treated with different concentrations of XN. This study revealed that nanomolar concentrations of XN, a unique compound with anticancer properties, exert stimulating effects on radioiodide uptake. In contrast to many other plant-derived phenolic secondary metabolites such as (iso-)flavonoids, which inhibit I^- uptake, XN might be an interesting candidate for more efficient radioiodide therapy of thyroid and perhaps other cancer expressing NIS such as breast cancer.

Keywords: FRTL-5 / Iodide uptake / Sodium-iodide-symporter / Thyroid cancer / Xanthohumol

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1 Introduction

Iodine is a rare but essential element for the biosynthesis of thyroid hormones thyroxine (T4) and triiodothyronine (T3), the only iodine containing hormones in vertebrates. As an adaptation to iodine's scarcity in the environment, a highly specialized system has evolved in the thyroid gland, which facilitates not only transport of iodide (I^-) against chemical gradient [1] but also accumulation in thyroglobulin-bound form for future needs.

Sodium-iodide-symporter (NIS), an integral plasma membrane glycoprotein basolaterally located [2] in the thyroid follicular cells, plays a crucial role in these processes. NIS mediates active uptake of one I^- simultaneously with two Na^+, whose translocation is maintained by the electrochemical gradient created by Na^+/K^+ ATPase activity, also located on the basolateral side of the follicular cells, facing the blood supply [3]. Structure/function characterization studies [4 – 8] revealed that NIS is a glycoprotein with 13 transmembrane regions showing high homology between humans, pigs, and rats as well as homology to other sodium-dependent transporters. NIS mRNA expression, protein content, and I^- uptake are up-regulated by thyrotropin (TSH) which was not only demonstrated in the rat thyroid-derived FRTL-5 cell line [9], but also in rats *in vivo* [10]. Moreover, no thyroidal I^- uptake is detected in humans whose serum TSH levels are suppressed [11]. I^- itself exhibits a transient inhibitory effect on NIS mRNA expression and accumulation of I^- in the thyroid *in vitro* and *in vivo* [5, 12].

NIS also mediates active I^- transport in nonthyroid tissues including salivary glands, gastric mucosa, lactating mammary gland, choroid plexus, and the ciliary body of the eye. TSH has no influence on nonthyroid I^- accumulation and, with the exception of the lactating mammary gland, nonthyroid I^- transporting tissues do not have the ability to

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Abbreviations: FBS, fetal bovine serum; **NIS**, sodium-iodide-symporter; **T3**, 3,3′,5-triiodo-L-thyronine; **T4**, thyroxine; **TSH**, thyrotropin; **XN**, xanthohumol

organify accumulated I^- [5]. The endogenous expression of NIS in mammary tissue is not only important because of its physiological significance in I^- supply of the suckling newborns *via* maternal milk, but also because of possible exploitation of radioiodide therapy in breast cancer treatment. Although more studies are necessary, expression of NIS in breast cancer has been documented both in experimental mice and humans [3].

In most thyroid cancers, I^- uptake is decreased compared to surrounding normal tissue due to both low NIS expression and alterations in NIS trafficking [13, 14]. I^- uptake is still sufficient to enable effective radioiodide therapy in many, but not all, cases. Therefore, understanding of the cause of diminished I^- uptake and searching for the substances able to increase I^- accumulation is advantageous [15].

Xanthohumol (XN), a prenylated chalcone from hops (*Humulus lupulus* L.), exhibits a broad spectrum of inhibitory mechanisms at the initiation, promotion, and progression stage of carcinogenesis [16]. Anticarcinogenic properties of XN are well described in numerous studies [16, 17] but there is no data concerning potential effects of XN on thyroid cancer and on the thyroid hormone axis in general. In this study, we analyze the influence of XN on I^- uptake in a cell culture model of normal, nontransformed thyrocytes. We found that XN up-regulates I^- uptake, which could be of therapeutic significance in the radioiodide treatment of thyroid or even mammary carcinoma and particularly beneficial in combination with anticancer properties of XN described.

2 Materials and methods

2.1 Chemicals

All chemicals were of analytical or biochemical grade. Cell culture medium and fetal bovine serum (FBS) were obtained from Biochrom AG (Berlin, Germany). XN, kindly provided by H. Becker (Universität des Saarlandes Saarbrücken, Germany), was dissolved in DMSO at a concentration of 100 mM and stored at −20°C. Iodide-125 (37 MBq) was obtained from Perkin Elmer (Boston, MA, USA) and α-^{32}P 2-deoxycytidine 5′-triphosphate from Hartmann Analytic (Braunschweig, Germany). Oligonucleotides were synthesized by MWG-Biotech AG (Ebersberg, Germany).

2.2 Iodide uptake assay

For iodide uptake assays, rat thyroid-derived FRTL-5 cells were splitted and seeded into 24-well plates (TPP AG, Trasadingen, Switzerland) with Coon's F12 medium optimized to grow rat thyroid cells containing 2.5 g/L $NaHCO_3$ and without L-glutamine. FBS (5%), six hormones [18], and 1 nM sodium-selenite were added to facilitate cell growth and adhesion. After 24 – h adaptation, on day 0 cells were treated once with XN (1, 10, 100, 1000, and 10000 nM) diluted in 1% DMSO (which was also added to control cells) and harvested during subsequent days for the I^- uptake assay. For acute treatment, XN was added directly to assay buffer. For the assay, the medium was removed and cells were washed with 0.5 mL HBSS (137 mM NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2$, 0.4 mM $MgSO_4$, 0.5 mM $MgCl_2$, 0.4 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 5.55 mM glucose, and 10 mM Hepes, pH 7.3) [15]. Then the cells were overlaid with HBSS containing 10 mM NaI and I-125 tracer to give a specific activity of 10 – 20 mCi · mmol^{-1}. NIS inhibitor $NaClO_4$ (10 μM) was used as negative control to calculate specific uptake. After 30 min at 37°C in a humid atmosphere, the radioactive medium was aspirated, cells were washed with ice cold HBSS, and accumulated iodide was extracted at −20°C with 1 mL of ethanol. Ethanol extracts were counted in an LKB Wallac 1277 γ counter. Protein concentrations in cell homogenates were determined by a modified Bradford protein assay (BioRad, Munich, Germany). Results represent the amounts of iodide accumulated *per* μg protein and are expressed as a percentage of control. Experiments were done in triplicates and repeated twice.

2.3 Northern blot analysis

FRTL-5 cells treated for 3 days with increased concentration of XN (0.1, 1, 10, 100, and 1000 nM) under the same conditions as described in Section 2.2 were homogenized in 5 mL of an SDS-containing Tris-based buffer (0.1 M Tris, 0.5 M LiCl, 10 mM EDTA, 1% SDS, and 5 mM DTT, pH 8.0). Total RNA was isolated from the cells with peqGold TriFast reagent (Peqlab Biotechnologie, Erlangen, Germany) according to the manufacturer instructions. Samples of 18 μg total RNA were separated by electrophoresis in denaturating agarose gels (2.2 M formaldehyde, 1.5% agarose), capillary transferred to nylon membranes (Nytran NY 12 N, Schleicher and Schuell, Dassel, Germany), and cross-linked by UV-irradiation. Hybridizations were performed, as described previously [19], with the following ^{32}P-labeled complementary DNA (cDNA)-probes: a 269-b NIS fragment of rat cDNA and as standard, a 1.1-kb fragment of human cDNA encoding glyceraldehyde-3-phosphate dehydrogenase. The nylon membranes were washed to a final stringency of 0.5 × SSPE/0.3% SDS (0.5 × SSPE = 75 mM NaCl, 5 mM Na-phosphate, 0.5 mM EDTA, pH 7.4) for 30 min at 60°C. Autoradiographic signals were analyzed and quantified by a phosphoimager (cyclone storage phosphor screen (Packard Instruments)). All data were corrected for variability in loading by normalization to the

values obtained with the glyceraldehyde-3-phosphate dehydrogenase.

2.4 Statistical analysis

Results are expressed as mean ± SD. Nonparametric one-way analysis of variance (Kruskal–Wallis test) was used for comparison between multiple groups, and the unpaired *t*-test was used to compare two groups (GraphPad Prism 4 software, GraphPad Software, Inc.). All *p*-values were two-tailed and a *p*-value less than 0.05 was considered to indicate statistical significance.

3 Results

We examined whether XN influences I^- uptake in rat thyroid-derived FRTL-5 cells and found significant time-dependent increase after XN stimulation. Figure 1 represents experimental data showing that acute treatment (day 0) with XN does not influence I^- uptake. An increase in I^- uptake was observed after 1 day of XN stimulation, reaching its maximum after 2 and 3 days. On day 4, I^- uptake stimulation was less pronounced but still up-regulated compared to control. The results of the statistical evaluation by the Kruskal–Wallis test indicated a significant influence of XN on I^- uptake ($p < 0.0001$). We observed clear time course for stimulation of uptake by the lower XN concentrations whereas high concentrations showed no increase of I^- uptake. Figure 1b shows the protein content after treatment with increasing dose of XN. After 1 day of stimulation with 100 μM of XN, cells detached from the plate and therefore no protein and I^- uptake were detected.

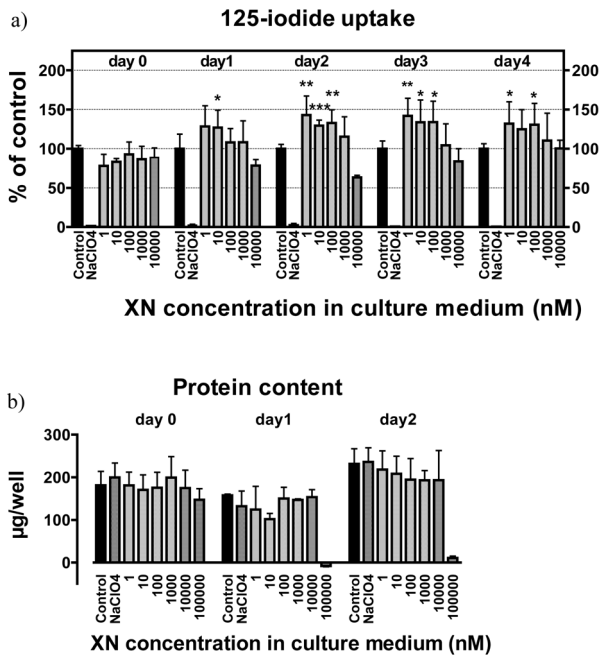

Figure 1. (a) Time course of stimulation of iodide-125 uptake in rat thyroid-derived FRTL-5 cells by XN. Values are expressed as percentage of control. Mean value of the untreated control cells was defined as 100% at each time point. NIS inhibitor $NaClO_4$ (10 μM) was used as negative control to calculate specific uptake mediated by NIS. Experiments were performed in triplicates and repeated twice. Kruskal–Wallis test indicated a significant influence of XN on I^- uptake ($p < 0.0001$). Unpaired *t*-test was used to compare the treatment group with its corresponding control group (*$p < 0.05$; **$p < 0.005$; ***$p < 0.0001$). (b) Protein content after treatment with increasing dose of XN (1 nM–100 μM). Results are derived from the same experiments presented in (a).

Table 1. Representative specific iodide uptake results after subtraction of $NaClO_4$ (10 μM) inhibited uptake which amounted less than 2 cpm/μg protein. Experiment was performed in triplicates and the values are presented as mean values ± SD

Day	Specific iodide uptake, cpm/μg protein
0	40.6 ± 2.6
1	36.9 ± 5.5
2	28.4 ± 1.9
3	30.3 ± 1.8
4	39.9 ± 6.2

Table 1 represents the absolute values of I^- uptake in non-treated control cells in one representative experiment. Basal I^- uptake showed transient decrease after 2 and 3 days but comparable on day 4.

As we observed time-dependent stimulation but no effect on day 0, we performed another experiment to investigate whether NIS mRNA expression in FRTL-5 cells is also up-regulated. The cells were grown for 3 days in a medium containing increasing concentrations of XN (0.1 nM–1 μM) and all components necessary for growth and function as already described. After performing Northern blot analysis, no difference in NIS mRNA expression was found between control cells and those treated with different concentrations of XN (Fig. 2).

4 Discussion

In this study, we present evidence that the natural flavonoid XN affects the *in vitro* 125-iodide uptake in the nontransformed rat thyroid-derived FRTL-5 cell line. Schröder-van der Elst *et al.* [20] reported that most flavonoids decrease, whereas myricetin increases iodide uptake by 35% after 4 days of incubation at 5 μM using the human follicular thyroid cancer cell line (FTC133), which was stably transfected with the human NIS. We observed significant increase of iodide uptake in the nontransformed rat FRTL-5

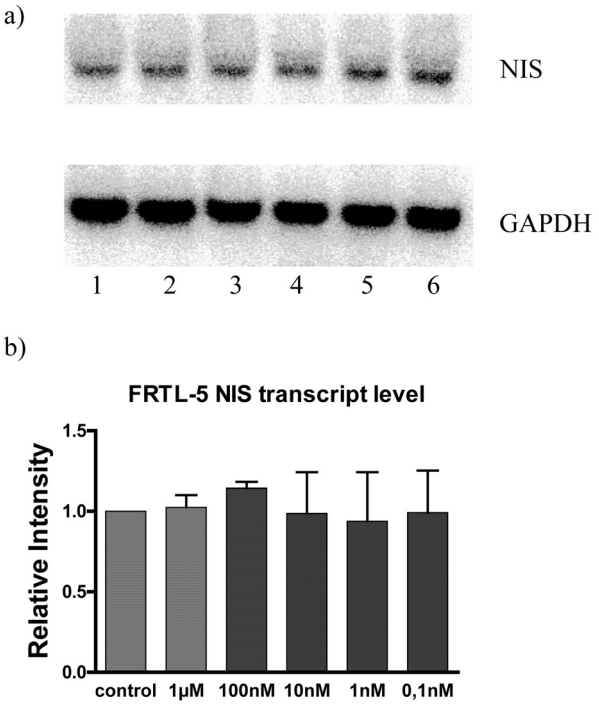

Figure 2. (a) Northern blot analysis of NIS mRNA expression after XN stimulation. FRTL-5 cells were grown for 3 days in 6H medium (containing TSH and sodium-selenite) in the presence or absence of increasing concentrations of XN: 1 – control; 2 – 1 μM XN; 3 – 100 nM XN; 4 – 10 nM XN; 5 – 1 nM XN; 6 – 0.1 nM XN. (b) Relative FRTL-5 NIS transcript level data from two independent experiments quantified by phosphoimager analysis in relation to GAPDH signals. Expression of the control on each membrane was set as 1.

cell line which was maximal after 2 and 3 days of incubation using a much lower concentration of XN (1 nM). The increase in iodide uptake was clearly time-dependent, raising questions concerning the mechanism of action of XN.

Iodide accumulation is the net result of the influx mediated by NIS and of the efflux mediated by pendrin and the apical iodide transporter. Because time-dependent stimulation but no effect of XN on iodide uptake on day 0 was observed, an increase in NIS mRNA expression was expected. Surprisingly, no changes in NIS transcript levels were observed between control cells and cells stimulated for 3 days with XN. Cells were grown in a medium containing TSH and harvested after 3 days because we observed clear increase in iodide uptake after 3 days. NIS half-life is approximately 5 days in the presence and approximately 3 days in the absence of TSH [5]. Schröder-van der Elst *et al.* [20] showed that the inhibitory effect on iodide influx exerted by some flavonoids (kaempferol, apigenin, luteolin) is a consequence of decreased NIS mRNA expression. The XN-triggered up-regulation of iodide influx reported in this experiment cannot be explained in the same way. Whether XN affects thyroid peroxidase (TPO) and alters protein iodination remains to be analyzed in appropriate models as FRTL-5 cells do not express functional TPO [21]. TSH is the primary hormonal regulator of thyroid function and stimulates iodide accumulation [22] in the thyroid. TSH also up-regulates NIS protein expression [10] and is required for targeting NIS to and/or retaining it at the plasma membrane. Cell polarization and spatial organization are also crucial for proper NIS activity, and NIS may be regulated by posttranscriptional events such as altered subcellular distribution [5]. XN might be involved in a posttranscriptional regulation of NIS, independent from or together with TSH. There is evidence that NIS function requires proper membrane-integrated TSH phosphorylation [5]; thus, a similar modulation of NIS phosphorylation pattern might be caused by XN. However, further studies are needed to reveal this mechanism of action.

Decreased I^- uptake on day 4 might be explained by TSH deficiency because there was no additional treatment with TSH during the experiment. This is consistent with observations presented in Table 1 where absolute values of I^- uptake in control cells were slightly decreased after 2 and 3 days of treatment with XN. Apparently, several processes overleap in their time course such as TSH-dependent stimulation, proliferation-associated expression, and low dose XN-stimulated expression of NIS transcript and functional protein. The treatment of the cells with >100 nM of XN is obviously not beneficial for I^- uptake as shown in Fig. 1. Iodide accumulation is most probably decreased because of negative effects of XN at higher concentration on whole cell metabolism and survival. Decreased protein content presented in Fig. 1b was due to cell detachment from culture dishes at 100 μM of XN after 1 day of exposure. Antiproliferative and cytotoxic effects of XN reported in different cancer cell lines (IC_{50} values in μM concentration range) [16, 23] might be also expected in nontransformed FRTL-5 cells.

Although our data clearly showed that XN up-regulates iodide accumulation in thyroid cells, it remains open whether this increase observed *in vitro* could affect pituitary–thyroid axis; *in vivo* studies are needed to clarify this. XN is, therefore, probably not applicable for the treatment of hypothyroidism but could be very interesting for enhancement of radioiodide therapy in thyroid cancer as a compound that has both antitumor and beneficial effects on radioiodide uptake. Although we showed that XN stimulates iodide uptake in nontransformed thyroid cells, we might expect a similar effect in thyroid carcinoma cells. Moreover, breast cancer could be also amenable to effective radioiodide therapy because it expresses endogenous NIS molecules too [24]. XN is a "broad-spectrum" cancer chemopreventive agent that acts by inhibiting the metabolic activation of procarcinogens, induction of carcinogen-

detoxifying enzymes, and by inhibition of tumor growth at an early stage [17]. All these findings revealed XN as a unique compound that has broad anticancer properties and the beneficial effects on radioiodide uptake described here, which makes it an interesting candidate for more efficient radioiodide therapy of thyroid and perhaps other NIS expressing cancers [25].

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