RESEARCH ARTICLE

# Xanthohumol, a prenylated chalcone from hops, modulates hepatic expression of genes involved in thyroid hormone distribution and metabolism

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In the present study, we analyzed the influence of xanthohumol (XN) on thyroid hormone (TH) distribution and metabolism in rats. A potent and selective competition of XN for thyroxine (T4) binding to transthyretin ($IC_{50} = 1\,\mu M$ at 1.7 nM [$^{125}$I]T4) was found in human and rat sera *in vitro*. Female rats treated orally with XN showed increased hepatic expression of T4-binding globulin and decreased transthyretin and albumin. Thyrotropin levels and hepatic type 1 deiodinase activity were moderately increased. Northern blot analysis revealed diminished expression of liver sulfotransferase (Sult1a1) and uridine-diphosphate glucuronosyltransferase (Ugt1a1) after XN treatment. The transcript levels of constitutive androstane receptor (CAR), known to be involved in regulation of enzymes metabolizing hormones, drugs and xenobiotics, was lower in rats treated with $>10$ mg XN/kg body weight *per* day. Immunoblot analysis indicates reduced amounts of CAR protein. The phenobarbital-inducible cytochrome P450 mRNA level was decreased in rats treated with $>10$ mg XN/kg/day, in agreement with reduced CAR protein. Although only moderate changes in TH serum levels were observed, the XN-dependent altered expression of components involved in TH homeostasis might be important not only for hormone metabolism, but also for hepatic phase I and II elimination of drug metabolites and xenobiotics.

**Keywords:**
Constitutive androstane receptor / Thyroid hormone / Thyroxine-binding globulin / Transthyretin / Xanthohumol

## 1 Introduction

Xanthohumol (XN) is a prenylated chalcone and the principal flavonoid from the hop plant (*Humulus lupulus* L.). The main dietary source of XN is beer, although its average concentration of about 0.1 mg/L is relatively low. Hop cone extracts and food supplements are also used therapeutically as mild sedatives to induce sleep and have been investigated

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**Abbreviations: ALB,** albumin; **BW,** body weight; **CAR,** constitutive androstane receptor; **Cyp,** cytochrome P450; **Dio1,** type 1 deiodinase; **PB,** phenobarbital; **SD,** Sprague Dawley; **Sult,** sulfotransferase; **TBG,** thyroxine-binding globulin; **TH,** thyroid hormone; **TSH,** thyrotropin; **TTR,** transthyretin; **T4,** thyroxine; **Ugt,** uridine-diphosphate glucuronosyltransferase; **XN,** xanthohumol

for their effect on menopausal symptoms [1–4]. In recent years, XN has been intensively studied as antioxidative, antimutagenic and chemopreventive agent [5–7]. In addition, XN ameliorates lipid and glucose metabolism [8, 9] *via* interaction with the nuclear farnesoid X receptor. These potentially beneficial characteristics may lead to medical application and have therefore received much attention in recent years. On the other side, because of increased popularity, there is a growing use of such alternative preparations without medical prescription, partly also due to intense marketing *via* the Internet, although very few studies on efficacy and safety have been performed until now.

Previous studies have shown that flavonoids may alter thyroid function [10–12]. We have demonstrated recently that XN has a beneficial effect on iodide uptake *in vitro* [13], but the potential effects of XN on thyroid hormone (TH) distribution and subsequent metabolism and elimination have not been described. In the healthy adult rat, THs are transported to target tissues primarily bound to transthyretin (TTR), the major serum distributor protein for TH in rodents [14]. This is in contrast to human serum, where thyroxine-binding globulin (TBG) binds L-thyroxine (T4) with highest affinity. Flavonoids are strongly and preferentially bound to TTR in most species, including man, but show no or only minor competition to TH binding to TBG, or to serum albumin (ALB) [15, 16]. These altered patterns of binding of THs to their serum distributor proteins might influence free hormone concentration and cellular bioavailability. This might be the case both for the prohormone T4 and the active TH receptor ligand T3, which have cell type specific distinct access to their target cells both during development and in the adult organism [17]. In maintaining TH homeostasis, TH metabolism plays a role as important as TH secretion by the thyroid gland. Several metabolic pathways interact to balance synthesis, bioactivation and inactivation of TH. The principal pathways of peripheral TH metabolism are deiodination, conjugation, ether bond cleavage, oxidative deamination and decarboxylation [18, 19]. The selenoenzyme deiodinase 1 (Dio1), one of the three deiodinases, which is highly expressed in liver, can both activate and inactivate T4. Dio1 catalyzes 5′-(''outer ring'') deiodination to produce active hormone T3 or 5-(''inner ring'') deiodination to generate the ''inactive'' metabolite rT3. Conjugation of TH involves glucuronidation or sulfation of the phenolic 4′-hydroxyl group, which increases water solubility and facilitates biliary excretion [20, 21]. Interestingly, when T4 is sulfated, outer ring deiodinaton becomes undetectable, while inner ring deiodination increases over 130-fold [22]. TH sulfation and glucuronidation is mediated by the phase II liver enzymes sulfotransferase (Sult) and uridine-diphosphate-glucuronosyltransferase (Ugt) [23–25]. Members of the Ugt1 family are particularly important for TH conjugation and biliary and urinary excretion [20].

Nuclear receptors play a crucial role in activation and control of genes involved in hepatic metabolism of hormones, drugs and xenobiotics. Constitutive androstane receptor (CAR) was originally described as activator of the cytochrome P450 2b subfamily (Cyp2b) [26, 27]. The list of CAR target genes has been recently expanded on other liver phase I and phase II drug metabolism enzymes involved in oxidative metabolism, conjugation and transport [28–31]. Recent studies have also demonstrated that CAR participates in molecular mechanisms that control energy homeostasis, lipid and TH metabolism [30, 32–34].

Based on our previous results with XN related to iodine uptake [13] and its potential to interact with nuclear hormone receptors [9], we performed the present study to investigate potential influences of XN on TH distribution among serum-binding proteins and metabolism in a rodent model.

## 2 Materials and methods

### 2.1 Chemicals and materials

XN was isolated as described earlier [5]. $^{125}$I–L-thyroxine (specific activity 135–165 μCi/μg) was purchased from Perkin Elmer (Billerica, MA, USA). 3,3′,5,5′-tetraiodo-L-thyronine (T4) was provided by Henning Berlin (Germany). Human purified TTR and TBG was prepared and generously supplied by Dr. Vivian Cody (Hauptman-Woodward Medical Research Institute, Buffalo, NY, USA). The Wistar rat serum pool was kindly provided by Dr. Alexander Patchev (Exp. Endocrinology, Charité, Berlin, Germany) and the human serum pool by Dr. Lutz Schomburg (Exp. Endocrinology, Charité). Anti-CAR antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

### 2.2 Analysis of T4 binding to serum proteins

Binding of $^{125}$I-labeled T4 to serum proteins TTR, ALB and TBG was assessed by non-denaturing PAGE, as previously described [14]. The human and rat sera samples (10 μL) were incubated in 1.5 mL Eppendorf tubes for 30 min at room temperature with 10 μL of [$^{125}$I]T4 (≈0.02μCi) diluted in 0.02 M phosphate buffer, pH 9.0 (100 μL total incubation volume), in the absence or presence of increasing concentrations of XN (1–100 μmol/L). Sixty-microliter aliquots of the incubated mixture were loaded on non-denaturing PAGE gels and run for 14 h at 50 V in a Tris-glycine native running buffer, pH 8.4. The temperature was maintained at 6°C by the Bio-Rad Protean II xi cooling electrophoresis chamber. Gels were sealed in a plastic transparent bag and exposed to phosphoimager plates overnight, before scanning. The distribution of radiolabeled T4 to individual binding proteins was analyzed by a Cyclone storage phosphor screen (Packard Instrument Company) and quantified by densitometric analysis using the ''Image J'' image

 

analysis software (Wayne Rasband, NIH, USA). Integrated density represents the sum of the values of the pixels in the image or selection. The same procedure was used for rat sera obtained after oral administration of XN.

### 2.3 *In vitro* T4-TTR competition-binding studies

The analysis of the capacity of XN to compete with T4 binding to purified human TTR was performed as described previously [35] with slight modifications. The assay mixture consisted of a 0.1 M Tris-HCl buffer, pH 8.0, containing 0.1 M NaCl and 1 mM EDTA, purified human TTR (2.5 μg/μL = 23 nmol/L), $^{125}$I-labeled T4 (47 000 cpm/tube ≈50 pmol/L) and competitor (XN) with increasing concentrations (0.001–10 μmol/L), in a total volume of 100 μL. Control incubations contained 1% DMSO, which served as the solvent. The incubation mixtures were allowed to reach binding equilibrium at room temperature for 30 min, and incubation was stopped by adding 0.5 mL of ice-cold dextran-coated charcoal. TTR bound and free [$^{125}$I]T4 were separated after 10 min of incubation at 4°C by 10-min centrifugation at 3000 × *g*. The decanted supernatant was counted in an LKB Wallac 1277γ counter (Perkin Elmer). Unspecific binding, determined by adding excess L-T4 (10 μmol/L), was subtracted to obtain specific binding data. All analyses were performed with data from at least three different experiments each performed in duplicate. Calculation of binding parameters was performed with GraphPad Prism Version 4 for Windows (GraphPad Software).

### 2.4 Animal experiments

Female Sprague Dawley (SD) rats were purchased from Charles River Wiga (Sulzfeld, Germany). Tissue samples and serum were derived from animal studies that have been described in detail earlier [36]. As a brief summary, in study 1, 4-wk-old female SD rats were treated with XN for a period of 28 days. Rats were fed a standard diet enriched with XN at a concentration of 0.5% to achieve a mean dose of approximately 500 mg/kg body weight (BW) *per* day. The food was prepared by adding isolated XN to a powdered standard rat chow at a concentration of 0.5%, followed by pelleting by the manufacturer. Alternatively, animals received XN by gavage at 1000 mg/kg BW *per* day in 1% starch suspension, in comparison with a vehicle-treated (1% starch suspension) control group ($n = 3$ *per* group). The daily intake of XN was calculated from the amount of food intake (g) and BW (g) assessed daily. Doses and plasma levels of XN cannot be directly compared due to the differences in application (*via* the chow or by gavage).

In study 2, 10-wk-old female SD rats were administered XN dissolved in drinking water containing 0.75% EtOH and 1.5% Tween-20 or solvent alone for 4 wk (control, 10 and 100 mg XN/kg BW/day – 6 *per* group). The XN-containing liquid was prepared freshly every second day. The stability of XN was checked by HPLC for extended periods >72 h, and no signs of degradation were observed. The daily dose of XN was adjusted to BW (g) assessed daily. All rats were housed in a climate-controlled room with a 12 h light/12 h dark cycle and were fed a standard diet (Altromin, Lage, Germany). Animals were given free access to diet and water. All animal experiments were approved by an ethical committee (35–9185.81/G-54/04 – Regierungspräsidium Karlsruhe).

### 2.5 Determination of mRNA levels

Total RNA was isolated from rat livers (control, 10 and 100 mg XN/kg BW/day – 6 *per* group) with peqGold TriFast reagent (Peqlab Biotechnologie, Erlangen, Germany) according to the manufacturer's instructions. For Northern blot analysis, samples of 18 μg total RNA were separated by electrophoresis in denaturing agarose gels (2.2 M formaldehyde, 1.5% agarose), capillary transferred to nylon membranes (Nytran NY 12 N, Schleicher & Schuell, Dassel, Germany) and cross-linked by UV-irradiation. cDNA fragments encompassing the open reading frames of rat TBG, TTR, ALB, Sult1a1, Sult2a1, Ugt1a1 and Cyp2b15 were amplified by PCR, subcloned, sequenced and randomly labeled with $^{32}$P-dCTP (Hartmann Analytic, Braunschweig, Germany) to high specific activities (≥$10^9$ cpm/μg). 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 Instrument Company). All data were corrected for variability in loading by normalization to the values obtained with 18S ribosomal RNA. For quantitative real-time mRNA analysis, cDNA probes were prepared by reverse transcription with rat liver RNA using Super-Script II system (Invitrogen). Real-time PCR was performed using SYBR Green Fluorescein Mix (ABgene House, Epsom, UK) with an iCycler iQ (Biorad) following the manufacturer's instructions and the amount of PCR products was calculated using ΔΔ $C_T$ method (normalized against β-actin or HPRT). The following primers were used: DIO1-f: 5′-TTG ACC AGT TCA AGA GGC TTAT-3′; DIO1-r: 5′-TGA TTT CTG ATG TCC ATG TTGT-3′; HPRT-f: 5′-AAG GAC CTC TCG AAG TGT TGG ATA; HPRT-r: 5′-CAT TTA AAA GGA ACT GTT GAC AACG; CAR-f: 5′-ATA TGG CAC TGA GAC TACC-3′; CAR-r: 5′-AAC AAC TGA CAG GAA TTA GG-3′; β-actin-f: 5′-AGC CAT GTA CGT AGC CAT CC-3′; β-actin-r: 5′-CTC TCA GCT GTG GTG GTG AA-3′.

### 2.6 Hormone assays

For total T4 determination, a competitive RIA was performed (DSL-3200 Active; Diagnostic System Laboratory,

 

TX, USA). Twenty-five microliter of each sample (serum or standard) were incubated for 60 min with 200 μL of tracer-buffer reagent in an anti-T4-coated tube at room temperature. Then all tubes were aspirated and washed except for total count tubes, and the radioactivity in each tube was measured for 1 min using an LKB Wallac 1277γ counter (Perkin Elmer). Standard concentrations of T4 ranged from 10 to 500 μg/L. Samples were measured in duplicate.

For total T3 determination, a similar competitive RIA was performed (DiaSorin RIA kit T3-CTK, Rome, Italy). For this technique, 25 μL of each sample (serum or standard) was incubated for 90 min with 200 μL of tracer-buffer reagent in an anti-T3-coated tube at room temperature and then aspirated, washed and measured for 1 min. Standard concentrations of T3 ranged from 0.5 to 7.5 ng/mL. Samples were measured in duplicate.

For thyrotropin (TSH) determination, a rat TSH RIA kit (ALPCO diagnostics, Windham, NH, USA) was used. Twenty-five microliter of each sample was mixed with 50 μL of antiserum (Ab Rat TSH) and incubated overnight (all incubation steps at room temperature). On day 2, 50 μL $^{125}$I TSH was added, mixed and incubated overnight. After 24 h, 100 μL of magnetic particles (SORB Ab FC) were added to each tube except ''total count tube'' to separate bound from free TSH, mixed and incubated for 1 h. The tubes were put on a magnetic plate and allowed to sediment for 15 min. Then, the supernatant was decanted and after one washing step, the radioactivity in each tube was measured using an LKB Wallac 1277γ counter (PerkinElmer). Standard concentrations of TSH ranged from 0.3 to 20 ng/mL. All samples were measured in duplicate.

### 2.7 5′-Deiodinase activity assay

The rat livers were pulverized in liquid nitrogen using a dismembrator (Braun, Melsungen, Germany). Aliquots were homogenized on ice in approximately 5 volumes of 20 mM HEPES (pH 7.0), containing 250 mM sucrose and 1 mM EDTA using a glass/Teflon homogenizer. The homogenates were centrifuged at 10 000 × *g* for 20 min, the supernatants were collected and pellets were resuspended in homogenization buffer supplemented with 1 mM dithiothreitol. Protein concentrations were determined by a modified Bradford assay using IgG as standard (Bio-Rad, München, Germany). The resuspended pellet fractions were assayed for deiodinase activities in a reaction mixture containing 20 μg protein as previously described [37]. Dithiothreitol (10 mM) served as the cofactor and [$^{125}$I]rT3 was added as tracer (50 000 cpm/tube, specific activity: 25 TBq/mmol, PerkinElmer). The substrate concentration was 1.0 μM rT3. Reaction volume was 100 μL, the reaction proceeded at 37°C for 1 or 2 h and the determinations were performed in triplicate. Conditions had been optimized such that final substrate deiodination was less than 15%. 5′-Dio1 and 5′-Dio2 activities were distinguished by the inclusion of 1 mM 6-*n*-propyl-2-thiouracil in the incubation mixture [38]. The fraction of iodide release blocked by 6-*n*-propyl-2-thiouracil was assigned to Dio1, residual background-corrected activity to Dio2. [$^{125}$I]rT3 was purified from free [$^{125}$I] by chromatography using Sephadex LH-20 (Sigma-Aldrich, Muenchen, Germany) before use.

### 2.8 Western blot

Fifty microgram of rat liver cytoplasmic protein fraction (after centrifugation at 13 000 × *g* for 15 min) were subjected to 15% SDS-PAGE and proteins were transferred to a nitrocellulose membrane (Protran, 0.45 μm, Schleicher & Schüll). A primary antibody against rat CAR (Santa Cruz) was used at a dilution of 1:250 and rat β-actin (DakoCytomation, Glostrup, Denmark) as a control at a dilution 1:2000, followed by secondary antibody (DakoCytomation) conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Bands were visualized using the ECL (enhanced chemiluminescence) detection system (Amersham Biosciences, Freiburg, Germany). Signals were quantified by densitometric analysis using the ''Image J'' image analysis software (Wayne Rasband, NIH,). Integrated density represents the sum of the values of the pixels in the image or selection.

### 2.9 Statistical analysis

All statistic calculations were performed with GraphPad Prism Version 4 for Windows (GraphPad Software). Unpaired Student's *t*-test was used to compare two groups. All *p*-values were two-tailed and a *p*-value of less then 0.05 was considered to indicate statistical significance.

## 3 Results

We have shown previously that isoflavones from soy interfere with TH binding to TTR [16]. Therefore, we were interested whether XN, which has some structural similarities with isoflavones would also affect T4 binding to TH distribution proteins. As summarized in Fig. 1, XN displaced [$^{125}$I]-labeled T4 from TTR in a dose-dependent manner, as shown in two different *in vitro* TH-binding assays (Fig. 1A and B). In human serum, T4 was displaced mainly to TBG and in rat serum to ALB (Fig. 1A). To obtain binding characteristics of XN, a competition-binding assay was performed using purified human TTR and L-T4 as a standard agonist. $IC_{50}$ values obtained were 0.1 μM for L-T4 and 1 μM for XN at 1.7 nM [$^{125}$I]T4 tracer concentration (Fig. 1B). To elucidate whether XN has an effect on T4 binding and distribution *in vivo*, sera of female rats treated for 4 wk with 500 mg/kg BW/day *via* the diet and with 1 g XN/kg BW/day by gavage were analyzed and compared with

 

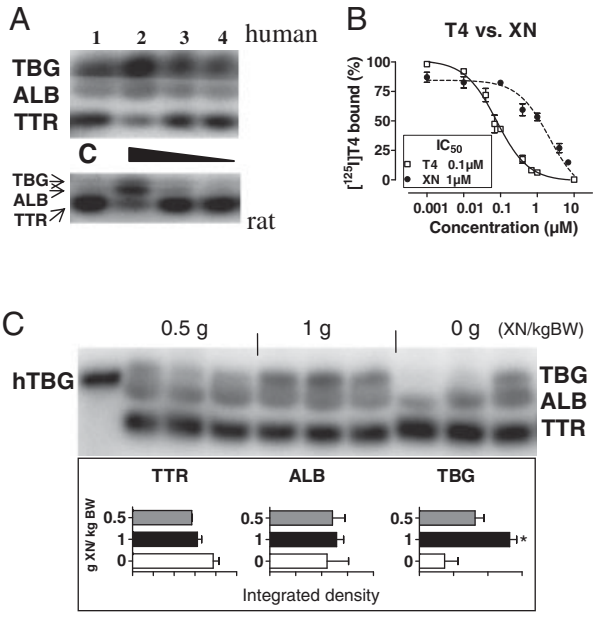


**Figure 1.** XN modulates T4 binding to TH serum transport proteins *in vitro* and *in vivo*: (A) autoradiographs representing effects of XN, added *in vitro*, on binding of [$^{125}$I]T4 to distribution proteins in human and rat serum. Lane 1 (C) represents control sera without added XN. In lanes 2–4, decreasing concentrations of XN (end concentrations 100, 10 and 1 μM) were added to the assay mixture. TBG – thyroxine-binding globulin; ALB – albumin; TTR – transthyretin. (B) Dose–response displacement of [$^{125}$I]thyroxine ([$^{125}$I]T4) from transthyretin (TTR) by L-thyroxine (T4) and xanthohumol (XN). Data points are mean values of at least three measurements in duplicate, with standard deviations represented by vertical bars. [$^{125}$I]T4–TTR binding data were normalized for each experiment to span the range from 0 to 100%. Values for 50% inhibitory concentration were 0.1 μM for T4, and 1 μM for XN at 1.7 nM [$^{125}$I]T4 tracer concentration. (C) Autoradiograph representing binding of [$^{125}$I]T4 to distribution proteins in sera of female SD rats after administration of 0.5 and 1 g XN/kg BW/day for 4 wk. Each group is represented by three different animals. hTBG – purified human TBG was used as a positive control to monitor mobility and high affinity binding of T4 in lane 1. Integrated density represents the mean density values. Error bars represent standard deviations. *Indicates the statistical significance for $p<0.05$ (unpaired Student's *t*-test).

a control group. As shown in Fig. 1C, less [$^{125}$I]-labeled T4 was bound to serum TTR in treated rats compared to control, while [$^{125}$I]T4 binding to TBG was increased. The most pronounced effect was observed in sera of rats treated with the highest dose.

Based on these results, we investigated whether only binding of T4 to distribution proteins was disrupted, or whether administration of XN also changed the hepatic expression of serum TH distribution proteins. Therefore, relative mRNA levels of TBG, TTR and ALB were measured using Northern blot analysis in livers of female rats treated with lower doses of XN *via* the drinking water. As shown in Fig. 2, expression of the TBG transcript was significantly increased and TTR transcript levels were significantly

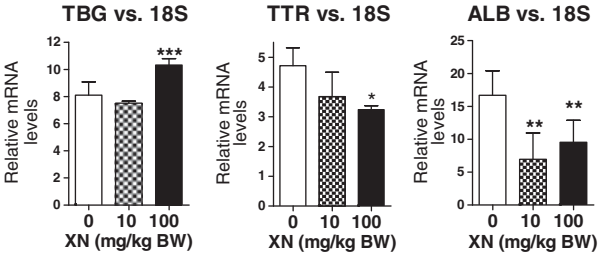


**Figure 2.** XN intervention modulates mRNA expression of TH serum transport proteins: Northern blot analysis of hepatic mRNA levels of thyroid hormone distribution proteins in female SD rats, treated with 10 and 100 mg XN/kg BW/day for 4 wk. Each group represents the mean relative mRNA expression value of six animals, normalized to 18S RNA. Error bars represent standard deviations. TBG – thyroxine-binding globulin; TTR – transthyretin; ALB – albumin. *Indicates the statistical significance for $p<0.05$, **for $p<0.01$ and ***for $p<0.001$ (unpaired Student's *t*-test).

decreased in rats treated with 100 mg XN/kg BW/day, whereas the lower concentration (10 mg/kg BW/day) had no significant effect on mRNA expression of both genes. Interestingly, ALB mRNA expression was strongly downregulated in rats of both treatment groups (10 and 100 mg XN/kg BW/day). However, no dose–response effect was observed.

To determine whether changes in expression and binding properties of distribution proteins after XN intervention modulate TH hormone status, we further measured T4, TSH and T3 levels. TSH and T3 serum levels (Fig. 3A) were moderately increased compared to control by administration of 100 mg XN/kg BW/day. Activity of liver Dio1, an enzyme crucial for production of active TH T3, was increased in both treatment groups. Real-time analysis of the Dio1 transcript also showed a trend of increase in expression (Fig. 3B).

Considering that the TH status depends not only on TH production but also on extrathyroidal metabolism and elimination, the influence of XN on gene expression of TH metabolizing enzymes in liver was investigated. For that purpose, transcripts of the following phase I and II enzymes were examined: Sult1a1, Sult1b1, Sult1c1, Sult2a1, Est6, Ugt1a1, Cyp2b15 and Cyp3a1. Northern blot analysis revealed that XN decreased hepatic Sult1a1 and Sult2a1 mRNA level (Fig. 4). The effect was significant for the 100 mg/kg BW/day group. A dose-dependent decrease in mRNA expression of Ugt1a1 enzyme, which has been shown to glucuronidate T4 [39], was found with both treatment groups. Finally, a dose-dependent downregulation of hepatic phase I enzyme Cyp2b15 mRNA levels was observed with both intervention groups. mRNA expression of all other genes was not influenced by the XN intervention (data not shown).

To investigate whether modulations in mRNA expression of phase I and II enzymes result from a direct effect of XN, or indirectly *via* influencing their transcriptional regulators, mRNA expression levels of nuclear receptors CAR, FXR,

 

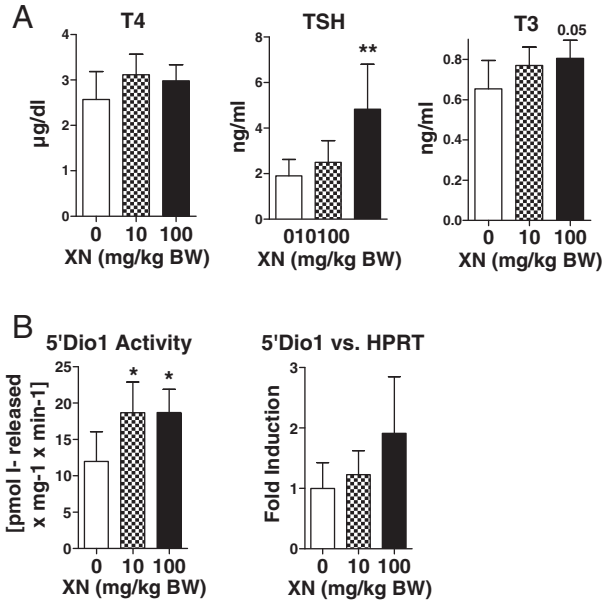


**Figure 3.** XN intervention increases serum TSH and T3 levels and hepatic Type 1 deiodinase (Dio1) activity: (A) thyroid hormone values measured in sera of female SD rats, treated with 10 and 100 mg XN/kg BW/day for 4 wk. Each group represents mean value ± standard deviation of six animals. **Indicates the statistical significance for $p<0.01$ (unpaired Student's *t*-test). T4 – thyroxine; TSH – thyrotropin; T3 – triiodothyronine. (B) Type 1 deiodinase (Dio1) activity and expression levels in female SD rats, treated with 10 and 100 mg XN/kg BW/day for 4 wk. Each group represents the mean value ± standard deviation of six animals. Dio1 activity data represent mean values derived from two determinations. Liver Dio1 mRNA expression values were obtained by quantitative real-time PCR analysis and normalized to HPRT. *Indicates the statistical significance for $p<0.05$ (unpaired Student's *t*-test).

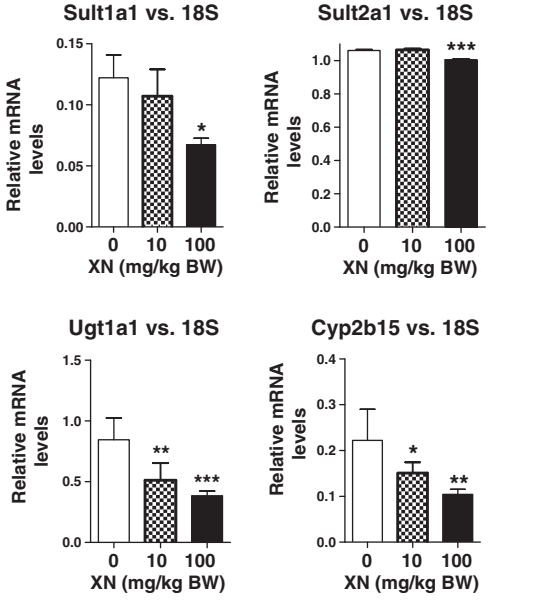


**Figure 4.** XN intervention reduces hepatic mRNA expression of phase I and II conjugation enzymes: Northern blot analysis of hepatic mRNA levels of Sult1a1, Sult2a1, Ugt1a1 and Cyb2b15 in female SD rats treated with XN for 4 wk as indicated. Each group represents the mean relative mRNA expression value of six animals, normalized to 18S RNA. Error bars represent standard deviations. Sult – sulphotransferase; Ugt – UDP glucuronosyltransferase; Cyp – cytochrome P450; *indicates the statistical significance for $^{*}p<0.05$, for $^{**}p<0.01$ and for $^{***}p<0.001$ (unpaired Student's *t*-test).

PXR and RXR were measured by quantitative real-time PCR analysis. As shown in Fig. 5A, rats fed 10 mg XN/kg BW/day expressed about threefold less CAR mRNA compared to control, while in the group fed 100 mg XN/kg BW/day, about twofold decrease was observed. Western blot analysis, using two random samples *per* group, showed a decreased amount of cytosolic CAR protein in rats treated with XN compared to control (Fig. 5B). There were no changes in expression of FXR, PXR and RXR in rats fed XN (data not shown).

## 4 Discussion

In this study, we showed that XN modulates expression of rat liver enzymes and proteins that are essential for maintaining TH homeostasis. XN affects expression of TH distribution proteins TTR, ALB and TBG in rat liver, directly interferes with binding of T4 to TTR, and downregulates the expression of phase I enzyme Cyp2b15 as well as phase II enzymes Sult1a1 and Ugt1a1. The nuclear receptor CAR, which was also downregulated, is a candidate regulatory factor for modulations in gene expression of phase I and II enzymes. A previous safety study on oral *ad libitum* administration of XN in drinking water at low dose ($5 \times 10^{-4}$ M, corresponding to 0.7 mg/kg BW/day) revealed no effect on a variety of hematological and biochemical parameters including free T4 serum levels [40], suggesting some safety margin of this compound.

Soy isoflavones [16] as well as some other natural and synthetic flavonoids [41, 42] interfere with TH binding to TTR. The present *in vitro* study revealed similar binding and competition potencies for XN compared to soy isoflavones. In human serum mixed with XN, radioactively labeled T4 was displaced from TTR mainly to TBG, consistent with TBG's highest affinity to bind TH [43, 44]. In contrast, [$^{125}$I]T4 was displaced from TTR to albumin in rat serum under *in vitro* conditions. This may be due to the fact that under normal physiological conditions, only low expression levels of medium affinity TBG, which is of minor importance for TH distribution compared to humans [14], are observed in rats [45].

These findings prompted us to investigate whether *in vivo* treatment with XN would cause alterations in T4 binding to TTR and the other serum distribution proteins. In fact, we observed a dose-dependent increase in T4 binding to TBG in all treated rats compared to control, whereas

 

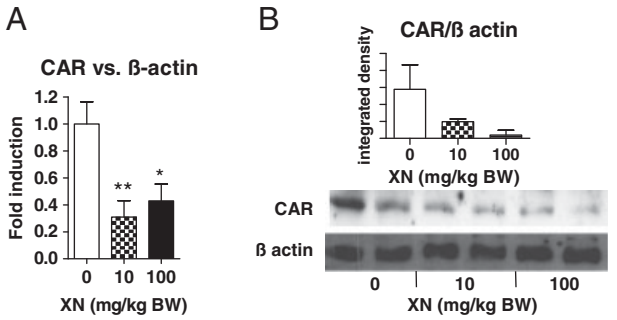


**Figure 5.** XN intervention reduces mRNA and protein expression of constitutive androstane receptor (CAR): (A) quantitative real-time PCR analysis of hepatic mRNA expression of CAR in female SD rats treated for 4 wk with 10 and 100 mg XN/kg BW/day. Each group represents the mean relative mRNA expression of six animals, normalized to β-actin. Error bars represent standard deviations. *Indicates the statistical significance for $^{*}p<0.05$, for $^{**}p<0.01$ (unpaired Student's *t*-test). (B) Western blot analysis of CAR protein from liver cytoplasmic extracts of female rats treated with XN for 4 wk as indicated. Two randomly chosen animals from each treated group are presented on the blot. β-actin was used as a loading control. The graph above represents the mean band intensities relative to β-actin.

less T4 was bound to TTR. However, these experiments did not elucidate whether XN causes only the displacement of T4 from TTR to the other binding proteins, or also additional effects on hepatic protein expression. Consequently, expression levels of TH distribution proteins in liver, which is their major site of production [46], were analyzed in samples of rats treated with lower doses of XN. The obtained results were in agreement with the binding data, demonstrating dose-dependent decrease in TTR mRNA expression in treated rats as well as an increase in TBG transcript levels. Moreover, the expression of ALB was also decreased, although it was not reflected in the binding assay data. ALB is very abundantly expressed comprising about 50% of all serum proteins, but the binding affinity of TH to ALB is the lowest compared to TTR and TBG [47]. Therefore, it is technically difficult to quantify even large changes in its expression and binding properties. ALB levels decrease in burns, liver disease, renal disease, stress and sepsis. One might speculate that XN causes metabolic changes similar to that in liver disease, or rather acute phase, but with exception of decreased ALB and Sult2a1 expression [48] no other typical hepatic acute phase marker was changed (data not shown). Whether XN-induced findings represent a kind of mild acute phase response or the whole liver metabolism is slowed down, and therefore TTR also is decreased, remains to be elucidated in further studies. However, acute phase and impaired liver status is typically associated with decreased serum T3 level, which contrasts to our observation. Whether these changes in XN-dependent distributor protein expression and TH binding alter free hormone concentrations in the blood and other body fluids requires more detailed and comprehensive acute kinetic and chronic exposure models including positive controls for interference with distributor proteins and hepatic metabolism.

TSH was significantly increased in rats treated with XN, but this increase is not strong enough to designate XN as a goitrogen. In addition, increased hepatic Dio1 activity was found. However, it is hard to reconcile increasing T3 serum levels with both increased serum TSH and hepatic Dio1 activity. Elevated serum TSH might indicate mild hypothyroidism registered at the pituitary thyrotrope cell level [16], which is difficult to reconcile with elevated serum T3. Possibly, hypothalamic TRH stimulation of thyrotropes might be impaired indicating a disturbed hypothyroid brain-CSF input, a consequence of impaired TH binding to TTR, which is the sole TH distribution protein in the cerebrospinal fluid. Elevated serum T3 is compatible with increased activity of Dio1, as observed in hyperthyroidism for this T3-responsive enzyme. Serum T3 might be higher in XN-treated rats because of lower degradation in the liver and increased production by peripheral deiodination, while the pituitary gland might sense impaired TRH stimulation and/or lower local T3 concentrations leading to a further increase in TSH. However, future studies focused on the hypothalamus–pituitary axis and TH binding in the cerebrospinal fluid are needed to understand this ''deranged'' TH homeostasis. Such constellations resemble pituitary TH resistance caused by mutations in the human TRβ receptor or are typical for defects in MCT8, the cell membrane transporter for T3, which is also expressed in the pituitary [49]. To explain elevated serum T3 levels, we investigated if some of the enzymes important for hepatic TH degradation were impaired after treatment with XN. Previous *in vitro* studies provided evidence for conjugation of XN by human recombinant SULT and UGT isoforms [50]. Here, we found decreased mRNA expression of phase II enzymes Sult1a1 and Ugt1a1 and phase I cytochrome P450 enzyme Cyp2b15 in livers of rats treated with XN. Ugt1a1 is a phase II enzyme involved in TH metabolism, which was downregulated by XN with clear dose dependence. Besides other endogenous compounds, Ugt1a1 glucuronidates predominantly T4 and rT3, but not T3 [51, 52]. T3 is, on the other side, a potent regulator of Ugt1a1 activity and transcription. With the exception of one *in vivo* study showing an increase in Ugt1a1 expression after T3 administration [53], other authors reported decreased activity and expression [54, 55], especially with vitamin A as co-regulator [56]. Although we observed an increase in serum T3 after administration with 100 mg XN/kg BW/day XN, we have no evidence that T3 causes this decrease in Ugt1a1 expression. It might rather be an effect on common transcription regulators of phase II enzymes Sult1a1, Sult2a1, Ugt1a1 as well as phase I enzyme Cyp2b15, which were all downregulated.

Hormones and xenobiotic agents that induce P450 expression also affect the expression of the SULT multigene family [57]. It was also shown that prenylated flavonoids from hops inhibit various Cyp enzymes *in vitro* [5, 58, 59]. The candidate common transcription regulator

 

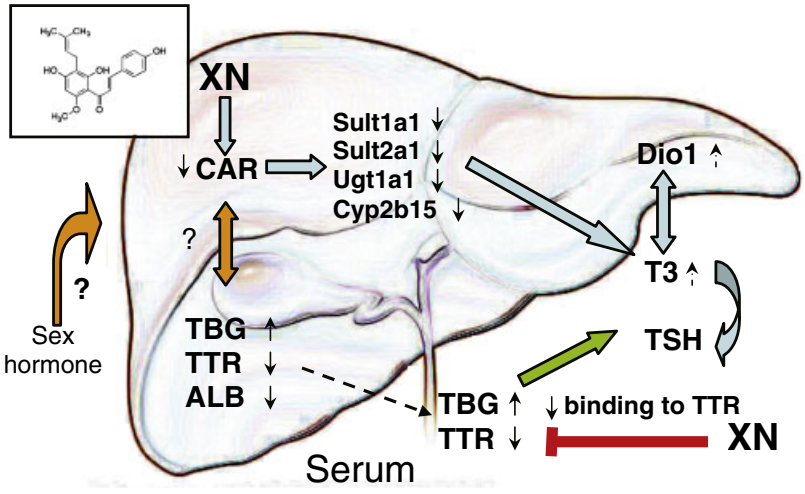


**Figure 6.** Scheme summarizing the effects of XN on TH levels as well as on TH distributing and metabolizing proteins in serum and liver.

for expression of all the above-listed phase I and II genes is the nuclear hormone and xenobiotic receptor CAR. The list of CAR target genes includes all phases of xenobiotic and hormone metabolism, such as oxidative metabolism, conjugation phase and transport phase [28, 30, 32, 60–66]. Interestingly, we observed no clear dose–response in downregulation of CAR transcript. One possible reason might be an individual variation in susceptibility to XN related to some endogenous cofactors such as sex hormones, knowing that these animals were not ovariectomized. There is one report showing sex-dependent regulation of Sult2a1 by phenobarbital (PB) in Wistar Kyoto, but not in Fischer rats [28]. Qatanani *et al.* suggested that CAR functions as global regulator of increased sulfation and glucuronidation induced by the antiepileptic reagent PB [32], which also induces Cyp2b1 gene expression in rat and mouse livers *via* CAR activation [28, 67–69]. XN effects on phase I and II enzymes as well as their regulator nuclear receptor CAR are obviously opposite compared to PB.

In conclusion, this study showed that XN affects TH distribution and alters expression of TH distribution proteins on one side and hepatic enzymes important for degradation and elimination of TH on the other (summarized in Fig. 6). Even though we could not yet identify the direct link between these two groups of effects, the fact that both distribution and elimination are important for TH homeostasis and that the liver is the central organ involved in these processes prompted us to report this data together. Though our study contributes to XN research with respect to its interference in the TH axis, it does not allow concluding if this substance is beneficial or adverse for human health. We cannot predict at this moment if the doses under investigation might affect human health but exposure level might increase following the current marketing efforts by industry. The present studies expand the list of XN antitumor properties as XN decreases CAR levels, which plays a pivotal role in liver tumor promoter activity of PB [70]. Decreased CAR might lead to decreased detoxification and elimination of some xenobiotics. However, induction of the phase II enzyme quinone reductase by XN has been shown *in vitro* and *in vivo* in mice [6, 71], but this inducing potential is more commonly attributed to activation of transcription factor Nrf2 [72]. Further studies in comparison to agents known to interfere with serum distribution and hepatic metabolism [19] are needed to shed more light on the mechanism of action of XN on the central parts of the TH axis.

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