

Retinol has specific effects on binding of thyrotrophin to cultured porcine thyrocytes

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Abstract

Retinoids are potential candidates for the treatment of thyroid cancer. However, one of the disadvantages of these substances is their dedifferentiating effect on normal non-transformed thyrocytes. To identify conditions under which no dedifferentiating effect of retinol on normal thyrocytes can be observed, we determined iodide uptake, protein iodination, expression of sodium–iodide symporter (NIS) mRNA and protein, and the binding of iodine-125-labelled bTSH in cultured porcine thyrocytes. Combination of TSH and $\leq 6.5 \mu\text{M}$ retinol increased iodide uptake and protein iodination compared with TSH alone over the entire incubation time, whereas TSH plus $\geq 13 \mu\text{M}$ retinol increased the uptake of iodine-125 only during the

first 12 h but decreased it after 30 h and longer. After ≥ 30 h incubation times with $\geq 13 \mu\text{M}$ retinol, the fraction of apoptotic cells was enhanced and proliferation decreased. The incubation with retinol enhanced the binding of [¹²⁵I]bTSH to thyrocytes, but did not influence expression of the NIS. With low retinol concentrations, the effect on the binding of TSH apparently predominated and retinol increased thyroid function; with higher concentrations the pro-apoptotic effect of retinol overlapped and a two-phased time course resulted. It can be concluded that low concentrations of retinol also exert differentiating effects in normal thyrocytes.

Journal of Endocrinology (2004) **183**, 617–626

Introduction

Retinoids influence proliferation, differentiation and apoptosis in many cell types *in vivo* and *in vitro*. Their main differentiating effects on transformed cells are the induction of iodothyronine 5'-deiodinase (Schreck *et al.* 1994, Schmutzler & Kohrle 2000) and of the sodium–iodide symporter (NIS) (Schmutzler *et al.* 1997, Schmutzler & Kohrle 1998, Filetti *et al.* 1999, Schmutzler & Kohrle 2000, Schmutzler 2001). In contrast to these positive effects on transformed cells, retinoid acid decreases the expression of the NIS in normal rat thyrocytes (Schmutzler *et al.* 1997, Schmutzler & Kohrle 1998, Filetti *et al.* 1999, Schmutzler & Kohrle 2000) and both the thyroid-stimulating hormone (TSH)-induced uptake of iodide and the release of organified iodine in normal human thyrocytes (Namba *et al.* 1993). High concentrations of retinol also exert dedifferentiating effects on normal thyrocytes (Fröhlich *et al.* 2001). These effects limit the value of retinoids for the treatment of thyroid carcinomas.

Retinoids are believed to act via nuclear receptors (Chambon *et al.* 1991, Chertow *et al.* 1996), which suggests that the effects on protein concentrations are

expected to occur within a range of hours. In keeping with this idea, the decrease in the expression of NIS protein has been observed after 24–36 h (Kogai *et al.* 1997). However, this might not be the only action of these substances: within 1 h, retinoic acid modulates gap junction permeability in neurones and in numerous mammalian cells (Brummer *et al.* 1991, Weiler *et al.* 1999) and markedly reduces the concentrations of inositol phosphate and diacyl glycerol in HL-60 cells (Geny *et al.* 1991).

Using normal porcine thyrocytes as a culture model, we aimed to detect conditions under which no dedifferentiating effects on normal thyrocytes were observed and to detect effects that may be initiated after short periods of incubation. We determined iodide uptake, iodination of protein, NIS mRNA, NIS protein expression and the number of NIS-immunoreactive cells after different periods of incubation with retinoids, retinoids plus TSH or TSH alone. The effects of retinol, other alcohols and retinoic acid on the binding of TSH to thyrocytes were also investigated. We confined our study mainly to the lower range of retinol concentrations under which differentiating effects on the human follicular carcinoma cells line FTC 238 had been detected (unpublished results).

Materials and Methods

Isolation of thyroid cells and culture conditions

Porcine thyroid glands were obtained from the local slaughterhouse, and cells were isolated as described previously (Wahl *et al.* 1992). For the determinations of NIS protein and NIS mRNA, 1.5×10^6 cells per well (12-well Costar No. 3512 culture plates) were seeded. For the measurement of iodide uptake, 0.13×10^6 cells were seeded per well (48-well culture plate, Costar No. 3548). In all experiments, the number of cells was sufficient for growing to confluence. NCTC (National Culture and Tissue Collection)-135 medium (standard and special retinol-free preparation, Gibco) supplemented with 2% of the serum substitute, Ultrosor G (BioSeptra, Villeneuve-la-Garenne, France), penicillin (200 IU/ml), streptomycin (0.2 mg/ml) and ciprofloxacin (Ciprobay, Bayer; 60 µg/ml) was used for the experiments. Standard medium contained about 0.6 µM retinol; these trace amounts of retinol are important because they decrease apoptosis and increase the rate of proliferation in porcine thyrocytes compared with retinol-free medium (Fröhlich *et al.* 2001). In Ultrosor G, no retinol was detectable by HPLC. Ultrosor G contains about 130 µg/l selenium, detected by HPLC, which is essential for thyroid function (Kohrle 1996, 1999). It also contains insulin (250 mg/l). The culture conditions were: 37 °C at 100% humidity in 5% CO₂ and 95% air.

Stimulation procedures

All-*trans* retinol and all-*trans* retinoic acid (Sigma), diluted in methanol, were added in concentrations from 0 to 30 nmol/well (0–40 µM). Concentrations less than 27 µM do not induce apoptosis or necrosis to a significant degree after incubations for up to 48 h (Fröhlich & Wahl 1999). Control cultures contained only methanol.

Routinely, before the stimulation with porcine TSH, with retinoids or with both together, the thyroid cells were cultured in standard medium or retinol-free standard medium with or without TSH (Sigma) for 18 h, to make certain that follicle and monolayer cultures were investigated; this time is not included in the times of incubation that are given. Unless otherwise indicated, the TSH concentration was 1 mU/ml. To investigate the influence of the retinol stimulation on the uptake of iodide, the following stimulation procedure was also used: firstly, the cells were cultured without TSH for 18 h, then stimulated with 13 µM retinol for 6 h, and subsequently 1 mU/ml TSH (without retinol) was added. Uptake of iodine-125-labelled iodide was measured 6, 12, 30 and 48 h after stimulation with TSH. Secondly, to evaluate the effect of repeated stimulation with retinol, thyrocytes were stimulated with TSH plus retinol for 18 h, thereafter kept in TSH-containing medium without retinol stimulation for

24–30 h, and then again stimulated with retinol (13 µM). The results from these experiments were compared with those from cultures stimulated with TSH only.

To estimate whether synthesis of new proteins influences the uptake of [¹²⁵I]iodide, thyrocytes were cultured in the presence of cycloheximide (Sigma), an inhibitor of translation. Cycloheximide was diluted in ethanol and concentrations of 1, 10 and 100 µg/ml were used. Cycloheximide in combination with 4 kBq [¹²⁵I]NaI/well was added to the culture medium together with TSH alone, TSH plus ethanol (solvent control) or TSH plus retinol.

Uptake of [¹²⁵I]iodide

Uptake of [¹²⁵I]iodide was determined with and without stimulation with different concentrations of TSH or retinoids according to the stimulation procedures. NaI (1 µM final concentration) traced with 4 kBq/well carrier-free [¹²⁵I]NaI (Amersham Pharmacia Biotech) was added to each culture 6 h before measurements were made. The cells were collected and washed with a 48-well cell harvester (Inotech IH 280, Inotech, Dottikon, Switzerland). Filtermats (Skatron 11731, Skatron, Lier, Norway) were transferred to counting tubes and their radioactivity measured. To test the specificity of NIS-mediated iodide uptake, controls were performed by the addition of 1 mM sodium perchlorate, a competing inhibitor of thyroid iodide transport.

Iodination of protein

Cells incubated with [¹²⁵I]NaI in six-well culture plates were detached from the support, rinsed with 2 ml ice-cold Earle's balanced salt solution (EBSS) buffer and centrifuged at 500 g for 4 min. The cells were rinsed again in 2 ml ice-cold EBSS and radioactivity counted in a multi-gamma counter. Thereafter, 0.5 ml 10 µM non-radioactive NaI and 0.5 ml fetal calf serum (Sigma) were added, followed by 2 ml ice-cold 20% (v/v) trichloroacetic acid (TCA). The mixture was centrifuged at 500 g for 5 min and the resulting pellet was resuspended, washed twice with 10% cold TCA and the radioactivity counted as protein-bound iodine.

Quantification of NIS mRNA by PCR

Cells from 12-well culture plates were removed by pipetting from the support and collected in PBS. After centrifugation at 400 g for 4 min, the pellets were deep-frozen at –80 °C. Total RNA was isolated using the NucleoSpin RNA II (Macherey Nagel) kit according to the manufacturer's recommendations. The concentrations of RNA preparations were calculated by measuring the absorbance at 260 nm. One microgram total RNA was used for cDNA synthesis using the 1st Strand cDNA Synthesis Kit for RT-PCR (AMV, Roche Molecular

Biochemicals). cDNA was diluted 1:6 and 1.8 µl were used as a template for the PCR in a total reaction volume of 20 µl. For quantification of mRNA, the LightCycler-FastStart DNA Master SYBR Green I Kit (Roche Molecular Biochemicals) was used. The porcine NIS-specific primers were: 5'-TCA TGT CGG CGG TAC AGG TG-3' (forward, 476–495) and 5'-G TCC AGA TCA CAG CCT TCA TGC-3' (reverse, 845–824). The respective primers for actin were 5'-ACC TTC AAC ACG CCG GCC AT-3' (forward, 10–29) and 5'-CCA CAC GGA GTA CTT GCG CT-3' (reverse, 654–635). PCR was performed in the LightCycler (Roche) with plasmid DNA as external standards. The standards were produced as follows: the respective PCR products were cloned into a pCR-script Amp SK (+) cloning vector (Stratagene) and transformed in *Escherichia coli*. Plasmids were isolated using the QIAprep Spin Miniprep Kit (Qiagen). Serial dilutions of the plasmids containing the cloned inserts were then used as standards for quantification of mRNA.

Determination of NIS protein

For localization of the NIS, peptide antibodies (termed NISs 1–5) from different parts of the human NIS (raised in rabbits) were used. NIS 1 antibody was directed to the amino acid sequence 262–280, NIS 2 antibody to sequence 468–487, NIS 3 antibody to sequence 495–515, NIS 4 antibody to sequence 560–579 and NIS 5 antibody to the sequence 629–643 of the C-terminal part of the molecule. NIS 1 and NIS 2 sequences are located in close vicinity to a transmembrane domain; they reacted only weakly in immunocytochemistry and western blot. The human NIS 3 sequence contains, in its posterior half, two hydrophobic amino acids more than the respective porcine sequence, and the NIS 4 peptide differs by one amino acid from porcine NIS (Selmi-Ruby *et al.* 2003). These two NIS antibodies worked best for the detection of immunoreactive protein in sections and homogenates, and were used in the following experiments.

Cellular localization of NIS by immunohistochemistry

NIS protein was localized after 42 h of culture with or without retinol. Thyrocytes from two six-well culture plates were mechanically removed from their support by pipetting and suspended in a small volume of PBS. The suspension was transferred to slides coated with chrome-gelatin. The slides were dried for 30–60 min at 40 °C. After preincubation with blocking solution (5% normal goat serum, 0.5% Triton X100, 1% BSA in 0.1 M PBS pH 7.4) for 30 min at room temperature, the slides were incubated with the anti-NIS antibodies (1:100) diluted in a solution of 5% normal goat serum, 0.5% Triton X100 in 0.1 M PBS pH 7.4 at 4 °C overnight. After rinses in 0.1 M PBS, the slides were incubated with biotin-labelled anti-rabbit IgG (goat, Dianova, 1:100) for 1 h at room

temperature and alkaline phosphatase-labelled streptavidin (1:200, Dako Diagnostica) for 40 min at room temperature. The substrate solution for the red reaction product contained 8 mg naphthol AS-MX phosphate (Sigma) in 0.2 ml DMFA (dimethylformamide), 3 mg levamisole (Sigma) and 10 mg fast-red TR salt (Sigma) in 10 ml 0.1 M Tris/HCl pH 8.2. Incubation time with the substrate solution was 1 h at room temperature.

Quantification of NIS in homogenates by western blotting

Cells for quantification of NIS protein were collected after 6 h, 18 h and 42 h in the presence or absence of retinol. Thyrocytes from two six-well culture plates were collected in PBS after mechanical removal from their support. After centrifugation at 400 g for 4 min, cells were washed three times with homogenization buffer (0.02 M triethanolamine hydrochloride, 1.5 mM EDTA, 1 mM MgCl₂, 5 mM mercaptoethanol, pH 7.4). Each pellet was homogenized in 100 µl homogenization buffer. An equal volume of SDS loading buffer (0.01% Bromphenol Blue, 18% glycerol, 10.5% mercaptoethanol, 2% SDS, 0.13 M Tris/HCl pH 6.8) was added and the mixture heated at 95 °C for 5 min. Electrophoresis was performed on a 9% SDS polyacrylamide gel (Protean Minicell Chamber) according to the method of Laemmli (1970); 30 µg protein were loaded per lane. High molecular weight markers (53 000–212 000 Da; Pharmacia) were used as standards. Protein bands were detected with Coomassie Blue.

Proteins were transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore) according to the method of Towbin *et al.* (1979). For all incubations, Tris-buffered saline (TBS) consisting of 0.02 M Tris and 0.9% NaCl, pH 7.4 was used. Membranes were preincubated in 5% BSA in TBS at 4 °C for 18 h. Subsequently, the membranes were incubated in the following solutions: antibody to NIS 3 or NIS 4 (1:1000 diluted in 1% BSA, 0.05% Tween 20 in TBS) for 2 h; biotin-labelled anti-rabbit antibody (1:1000, goat, Dako) in TBS for 1 h and alkaline phosphatase-labelled streptavidin (1:2000, Dako) in TBS for 1 h. Between the incubations with the NIS antibody, with biotin-labelled anti-rabbit antibody and with alkaline phosphatase-labelled streptavidin, the blots were washed three times for 5 min with 0.1% BSA in TBS. The bands were visualized with Sigma Fast BCIP/NBT tablets (1 tablet for 100 ml aqua dest). The band densities were measured from scanned images of each western blot and quantified with NIH image-managing software (Scion).

Incorporation of [³H]thymidine into thyrocytes

Cultures (5 × 10⁵ cells/750 µl) with or without retinoid were incubated with 37 kBq [6-³H]thymidine/ml (specific activity 935 GBq/mmol; Amersham) for 24 h.

The cells were harvested with a Cell Harvester (Skatron Instruments) on filtermats (printed filtermat A 1205-401, Wallac, Turku, Finland).

Dried filtermats were put in sample bags (Sample Bags 1204-411, Wallac), scintillant (Ultima Gold Scintillant, Packard) was added and the bags were welded closed. Samples were measured in a Betaplate 1205 (Wallac).

Detection of apoptotic cells by annexin V labelling

The cells were harvested 48 h after addition of the respective retinoid. To obtain single cells, cells were passed through a 0.025 mm mesh sieve (Catalogue No. 1-4301, Weisse & Eschrich, Ludwigsstadt/Bayern, Germany) and centrifuged at 400 g for 6 min. The cells were then washed with 0.1 M PBS pH 7.4, and apoptotic cells were detected with the Apoptosis Detection Kit (R&D Systems): 100 µl 10⁶ cells/ml suspended in binding buffer were incubated with 10 µl annexin V-fluorescein isothiocyanate (FITC) (10 µg/ml) and 10 µl propidium iodide (50 µg/ml) for 15 min at room temperature in the dark. The FITC signal was detected at 488 nm, that of propidium iodide at 670 nm.

Binding of [¹²⁵I]bTSH (bovine TSH) to thyrocytes

Thyrocytes (130 × 10³/200 µl; 48-well culture plate, Costar No 3548) were cultured for 3 days in standard NCTC medium in the presence of dibutyryl-cAMP (0.4 mM; Roche) to aggregate to right-side right-follicle-like structures. On day 3, [¹²⁵I]bTSH (0.4 kBq/well) and 10 mU TSH (Sigma) for measurement of non-specific binding were added and, 60 min later, 13 µM or 27 µM retinol, or all-*trans* retinoic acid, or methanol, ethanol, pentanol or decanol 13 µM each was added. Cells were harvested after 30, 60, 120 and 240 min, and bound radioactivity was measured.

The [¹²⁵I]bTSH, purified by affinity chromatography using a solid-phase-bound TSH receptor, with a specific activity of approximately 2.22 MBq/µg, was a gift from Dr Steinmaus, Medipan, Berlin. Commercially available 'crude' preparations of TSH are unable to displace the radiolabelled TSH from its receptor (Szkudlinski *et al.* 1996). As a consequence, the non-specific TSH binding could not be determined.

Statistics

Experiments were performed at least three times. Test values are expressed as means ± S.E.M. For the [¹²⁵I]iodide uptake studies, differences between various settings were analysed using one-way analysis of variance. Multiple comparisons were taken into account with the Student–Newman–Keuls procedure. Statistical significance was assumed at *P* < 0.05. For the histograms presented in Figure 1, the original values were normalized in order to elimi-

nate the influence of variations in uptake of [¹²⁵I]iodide between experiments and different times of incubation (6–48 h) with [¹²⁵I]NaI. The radioactivity of the stimulated cultures was standardized against unstimulated cultures (without TSH and without retinol). These unstimulated cultures were set as 100% or 1.

Results

Retinol (0.6 µM) as a component of the NCTC 135 medium (standard medium) was termed 'physiological concentration'; the term 'stimulation with retinol' refers to concentrations of retinol greater than 3.25 µM that were added to the medium.

Uptake of [¹²⁵I]iodide, protein iodination, NIS mRNA and NIS protein were determined after short (6–12 h) and long (30–48 h) periods of incubation in order to differentiate between short- and long-term effects of retinol. In contrast to procedures in other studies of retinoids or NIS (Arai *et al.* 1991, Kogai *et al.* 1997, Schmutzler *et al.* 1997), thyrocytes (except controls) were exposed to TSH immediately after seeding.

Uptake of [¹²⁵I]iodide into thyrocytes

During the entire time of culturing, uptake of iodide into thyrocytes was greater in cultures treated with TSH than in cultures without TSH. In the absence of retinol, iodide uptake was maximal after stimulation with 1 mU/ml TSH; in the presence of retinol, 0.5 mU/ml and 1 mU/ml TSH were similarly effective. In other words, retinol increased uptake of iodide by thyrocytes in the presence of suboptimal concentrations of TSH.

Increases in iodide uptake were observed in the presence of 3.25 µM and 6.5 µM retinol plus TSH compared with TSH alone over the entire incubation time of 48 h. The increase after stimulation with 6.5 µM retinol plus TSH was statistically significant. In the presence of 13 µM retinol, a biphasic effect was noted (Fig. 1a). This biphasic effect consisted of a significant increase in iodide uptake over the first 12 h of incubation and a subsequent significant decrease in iodide uptake after incubations of 30 h and longer. A similar biphasic time course was also noted with TSH plus 27 µM retinol (data not shown). At concentrations greater than 40 µM retinol, iodide uptake was decreased over the entire period of incubation. All-*trans* retinoic acid, whether alone or in combination with TSH, did not increase the uptake of iodide. In the absence of TSH (monolayer cultures), the stimulation produced by retinol was significantly lower than that in TSH-treated (follicle) cultures (Fig. 1a, last group of columns).

To determine whether the effects of pharmacological doses of retinol were dependent on the 'physiological' concentrations of retinol in the standard NCTC 135 medium, cells cultured in retinol-free NCTC medium were tested and compared with cells cultured in standard

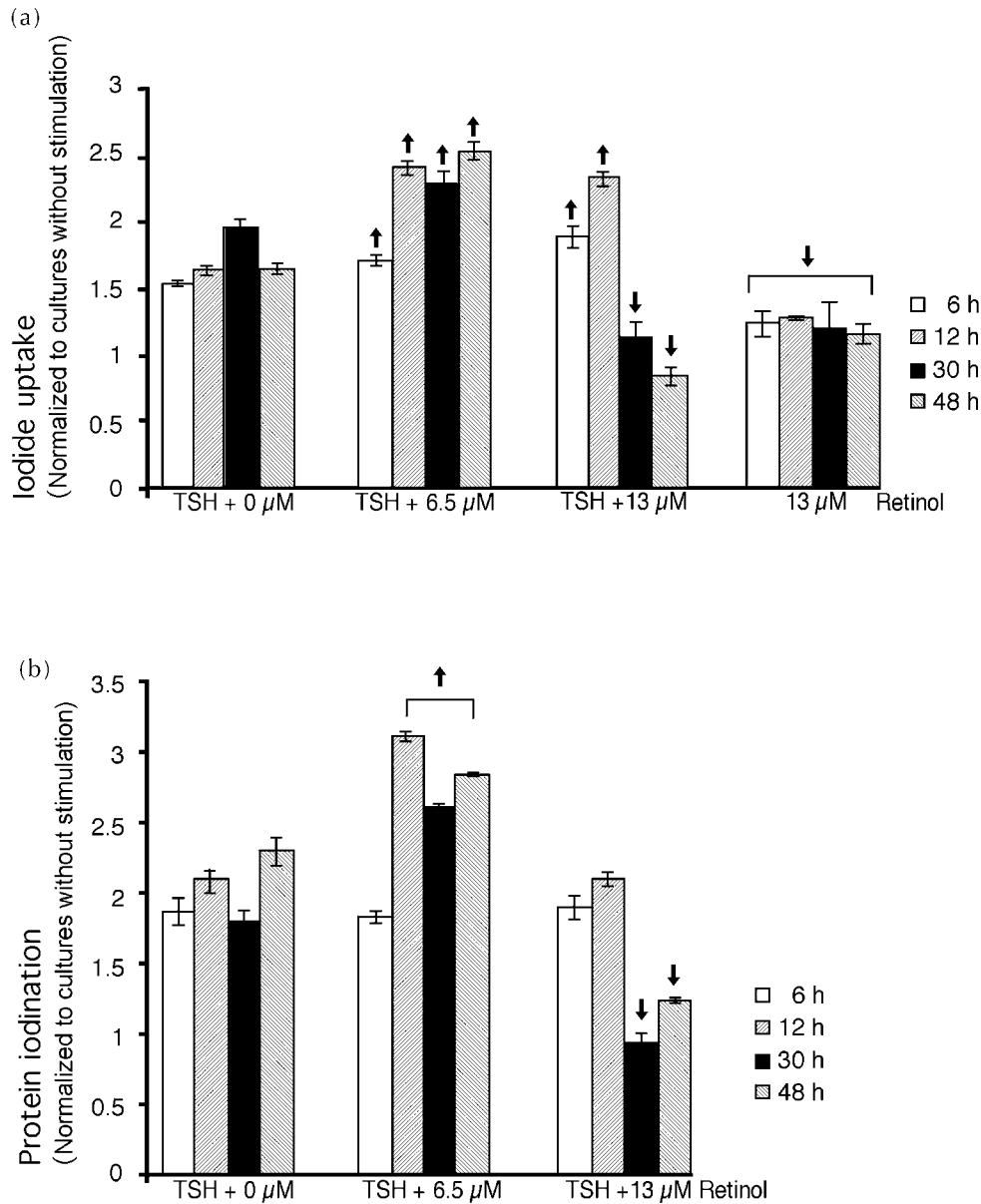


Figure 1 (a) Uptake of [125 I]iodide and (b) protein iodination, after short and prolonged incubation with TSH (1 mU/ml) alone, combinations of TSH and retinol, or retinol alone ($n=4$). Cultures without TSH were taken as the reference. Cells were precultured for 18 h in standard medium. Values are expressed as means \pm S.E.M. (a) The increase in uptake of [125 I]iodide was significantly greater in thyrocytes cultured with TSH plus retinol compared with TSH alone; it was restricted to short incubation times in the presence of at least 13 μ M retinol, but lasted for the entire period of incubation in the presence of 6.5 μ M retinol. Significant ($P<0.05$) differences in [125 I]iodide uptake compared with that in the presence of TSH alone: \uparrow increase; \downarrow decrease. (b) Iodination of protein was increased significantly only in incubations with 6.5 μ M retinol plus TSH.

NCTC medium: the basal uptake of iodide in retinol-depleted medium was 23% less than that in medium with physiological concentrations of retinol. Cells reacted to TSH alone and to retinol alone to an almost similar degree as in the standard NCTC medium. However, in retinol-

free medium uptake of iodide in the presence of TSH plus retinol was at all times significantly less than that after stimulation with TSH alone.

To test the specificity of the iodide uptake, perchlorate (1 mM) was included. Iodide uptake in these cultures

($n=6$) was significantly reduced compared with that in the unstimulated cultures: in retinol-containing medium to about 15% after 6 h and to 5% after 12 h, and in medium without retinol to 18% after 6 h and to 45% after 12 h.

Iodination of protein

Determination of protein iodination examines whether not only the uptake but also subsequent steps in the synthesis of thyroid hormone are influenced by the retinol treatment. The time-dependent iodination of protein followed a time curve similar to that of the iodide uptake for $6.5 \mu\text{M}$ retinol plus TSH and was significantly increased (Fig. 1b). The increase in iodinated protein in the presence of $3.25 \mu\text{M}$ retinol plus TSH was found to be lower than in the presence of $6.5 \mu\text{M}$ retinol plus TSH and was not significant. In the case of TSH plus $13 \mu\text{M}$ retinol, iodination of protein was slightly but not significantly increased after the first 12 h of incubation and significantly decreased after longer incubation times. In the presence of greater concentrations of retinol, protein iodination was decreased over the entire period of incubation.

Identification of effects on translation, proliferation and apoptosis

We investigated whether the effect of stimulation with $13 \mu\text{M}$ retinol for a short period of time was caused by any of the known actions of the retinoids, by determining NIS mRNA and NIS protein levels, by performing stimulations in the presence of an inhibitor of translation, and by evaluating the incorporation of [^3H]thymidine and the proportion of annexin-V-labelled cells.

NIS mRNA NIS mRNA content in the presence of TSH for longer than 6 h was significantly greater than that in unstimulated cultures. Stimulation with TSH plus retinol did not further enhance NIS mRNA. No significant differences in the amounts of actin mRNA were noted between the cultures. The levels of NIS mRNA in cells cultured in standard and in retinol-free NCTC medium were roughly similar.

NIS protein In the western blots, one band with an apparent molecular mass of about 80 kDa was detected (Fig. 2a). Significant differences were noted between samples that were or were not treated with TSH, but not between cultures that did or did not undergo stimulation with retinol (Fig. 2b). The content of NIS protein in cultures with retinol-depleted standard medium and standard medium was roughly similar. To confirm that the 80 kDa protein band in the western blot represented NIS protein, the same antibody was used to localize the protein in sections of normal porcine thyroid glands. The product of the antibody reaction was localized at the basal part of the thyroid cells (Fig. 3a), as would be expected for NIS

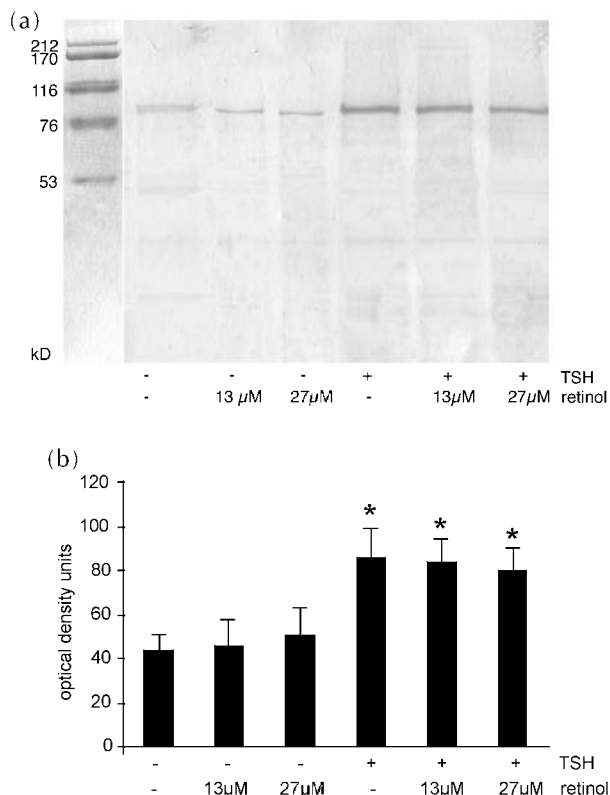


Figure 2 (a) Detection of NIS protein with NIS 3 antibody by western blotting in thyrocytes cultured in standard medium. (b) Evaluation of the band at about 80 kDa by densitometry. Cultures were stimulated with or without TSH and different concentrations of retinol for 42 h. There were significant increases ($*P<0.05$) in NIS protein in the TSH-treated cultures, but no marked influence of retinol could be detected. Means \pm S.E.M. are given.

protein. In cultures, the fraction of NIS-immunoreactive cells was low in the absence of TSH or of retinol (Fig. 3b). The fraction increased from $6 \pm 1.5\%$ ($n=3$) in cultures without TSH and without retinol to $12 \pm 2.1\%$ ($n=3$) in cultures with TSH, and to $18 \pm 3.4\%$ ($n=3$) in cultures on stimulation with retinol ($13 \mu\text{M}$) plus TSH. These increases, however, were not significant.

Inhibition of translation by cycloheximide In the presence of $100 \mu\text{g/ml}$ cycloheximide, iodide uptake in TSH-treated samples was reduced to $110 \pm 4\%$ that of the unstimulated control ($n=4$). Iodide uptake was also reduced in the presence of $1 \mu\text{g/ml}$ and $10 \mu\text{g/ml}$ cycloheximide in the samples treated with TSH. In all cycloheximide-treated samples, iodide uptake in the presence of TSH plus $6.5 \mu\text{M}$ or $13 \mu\text{M}$ retinol was about 10–30% greater than that in the samples treated with TSH alone.

Proliferation and apoptosis After 48 h in the presence of $13 \mu\text{M}$ retinol (corresponds to 62 h in culture), effects

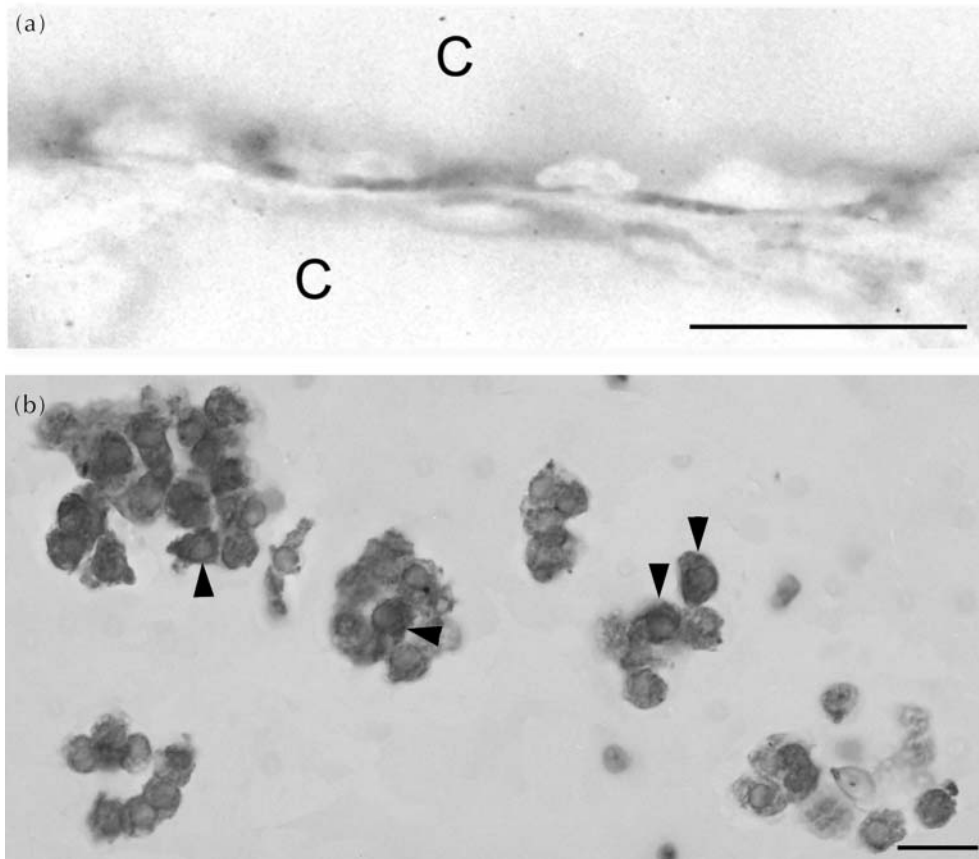


Figure 3 Immunocytochemical detection of NIS protein with NIS 3 antibody in (a) intact thyroid glands and (b) thyrocytes cultured in standard medium stimulated with 13 μM retinol for 42 h. NIS protein was localized in the basal portion of the follicle cells (a). Several NIS-immunoreactive cells are seen in the cultured thyrocytes (arrowheads). C, Colloid in the follicle lumen. Bars represent 20 μm.

on proliferation and apoptosis of thyrocytes were detected. Incorporation of [^3H]thymidine was decreased from $100 \pm 17\%$ in retinol-untreated cultures to $78 \pm 5\%$ in retinol-treated cultures. No decrease in incorporation of [^3H]thymidine was noted at up to $6.5 \mu\text{M}$ retinol.

The fraction of apoptotic cells was $11 \pm 0.5\%$ in cultures without retinol and TSH, $5 \pm 0.8\%$ in cultures with TSH and without retinol, $9 \pm 0.7\%$ in cultures with TSH plus $6.5 \mu\text{M}$ retinol, and $28 \pm 2\%$ in cultures with TSH plus $13 \mu\text{M}$ retinol. The increase in apoptotic cells in the presence of TSH plus $13 \mu\text{M}$ retinol was significant both compared with the unstimulated cultures and compared with cultures in the presence of TSH alone.

Identification of reversible membrane effects

The established retinoid effects on proliferation and apoptosis could not explain the initial increase in iodide uptake observed during the first 12 h of incubation. We therefore investigated the influence of retinol on the

interaction of TSH with the thyrocyte membrane, by preincubating cells with retinol before the application of TSH alone, repeatedly stimulating the cells with TSH plus retinol, and measuring the retention of [^{125}I]bTSH by the thyrocytes.

The increases in iodide uptake and in protein iodination were also observed after sequential incubation with retinol plus TSH and could be induced repeatedly, suggesting changes in the thyrocyte membrane.

Binding of [^{125}I]bTSH

The binding of [^{125}I]bTSH to thyrocytes in the presence of retinol was greater than that in the presence of other control substances such as retinoic acid and alcohols of longer chain lengths, namely pentanol and decanol. Methanol together with retinol (6.5 , 13 and $27 \mu\text{M}$) caused the greatest effects; after incubation with $27 \mu\text{M}$ retinol, the increase in TSH binding was significant (Fig. 4).

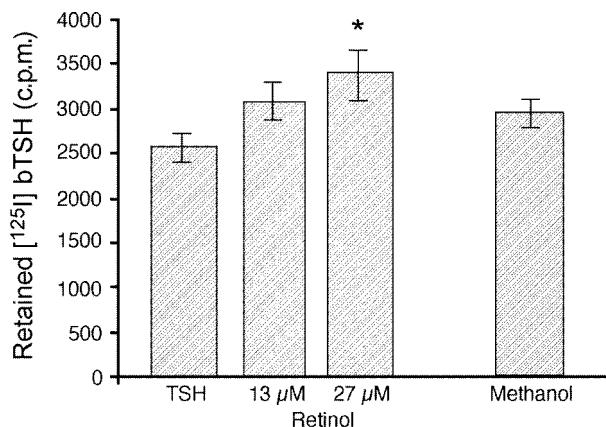


Figure 4 [¹²⁵I]bTSH retained 4 h after the removal of the stimulus. Thyrocytes cultured in standard medium were stimulated with 13 μM methanol, 13 μM retinol or 27 μM retinol ($n=6$). TSH was retained to a greater extent in the presence of all alcohols than in the controls. The increase in retention is significant ($*P<0.05$) for 27 μM retinol. Means \pm S.E.M. are given.

Discussion

Combinations of TSH and 3.25–6.5 μM retinol were able to increase the uptake of iodide and the iodination of protein. This increase was significant in the case of 6.5 μM retinol. With greater concentrations, the significant increase in the first 12 h of incubation was followed by a significant decrease caused by the induction of apoptosis by concentrations of 13 μM retinol or more. Retinol increased not only iodide uptake, but also protein iodination, suggesting that the stimulation can lead to an increased release of the active thyroid hormones, a process that is difficult to determine in culture. The enhancing effect of retinol on thyroid function did not need the simultaneous presence of TSH and retinol, but required physiological concentrations of vitamin A in the medium. The requirement for basal concentrations is not unexpected, firstly because vitamin A deficiency affects the cell function in several cell types (Butera & Krakowka 1986, Hunt 1986, Bowman *et al.* 1990, Twining *et al.* 1997), and secondly because retinoids influence the concentration of their receptors (Wan *et al.* 1994, Tanaka *et al.* 2001). This differentiating effect on normal thyrocytes differs from the dedifferentiating effect described after stimulation with retinol in greater concentrations or for a longer time, or both (Arai *et al.* 1991, Namba *et al.* 1993, Fröhlich *et al.* 2001).

Mode of action of retinol

The mode of action of retinol was characterized in part.

More NIS mRNA and protein were detected in the homogenates of TSH-stimulated cells than in samples that did not undergo stimulation with TSH. This indicates

that, as in both human and rat thyrocytes (Schmutzler *et al.* 1997, Spitzweg *et al.* 1999, Kogai *et al.* 2000), TSH induced the synthesis of NIS protein in porcine thyrocytes. According to the findings from immunocytochemical staining, the increase in NIS protein that we observed was caused by an augmentation of NIS-immunoreactive cells; their fraction increased to around 20%, which corresponds to the ratio observed in intact human thyroid glands (Saito *et al.* 1998, Filetti *et al.* 1999). Retinol alone exerted no effect on the amount of NIS protein. In this respect, retinol acts differently than retinoic acid, with which a decrease in NIS expression in rat thyrocytes has been reported (Schmutzler *et al.* 1997). This appears to indicate that, in thyrocytes, retinol does not act predominantly via its metabolite, retinoic acid.

An action of retinol on the configuration of the TSH receptor appears likely, because our preincubation experiments with retinol showed that retinol can increase the binding of [¹²⁵I]bTSH to the TSH receptor. Petersen *et al.* (1979) observed increases in the iodide uptake of thyrocytes after incubations with Triton X and postulated changes in the access of TSH to its receptor as the cause for this enhancement. As a hydrophobic substance, retinol is also likely to interact with the plasma membrane. This interaction, for instance, could change the state of aggregation of the receptor. It has been shown by Davies *et al.* (2002) that monomers are the active and oligomers the inactive form of the receptor. Retinol might induce monomerization of the receptor and thereby increase its activity. This activation could result in an increased binding of TSH to the thyrocytes. A finding that corroborates this idea was our observation that, in the absence of retinol, TSH exerted its greatest effect on the uptake of iodide in concentrations of 1 mU/ml, whereas in the presence of retinol TSH showed the same effect at 0.5 mU/ml. The concentrations of retinol needed to cause such an effect *in vivo* are difficult to deduce from the situation *in vitro*. An increase in the binding of [¹²⁵I]bTSH was seen with retinol in concentrations of 6.5 μM and more. This effect was statistically significant only for 27 μM retinol, but it is likely that the same mechanism also occurs with lower concentrations of retinol.

It can be concluded that retinol can cause differentiating effects not only in transformed cells, but also in normal thyrocytes, under the prerequisite that either the concentration of retinol is not high or exposure to the retinol is restricted to a short time. With longer incubation times in the presence of at least 6.5 μM retinol, the differentiating effects appear to be counteracted by the induction of apoptosis. Correspondingly, after longer incubation times with higher concentrations of retinol, the function of normal thyrocytes decreased as has also been reported in previous studies (Arai *et al.* 1991, Namba *et al.* 1993, Fröhlich *et al.* 2001). As one mechanism of the action of retinol, we postulate a retinol-induced change in the state of aggregation or the conformation, or both, of the

TSH receptor. This effect is unique for retinol and was not observed after incubations with retinoic acid. Our data suggest that, from the perspective of the metabolism of normal thyrocytes, retinol may be more suitable than retinoic acid for the treatment of thyroid carcinomas. Retinol may also be a potential substance for chemoprevention for a certain population. Patients with follicular adenomas that do not take up iodide ('cold' nodules) might represent a population suitable for chemopreventive treatment with retinol; these patients are at a greater risk of developing thyroid carcinomas and most often have their thyroid removed as a prophylaxis (Rios *et al.* 2004). Serum concentrations of 3.25 µM retinol or greater are not uncommon in humans (Michaelsson *et al.* 2003) and a further increase in the concentration might not be very dangerous, because retinol is relatively non-toxic. The integration of retinol into liposomes is easy (Lee *et al.* 2002). The relatively superficial location of the thyroid might even make a topical treatment with retinol possible; transdermal application is routinely used in hormone replacement therapy with oestrogen or gestagen (e.g. Shulman & Harari 2004). It has also been suggested that local treatment with retinol may prevent the progression of cervical dysplasia CIN III to cervical carcinomas (Volz *et al.* 1995). The possibility that application of retinol could re-induce the uptake of iodide in the 'cold' nodules of these patients should be investigated.

Acknowledgements

We thank Beate Aichele (western blot), Inge Benz (cell culture) and Elke Maier (immunocytochemistry) for their excellent technical assistance and Walter Renn for the statistic evaluation.

Funding

This study was supported by grant No. CMKP 501-1-1-01-10/99 to B C. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received in final form 28 August 2004
Accepted 14 September 2004