Action of thiazolidinediones on differentiation, proliferation and apoptosis of normal and transformed thyrocytes in culture

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Abstract

Differentiating drugs may be able to re-sensitize thyroid carcinomas to radioiodine therapy. Substituted thiazolidinediones (TZDs) belong to the group of oral anti-diabetic drugs that also possess anti-proliferative and pro-apoptotic effects and, potentially, differentiating effects on several cancer cell lines. Some of the effects are mediated via the peroxisome proliferator-activated receptor gamma (PPAR-γ). We investigated the effect of troglitazone, rosiglitazone and pioglitazone on differentiation in normal porcine thyrocytes and in the follicular carcinoma cell lines FTC 133 and FTC 238. Differentiation was investigated by measuring 125I uptake and the expression of sodium-iodide symporter and thyroglobulin proteins. The TZDs were tested in the presence of retinol and retinoic acid. Additionally, proliferation was evaluated by [3H]thymidine uptake and cell number and apoptosis by annexin V-labeling. Controls included tocopherol and unsubstituted thiazolidinedione and co-incubation of the TZDs with the PPAR-γ antagonist GW9662. PPAR-γ and retinol X receptor (RXR)-α were investigated by immunocytochemistry, Western blot and RT-PCR. Cells derived from the metastasis showed greater responses than cells derived from the primary tumor. Troglitazone showed greater effects than the other TZDs. Troglitazone significantly increased 125I uptake and apoptosis and decreased [3H]thymidine uptake and cell number. The amount of the sodium iodide-symporter in the membrane fraction was significantly increased, while that of thyroglobulin was not influenced by the treatment. Inclusion of antagonist did not abolish these effects. No synergistic effect with any retinoid was detected. All transformed cells expressed PPAR-γ and RXR-α but TZDs did not change their expression.

Troglitazone appears to be suited for the re-differentiation treatment of dedifferentiated thyroid carcinoma because its action is twofold. On the one hand it increases differentiation and on the other hand it inhibits proliferation.

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Introduction

The goal for the use of differentiating substances in thyroid cancer is the induction of iodide uptake in the cells to make them susceptible to radioiodine therapy. Only retinoids have shown some efficacy in the most recent clinical trials (Simon et al. 2002). However, retinoids have several disadvantages in thyroid cancers: not all tumors respond to the treatment (Simon et al. 1996, 1998, Grunwald et al. 1998, Schmutzler & Kohrle 2000), retinoids may promote proliferation in de-differentiated thyroid cells (Kurebayashi et al. 2000) and retinoids have dediffererntiating effects on normal thyrocytes (Namba et al. 1993, Fröhlich et al. 2001). Therefore, substances with less adverse reactions are needed. Substituted thiazolidinediones (TZDs) are a powerful and clinically important new class of oral anti-diabetic agents that act by improving insulin sensitivity (Lebovitz 2002). They act predominantly on the metabolism of adipocytes but also possess other
effects: they have an anti-inflammatory action, they improve the function of pancreatic β-cells, they cause dilatation of blood vessels and they decrease proliferation and increase apoptosis in transformed cells (Fujiwara & Horikoshi 2000, Marx et al. 2001). The latter effects are especially important because they may represent a new indication for these substances. A reduction of the cell number after treatment with TZDs was reported in transformed cells of lung, breast, colon, prostate and thyroid (Elstner et al. 1998, Kubota et al. 1998, Okuno et al. 1998, Kitamura et al. 1999, Tsubouchi et al. 2000). Fewer studies report an increase in differentiation such as morphological changes in colon carcinoma cell lines (Kawa et al. 2002) and expression of differentiation markers of the transformed cells (Sarraf et al. 1998, Kitamura et al. 1999). An anti-tumor action of the TZDs has been demonstrated in some clinical trials (Demetri et al. 1999, Mueller et al. 2000), in others no convincing effect was seen (Kulke et al. 2002, Burstein et al. 2003, Debrock et al. 2003). In these trials, no patients with thyroid carcinomas were included. The actions of TZDs on adipocytes are mediated through activation of peroxisome proliferator-activated receptor gamma (PPAR-γ). PPAR-γ itself may also be involved in the anti-tumor effects because other ligands of PPAR-γ also inhibit growth in cancer cells (Yee et al. 1999, Elnemr et al. 2000, Satoh et al. 2002, Yoshizawa et al. 1998, Yoshimura et al. 2003). The involvement of PPAR-γ in the regulation of proliferation and apoptosis in cancer cells has not been shown consistently: PPAR-γ antagonists, in some but not all studies, antagonized the actions of PPAR-γ ligands on transformed cells (Clay et al. 2002, Desmond et al. 2003) and an action of PPAR-γ ligands both dependent and independent of PPAR-γ activation in the same cells can also occur (Berge et al. 2001). The involvement of PPAR-γ may be important because it forms heterodimers with the retinoid X receptor (RXR) (Lenhard 2001) and the availability of this receptor may influence the efficacy of PPAR-γ activation. The specific ligand for the RXR is 9-cis retinoic acid (Heyman et al. 1992). Retinoids decrease cell proliferation, induce apoptosis and increase differentiation in transformed cells (Niles et al. 1990), and a synergism between TZDs and retinoids would increase the efficiency of the treatment. In transformed cells such a synergism has not been shown consistently (Sato et al. 2000, Kawakami et al. 2002).

We compared the effect of troglitazone, rosiglitazone and pioglitazone (their structures are depicted in Fig. 1) on differentiation, proliferation and apoptosis of normal and transformed thyrocytes in the presence of infra- and supraphysiological concentrations of retinol and 9-cis and all-trans retinoic acid. The study was aimed especially to reveal a potential synergism with retinoids and to investigate the effect on iodide uptake. The latter is a decisive clinical parameter because thyroid carcinomas often lose their ability to take up radioiodide and thus the efficacy of radiation therapy is lost. To address the involvement of PPAR-γ, co-incubation with the PPAR-γ antagonist GW9662 was performed and PPAR-γ and RXR-α mRNA and PPAR-γ protein were determined.

Materials and methods

Isolation of thyroid cells and culture conditions

Porcine thyroid glands were obtained from the local slaughterhouse, and cells were isolated as previously described (Wahl et al. 1992). These normal thyrocytes were seeded at a density of 1.2 $\times$ 10^5/cm^2 and cultured...
in NCTC-135 medium (Gibco) supplemented with 3% synthetic serum Ultroser G (BioSepra, Villeneuve-La-Garenne, France) and penicillin (500 000 U/ml), streptomycin (50 mg/ml) and cibrofloxacin (Cibrobay, Bayer Vital, Leverkusen, Germany; 60 μg/ml). The human thyroid follicular carcinoma cell lines FTC 133 and FTC 238 were obtained from ECACC (European Collection of Cell Cultures, Salisbury, UK) and seeded and cultured at 37°C in 5% CO2 and 95% air. NCTC-medium with no retinol (special preparation, Gibco), FTC 133 stems from the primary tumor, the more invasive FTC 238 from a metastasis of the same patient (see Goretzki et al. 1990 for characterization). All cells were cultured at 37°C in 5% CO2 and 95% air. NCTC-medium with no retinol (special preparation, Gibco), normal concentration (0.6 μM) and supra-physiological concentrations (1.8 and 3.6 μM) of retinol were used.

Stimulation protocols
All substances except retinoids were diluted in DMSO to a final concentration of 0.04%. This concentration did not cause toxic effects in the cells. Retinoids were dissolved in methanol. The following concentrations were tested: troglitazone (Calbiochem): 0.1, 1, 5 and 10 μM; rosiglitazone (SmithKline Beecham) and pioglitazone (Calbiochem (Merck Biosciences GMBH), Schwalbach, Germany): 5, 7.5, 10 and 50 μM; α-tocopherol (ICN Biomedicals, Eschwege, Germany): 5 and 10 μM; all-trans retinol (Sigma): 13 and 27 μM; 9-cis retinoic acid (Sigma): 1 and 5 μM; all-trans retinoic acid (Sigma): 1 and 5 μM. The inhibitor GW9662 (Calbiochem) was added to a final concentration of 5 μM (Gupta et al. 2001). Prior to the stimulation cells were cultured in the respective media (containing 0, 0.6, 1.8 or 3.6 μM retinol) for 18 h.

Iodide (125I) uptake
NaI (1 μM final concentration) traced with 4 kBq/well carrier-free Na125I (Amersham Pharmacia Biotech) was added to each culture 6 h prior to measurement. The cells were collected and washed with a 48-well cell harvester (Inotech IH 280, Inotech, Dottikon, Switzerland). Filtermats (Skatron 11731, Skatron Instruments, Lier, Norway) were transferred to counting tubes and their radioactivity measured.

[3H]Thymidine uptake
Cultures (5 x 10^5 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). Dried filtermats were put in sample bags (sample bags 1204-411, Wallac), scintillant (Ultima Gold Scintillant, Packard) was added and the bags were welded together. Samples were measured in a Betaplate 1205 (Wallac, Turku, Finland).

Determination of cell number and viable cells
Cells were harvested by incubation with 0.025% trypsin (Sigma) for 5 min. After centrifugation at 400 g at 4°C for 5 min, the cells were re-suspended in 10 ml medium. Cell number and cell viability were determined in 20 μl of this suspension in an automatic mode based on the Electrical sensing zone method (cell counter and analysis system, CASY Technology, Schärfe Systems, Reutlingen, Germany).

Immunohistochemistry
For these experiments, cells were cultured on cover slips coated with poly-L-lysine. The following primary antibodies were used: anti-PPAR-γ antibody (goat, Santa Cruz Biochemicals), anti-thyroglobulin (TG) (mouse, Novus Biologicals, Littleton, CO, USA), anti-sodium iodide symporter (NIS) (rabbit, antibody directed against amino acid sequence 560–579 of the human NIS; NIS 4; Dr Czarnocka, Warsaw, Poland). The cells were cultured for 42 h. Thereafter the coverslips were pre-incubated with blocking solution (5% normal serum (rabbit for PPAR-γ and goat for TG and NIS), 0.5% Triton X-100, 1% BSA in 0.1 M PBS pH 7.4) for 30 min at room temperature (RT) and subsequently incubated with anti-PPAR-γ antibody (1:100), anti-TG antibody (1:100), and anti-NIS antibody (1:150) diluted in a solution of 5% normal serum, 0.5% Triton X-100 in 0.1 M PBS pH 7.4 at 4°C overnight. After rinses in 0.1 M PBS, incubation with biotin-labeled secondary antibody (anti-goat for PPAR-γ, rabbit, Dianova, 1:100; anti-mouse for TG (goat, Sigma, 1:100); anti-rabbit for NIS (goat, Dianova, Hamburg, Germany, 1:100)) for 1 h at RT and alkaline phosphatase-labeled streptavidin (1:200, DAKO Diagnostica, DakoCytomation, Hamburg, Germany) for 40 min at RT followed. The substrate solution for the red reaction product contained 8 mg naphthol AS-MX phosphate (Sigma) in 0.2 ml dimethyl formamide, 3 mg levamisole (Sigma) and 10 mg fast red RT salt (Sigma) in 10 ml 0.1 M Tris/HCl pH 8.2. Incubation time with the substrate solution was 1 h at RT for PPAR-γ and 20 min for TG and NIS.

Western blot
For the Western blot analysis the same antibodies as for immunocytochemistry were used. Cells were
collected after 24 h and 42 h. Either whole cell extracts or membrane fractions of the cells were tested. Thyrocytes were collected in PBS after mechanical removal from their support and collected in 1 ml homogenization buffer (0.02 M triethanolamine hydrochloride, 1.5 mM EDTA, 1 mM MgCl₂, 5 mM mercaptoethanol, pH 7.4). The cells were centrifuged for 6 min at 1000 g and 4 °C, resuspended in 100 μl homogenization buffer, and centrifuged again. For whole cell extracts this supernatant was used. To produce membrane fractions the supernatant was centrifuged at 150 000 g for 30 min and 4 °C and the pellet represented the membrane fraction. The pellet was resuspended in 45 μl homogenization buffer. An equal volume of SDS loading buffer (0.01% Bromophenol Blue, 18% glycerol, 10.5% mercaptoethanol, 2% SDS, 0.13 M Tris/HCl pH 6.8) was added to the supernatant and the membrane fraction and the mixtures were heated at 95 °C for 5 min. Electrophoresis was performed on a 9% SDS polyacrylamide gel (Protein Minicell Chamber, Protein MiniCell Bio-Rad Laboratories, München, Germany) according to the method of Laemmli (1970); 30 μg protein were loaded per lane. High molecular mass markers (53 000–212 000 Dalton, Pharmacia) were used as standards. Proteins were transferred to PVDF membranes (Immobilon-P, Millipore, Eschwege, Germany) 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 pre-incubated in 5% BSA in TBS at 4 °C for 18 h. Subsequently, the membranes were incubated in the following solutions: PPARγ antibody, TG antibody or NIS 4 antibody (1:1000 diluted in 1% BSA, 0.05% Tween 20 in TBS) for 2 h; biotin-labeled secondary antibody (1:1000, see Immunocytochemistry) in TBS for 1 h and alkaline phosphatase-labeled streptavidin (1:2000, DAKO) in TBS for 1 h. Between the incubation with the primary antibody, the secondary antibody and with alkaline phosphatase-labeled streptavidin, the blots were washed for 3 × 5 min with 0.1% BSA in TBS. The bands were visualized with Sigma Fast BCIP/NBT tablets (1 tablet for 100 ml distilled water). To semiquantify the amount of NIS in the samples, the blots were scanned and the intensity quantified by densitometry with the NIH image software Scion Image (NIH, Bethesda, CA, USA).

RT-PCR

Cells were removed by pipetting from the support and were 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 optimized phenol guanidium isothiocyanate extraction method peqGOLD TriFast (peqlab, Erlangen, Germany) according to the manufacturer’s directions. Total RNA treated with RNase-free DNase I was transcribed into cDNA using AMV reverse transcriptase and the first strand cDNA kit from Roche Diagnostics (Mannheim). Quantitative PCR was carried out using SYBR Green I Dye on a high speed thermalycler with integrated microvolume fluorometer (LightCycler, Roche Diagnostics, Mannheim) according to the manufacturer’s directions. PCR primers for PPARγ1 were: 5′-GCC AAC AGC TTC TCC TTC TC-3′ (sense) and 5′-AGA ACA GAT CCA GTG GTT GC-3′ (antisense) and for RXR-α: 5′-AGG CGA TGA GCA GCT CAT TC-3′ (antisense) and 5′-TAC ACC TGC CGC GAC AAC AA-3′ (sense). PCR primers for glucose-6-phosphate dehydrogenase were: 5′-ACT AGA TGC GTG CAC CAG AG-3′ (sense) and 5′-GGG CAG CAG TCA GCA CAA TG-3′ (antisense) and for glyceraldehyde 3-phosphate dehydrogenase: 5′-TGG CAG CAG TCA GCA CAA TG-3′ (antisense) and 5′-ACT AGA TGC GTG CAC CAG AG-3′ (sense).

Apoptosis

Apoptotic cells were detected cytofluorometrically (FACS Calibur, Becton-Dickinson, Heidelberg, Germany) after 5–12 h of cell culture in the presence of 5–10 μM troglitazone and 10 μM rosiglitazone based on the surface binding of fluoresceinisothiocyanate (FITC)-conjugated annexin V (Annexin V-Fluos staining kit, Roche; 1:40, incubation for 10 min in the dark) and the incorporation of propidium iodide dye. FITC signal was detected at 488 nm, that of propidium iodide at 670 nm. Cultures were treated with apoptosis-inducing staurosporine (5 μM, Roche) as a positive control (Qiao et al. 1996).

Statistics

Experiments were performed at least three times. Test values are expressed as means ± standard error of means (S.E.M.), differences between various settings were analyzed 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.

Results

The following TZDs, troglitazone, rosiglitazone and pioglitazone, were investigated. All TZDs are highly
hydrophobic molecules based on 2,4-thiazolidinedione (Fig. 1). To estimate the effect of hydrophobic molecules in general and because of the apparent similarity of one part of the troglitazone molecule to tocopherol, this latter substance was included in the investigation. Like other lipophilic molecules, tocopherol (especially γ-tocopherol) is also an activator of PPAR-γ (Campbell et al. 2003). Controls included co-incubations with the PPAR-γ antagonist GW9662 and with the dimerization partner of PPAR-γ, the ligand for the RXR 9-cis retinoic acid and other retinoids. DMSO and methanol did not change any of the parameters tested. The cells were investigated at 4 h, 16 h, 40 h, 64 h and 88 h.

Iodide uptake

In normal porcine thyrocytes, 5 μM troglitazone and 10 μM rosiglitazone or pioglitazone caused the maximum effect on iodide uptake. Their effect was related to the basal supply with all-trans retinol in normal thyrocytes. Supra-physiological concentrations of 1.8 μM retinol in the medium caused a significantly higher iodide uptake. In transformed cells, neither of the cell lines showed an increase in iodide uptake after stimulation with thyroid-stimulating hormone (TSH) alone. Optimal responses were obtained at the same concentrations of the TZDs as for normal thyrocytes. FTC 238 reacted better to the TZD stimulus than did FTC 133 cells. The TZDs caused an increase in iodide uptake irrespective of the retinol concentrations present in the medium (Fig. 2a). Troglitazone was more efficient than pioglitazone and rosiglitazone. Co-incubation with the PPAR-γ antagonist GW9662 did not counteract this effect. Both tocopherol and 1.8 μM retinol alone enhanced iodide uptake (Fig. 2b) whereas the un-substituted TZD had no effect. No synergistic action of any TZD with either retinol in pharmacological concentrations (≥13 μM) or with 9-cis retinoic acid (1 μM) was detected. After multiple passages the ability to increase iodide uptake in the presence of either retinoids or TZDs was decreased or lost.

[^H]Thymidine uptake

In normal thyrocytes at both subnormal and supra-physiological basal concentrations of retinol,[^H]thymidine uptake was not significantly changed by the addition of TZDs. In transformed cells all TZDs decreased[^H]thymidine uptake in the cell lines (Fig. 3a); the basal concentration of retinol in the medium did not influence this effect to a great extent. Higher concentrations of the TZDs caused greater inhibitions of[^H]thymidine uptake but the cells also showed an abnormal morphology. Retinoic acid alone decreased and tocopherol increased[^H]thymidine uptake; 1.8 μM retinol and the un-substituted TZD caused no significant changes in[^H]thymidine uptake. No synergistic action between the substances was seen. The effect of the TZDs was not counteracted by incubation with the PPAR-γ antagonist GW9662 (Fig. 3b). Even after multiple passages the transformed cells still decreased[^H]thymidine uptake in the presence of the TZDs. The decrease in[^H]thymidine uptake caused a reduction in the number of cells after incubation for longer than 24 h in the presence of troglitazone (slope a = −1.66), rosiglitazone (a = −1.5) or 9-cis retinoic acid (a = −1.5). Over the entire incubation time this decrease was greater than after treatment with retinol and with pioglitazone (a = −0.6 and a = 0.3, Fig. 4). The fraction of viable cells in untreated and stimulated cells was 79 ± 5%.

Expression of differentiation marker proteins (Na^+/I^- symporter (NIS) and thyroglobulin (TG))

In normal thyrocytes, no marked effects of the TZDs on the expression of TG and the NIS were detected. In transformed cells immunocytochemical staining showed that all FTC 133 and FTC 238 cells showed reactivity with thyroglobulin antibody and 50%–75% of all cells showed reactivity with anti-NIS antibody. Neither cell line reacted in an obviously different way in these stainings and no pronounced increase in the reactive cells after TZD treatment was seen. In the Western blots incubation with the TZDs or tocopherol increased the expression of thyroglobulin and of NIS in the FTC 133 and FTC 238 cells. This effect was in the same order of magnitude as the incubation with TSH when whole cell extracts were tested. The differences were not statistically significant. In the membrane fraction of the cells NIS was absent in most samples; only under treatment with troglitazone was a weak band visible in the FTC 238 cells (Fig. 5). In some experiments there was a very slight increase in NIS protein in the membrane fraction after treatment with tocopherol. Co-incubation of the TZDs with retinol or retinoic acid did not further increase the intensity of the bands.

Expression of PPAR-γ mRNA, and RXR-α mRNA and protein in the transformed cells

mRNA for PPAR-γ was detected in all incubations with FTC cells, and the amount of PPAR-γ was not
changed in the presence of 5 μM troglitazone and 10 μM rosiglitazone. In the presence of higher concentrations the amount of PPAR-γ was decreased (Fig. 6b). PPAR-γ immunoreactive protein was detected in normal thyrocytes and in transformed cells. More than 70% of the cells contained the protein in variable degrees (Fig. 6a). However, in the Western blot only a weak band could be detected. RXR-α mRNA was also detected in both transformed cell lines but no changes under TZD and retinol treatment were observed. The protein was expressed only in a minority (20 ± 10%) of the cells.

Figure 2 Uptake of $^{125}$I in normal porcine thyrocytes (solid bars) and in the follicular cell carcinoma cell line FTC 238 (open bars) under various treatments. Iodide uptake in unstimulated cultures is represented as 100%. Significant changes are marked by asterisks. (a) In normal thyrocytes only troglitazone (5 μM) in combination with supra-physiological concentrations of retinol (1.8 μM) caused a significant increase in iodide uptake. In the FTC 238 cells iodide uptake is markedly increased in the presence of troglitazone irrespective of the retinol concentrations in the medium. (b) Experiments were performed in medium with a normal concentration of retinol (0.6 μM). Pioglitazone (10 μM, open bar) and troglitazone (5 μM, open bar) alone and in the presence of the PPAR-γ antagonist GW9662 (5 μM, hatched bar) increased iodide uptake in the FTC 238 cells significantly, whereas no significant increase was seen after incubation with rosiglitazone (10 μM). Retinol (1.8 μM) and tocopherol (Toco; 10 μM) also increased iodide uptake.
Annexin-V-labeling as an indication for apoptosis

In normal thyrocytes, the cells were very resistant to TZDs; up to 50 µM troglitazone did not increase apoptosis significantly. In transformed cells, incubation with staurosporine, troglitazone and rosiglitazone induced apoptosis in the transformed thyrocytes to different degrees. The transformed cells were about ten times more sensitive to the TZDs compared with normal thyrocytes. The pro-apoptotic effect of troglitazone was stronger than that of retinol at equimolar concentrations, and the effect of rosiglitazone and retinoic acid was intermediate between retinol and troglitazone (Fig. 7). Pioglitazone did not increase the number of annexin-labeled cells.

Discussion

Of the substituted TZDs tested, the efficacy on differentiation, proliferation and apoptosis decreased from troglitazone to pioglitazone. The solvents DMSO and methanol had no or only a minimal effect on the parameters tested. Troglitazone increased differentiation, decreased proliferation and cell number and increased apoptosis in the differentiated follicular thyroid carcinoma cell lines tested. The cell line derived from the metastatic lesion responded better to the treatment compared with the cells derived from the primary thyroid carcinoma. This fact is important because surgery is still the best treatment for primary thyroid carcinomas and patients receiving radioiodine therapy most often have advanced/metastatic thyroid...
carcinomas. Differentiating substances, therefore, ideally should be most effective on cells in metastases. This was seen for the TZDs. These substances had the additional advantage that no de-differentiating effect on normal thyrocytes was observed. In the study we used similar concentrations to those causing optimal effects on glucose metabolism \textit{in vitro} (Teboul \textit{et al.} 1995) and to the plasma concentrations seen in the treatment of diabetes patients. It is very unlikely that TZDs acted in a toxic manner on normal or transformed thyrocytes because, although the number of cells decreased to two thirds compared with unstimulated cultures, no differences in the viability between cells with and without treatment with TZDs was noted. It is more likely that the thyrocytes were arrested in the G1-phase, a mechanism that has been suggested for the action of differentiating substances in general and for the glitazones in particular (Sato \textit{et al.})

\begin{figure}
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\includegraphics[width=\textwidth]{Figure4.png}
\caption{Number of cells following up to 6 days in the presence of the stimulant. Treatment with pioglitazone (▲, 0.3) does not cause a decrease in cell number. All other substances show a linear decrease in cell number, the slope of the decrease being greater for troglitazone (■, 1.66) than for all-trans retinol (●, 0.6).}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{Figure5.png}
\caption{Expression of thyroglobulin protein and Na/I symporter (NIS) protein in the follicular cell carcinoma cell line FTC 238. (a) Whole cell extracts: troglitazone (Tro) induces small increases in thyroglobulin and NIS immunoreactive proteins (respective bands are indicated by arrows). (b) Membrane fraction: a marked increase in the amount of NIS protein is seen. C, control.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{Figure6.png}
\caption{Expression of PPAR-γ protein and PPAR-γ mRNA in the follicular cell carcinoma cell line FTC 238. (a) Immunoreactive protein for PPAR-γ can be localized in the cytoplasm of the majority of the cells. Scale bar: 20 µm. (b) Troglitazone at 5 µM has no marked effect on the amount of PPAR-γ mRNA but a higher concentration of troglitazone causes a decrease in its expression. mRNA content of unstimulated cells is represented as 100%.}
\end{figure}
They influence the transcription of regulators of the cell division cycle; troglitazone has been reported to be more effective in this respect than rosiglitazone (Bae et al. 2003). In our study troglitazone also decreased cell number to a greater extent than rosiglitazone.

One of the established modes of action of the TZDs is the activation of PPAR-\(\gamma\) (Otto et al. 2002). Although the FTC cell lines expressed this receptor, our data suggest that PPAR-\(\gamma\) is not involved in the action of TZDs because we did not detect any stimulation of PPAR-\(\gamma\) mRNA expression after stimulation with the TZDs. This argues against an involvement of PPAR-\(\gamma\) in the action because expression and activity of this receptor are closely correlated (Knouff & Auwerx 2004). In addition, co-incubation with the PPAR-\(\gamma\) antagonist GW9662 did not reverse the effects of the TZDs. There was also no induction of RXR-\(\alpha\), the heterodimerization partner of PPAR-\(\gamma\). Another indication for the lack of implication of PPAR-\(\gamma\) in these effects is that troglitazone, the TZD with the lowest affinity to PPAR-\(\gamma\) (Young et al. 1998) was the most effective in our study. It may be speculated that the transformed thyrocytes used in this study possess a higher differentiation than the cell lines where PPAR-\(\gamma\) was involved in the action of the TZDs. In malignant thyrocytes the involvement of PPAR-\(\gamma\) in the action of TZDs is not clear. Ohta et al. (2001) demonstrated an anti-proliferative effect of troglitazone by activation of PPAR-\(\gamma\) but no correlation between effects mediated by PPAR-\(\gamma\) and expression of PPAR-\(\gamma\) carcinoma cells was demonstrated (Klopper et al. 2004). The experiments by Huang et al. (2005) in breast cancer cells showed that troglitazone can repress cyclin D1 independently from PPAR-\(\gamma\) activation. Despite the lack of activation of PPAR-\(\gamma\) we observed an increase in iodide uptake and other effects in the FTC cell lines. The potency of the substances was different from that on glucose metabolism (Table 1) and appears to be specific for the structure of troglitazone (Fig. 1). The specific situation of troglitazone was obvious in the detection of NIS protein in the transformed cell lines; however, only the incubation with troglitazone and not that with TSH led to an increase in iodide uptake. The lack of direct correlation between NIS expression and iodide uptake is not unusual. It has been reported repeatedly and appears, in part, to be caused by an incorrect location of the NIS protein in the transformed cells. The correct location in the membrane is rarely observed in thyroid cancer cells (Trouttet-Masson et al. 2004). This causes the obvious lack of correlation between differentiation markers at the mRNA or protein level and the uptake of iodide. The expression of mRNA and/or protein of thyroid peroxidase and 5’-deiodinase appear to indicate

Table 1 Potency of various TZDs and tocopherol on the metabolism of transformed thyrocytes in our study compared with reported data on their action on glucose metabolism in 3T3-L1 adipocytes (Young et al. 1998)

<table>
<thead>
<tr>
<th>Substance</th>
<th>Increase of differentiation</th>
<th>Decrease of proliferation</th>
<th>Increase of apoptosis</th>
<th>Potency on glucose metabolism</th>
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<tr>
<td></td>
<td>Iodide-uptake</td>
<td>Thymidine uptake</td>
<td>Cell number</td>
<td>Annexin-labeling</td>
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<td>Pio:glitazone</td>
<td>++</td>
<td>--</td>
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<td>--</td>
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<td>Ros:glitazone</td>
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<td>Tocopherol</td>
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Changes related to unstimulated controls: --, 0–19%; +, 20–39%; ++, 40–59%; ++++, 60–79%; ++++, 80–99%.

EC\(_{50}\) (nM): concentration of the half-maximal effect of the compounds.
an increase in differentiation but in the case of NIS the sublocalization of the protein has also to be determined. In the troglitazone-stimulated cells the increase in NIS protein was very small but occurred predominantly/exclusively in the membrane fraction of the homogenate. Therefore, it is likely that troglitazone acted predominantly on the post-translational level. As NIS protein in the membrane fraction could not be demonstrated in either the rosiglitazone- or the pioglitazone-treated cells, the specific structure of troglitazone appears to be responsible for this effect. The first moiety of the molecule is similar to tocopherol and tocopherol also showed increases of NIS protein in the membrane fraction although less consistently. However, these increases were too low to permit a clear statement. We presume that the alteration of the NIS localization is a PPAR-\(\gamma\)-independent and troglitazone-specific mode of action. Other PPAR-\(\gamma\)-independent actions of troglitazone have been identified by Palakurthi et al. (2001) and by Takeda et al. (2001). These authors demonstrated an inhibition of translation initiation and suggest an activation of the MEK/ERK pathway as alternative or additional regulatory mechanisms of the TZDs. It may be possible that one of these mechanisms is also active in thyrocytes.

TZDs in this study acted in a similar manner as other differentiating substances such as retinoids, histone deacetylase inhibitors and DNA methyltransferase inhibitors. All these substances not only decrease proliferation and induce apoptosis but also increase the differentiation of the transformed cells. Retinoic acid, valproic acid and depsipeptide are efficient in the differentiation of the transformed cells. Retinoids and tocopherol also showed increases of NIS protein in the membrane fraction although less consistently. However, these increases were too low to permit a clear statement. We presume that the alteration of the NIS localization is a PPAR-\(\gamma\)-independent and troglitazone-specific mode of action. Other PPAR-\(\gamma\)-independent actions of troglitazone have been identified by Palakurthi et al. (2001) and by Takeda et al. (2001). These authors demonstrated an inhibition of translation initiation and suggest an activation of the MEK/ERK pathway as alternative or additional regulatory mechanisms of the TZDs. It may be possible that one of these mechanisms is also active in thyrocytes.

TZDs in this study acted in a similar manner as other differentiating substances such as retinoids, histone deacetylase inhibitors and DNA methyltransferase inhibitors. All these substances not only decrease proliferation and induce apoptosis but also increase the differentiation of the transformed cells. Retinoic acid, valproic acid and depsipeptide are efficient in thyroid cancer cell lines (del Senno et al. 1993, Schmutzler et al. 1997, Kurebayashi 1993, Kitazono et al. 2001, Fortunati et al. 2004). Retinoids have already entered clinical trials (Simon et al. 2002) but have exhibited only limited success; in addition, depsipeptide is afflicted with severe adverse effects, especially nausea, vomiting and anorexia (Marshall et al. 2002). The TZDs have the advantage that they have already successfully passed the pre-clinical tests as anti-diabetic drugs and do not show severe adverse effects in diabetes patients. Since the concentrations used in this study were in the same order of magnitude, it is unlikely that these doses will act in a toxic manner in patients with thyroid carcinomas. Another positive feature of the TZDs is that no negative influence on normal thyroid cell function was seen in this study. The promising effects of the TZDs on transformed thyroid cells in vitro encourage the study of their effects in vivo within a clinical trial. Troglitazone was the most effective substance regarding both differentiation and reduction in cell number. The only problem may be that troglitazone can cause hepatotoxicity in some patients, which led to its withdrawal from the market for the treatment of diabetes. Meanwhile, troglitazone has been studied in a clinical trial for other indications e.g. polycystic ovary syndrome (Legro et al. 2003). According to our in vitro study, TZDs appear to be suited for the re-differentiation treatment of dedifferentiated thyroid carcinomas but not for the treatment of anaplastic thyroid carcinomas because cells after multiple passages still decreased proliferation but did not increase iodide uptake.

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