EFFECTS OF RECEPTOR-SELECTIVE RETINOIDS ON CYP26 GENE EXPRESSION AND METABOLISM OF ALL-TRANS-RETINOIC ACID IN INTESTINAL CELLS

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ABSTRACT:

Retinoids mediate most of their function via interaction with retinoid receptors [retinoic acid receptors (RARs) and retinoid X receptors (RXRs)], which act as ligand-activated transcription factors controlling the expression of a number of target genes. The complex mechanistic pattern of retinoid-induced effects on gene expression of CYP26 and intestinal metabolism of all-trans-retinoic acid (RA) was investigated here by studying the effects of retinoid ligands with relative selectivity for binding and transactivation of the retinoid acid receptors, RARs and RXRs, in human intestinal Caco-2 cells. We show here that CYP26 is expressed in human duodenum and colon. In Caco-2 cells not only all-trans-RA but also synthetic agonists of the RAR induced intestinal CYP26 gene expression and all-trans-RA metabolism as well. The RARα ligand Am580 induced the CYP26 gene expression more than the RARβ ligand CD2019 or the RARγ ligand CD437 suggesting the highest specificity for RARα on intestinal CYP26 gene regulation. RAR ligands alone did not induce CYP26 gene expression or RA metabolism in Caco-2 cells at all. But together with the RARα ligand, Am580, there were enhanced effects on the induction of CYP26 gene expression and on the induction of the metabolism of all-trans-RA. We conclude that gene regulation of CYP26 and the metabolism of all-trans-RA in intestinal cells is regulated through RAR and RXR heterodimerization. When coadministered, RAR agonists showed the highest potency for CYP26 gene regulation. Receptor-selective retinoids showed enhanced effects on induction of CYP26 gene expression and all-trans-retinoic acid metabolism.

The oxidative metabolite of vitamin A alcohol (retinol), all-trans-retinoic acid (RA), plays a key role in growth and cell differentiation of the epithelial tissue. Retinol is formed through the hydrolysis of dietary retinyl esters and is taken up by the mucosa cells. There, retinol is bound by CRBPII and converted via lecithin-retinol acyltransferase or acyl-CoA: retinol-acyltransferase to retinyl esters (for reviews, see Nau and Blaner, 1999).

Recently we have shown that in the enterocyte dietary retinol, when it is absorbed in the small intestine, has an alternative pathway. Retinol is oxidized by enterocytes to all-trans-RA, which can be further metabolized mainly through the enzymatic activity of CYP26 to polar metabolites although other CYP-enzymes like CYP1A1 or CYP3A are also involved, in particular when they are induced (Lampen et al., 2000). CYP26 was cloned by different groups (Fujii et al., 1997; Ray et al., 1997; White et al., 1997). It seems to have an important role in regulating the RA-tissue concentration, which could have an impact on RA-regulated intestinal physiology. To our knowledge, CYP26 has not been detected in human intestinal cells so far. Nuclear receptors called retinoic acid receptors (RARs) and retinoid X receptors (RXRs) regulate most actions of retinoids. Six bona fide retinoid receptors were discovered that fall into two classes, the RARs α, β, γ and the RXRs α, β, γ (Petkovich et al., 1987; Mangelsdorf et al., 1990). As with all nuclear receptors, they contain the typical domains including a DNA- and a ligand-binding domain, and their ligand-binding domain shows low amino acid homologies. Consequently, the ligand specificities of the receptors differ. RARs bind all-trans-RA and 9-cis-RA whereas RXRs bind only 9-cis-RA. In addition, so-called RXR-selective retinoids, a class of synthetic retinoids that preferentially bind to RXRs with high affinity, have been developed: AGN191701 (Lehmann et al., 1992), CD3159, or CD2608 (Kochhar et al., 1996). Other synthetic ligands bind with some selectivity to RARs: Am580 to RARα (Delescluse et al., 1991), CD2019 to RARβ, and CD437 to RARγ (Bernhard et al., 1992; Darmon et al., 1989).

To convert a retinoid signal into transcriptional activation of a gene, the RARs function, however, as heterodimers with RXRs. The RXR-RAR heterodimers were found to bind effectively to their DNA recognition sequences, the retinoic acid response elements (Kliwer et al., 1992). We hypothesized that CYP26 is expressed in the human intestine, and it may be regulated by RA mediated by RAR and RXR. Furthermore we hypothesized that heterodimerization is an important step in that regulation and that consequently other RXR or RAR agonist besides all-trans-RA or 9-cis-RA may have an influence on CYP26 gene expression and/or intestinal RA metabolism.

Experimental Procedures

Cell Culture and Reagents. Caco-2 cells were obtained from European Collection of Cell Cultures (Salisbury, UK) and kept in Dulbecco’s modified...
Eagle’s medium supplemented with 10% fetal calf serum (Boehringer, Ingelheim, Germany) at 37°C in a humidified atmosphere of 5% CO2-air.

**Human Biopsy Samples.** Human duodenum and colon samples (biopsy probes) from five patients, respectively, were obtained from the Klinik für Abdominal und Transplantationschirurgie (Medizinische Hochschule Hannover, Hannover, Germany), and the collection of human intestinal samples for research was approved by the Ethical Committee of the Medizinische Hochschule Hannover.

**Materials and Reagents.** All-trans-retinol and all-trans-RA were purchased from Sigma (Deisenhofen, Germany), 13-cis-4-oxo and all-trans-4-oxo-RA were kindly provided by Hoffmann-La Roche (Basel, Switzerland). Lyophilized analytical grade bovine serum albumin was purchased from Sigma. All other chemicals were purchased from Merck (Darmstadt, Germany) or Sigma Chemicals in the highest available purity.

The RXR agonist used: Am580 (CD336; [4-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenylcarboxamido)benzoic acid]), CD2019 (2-(1-methylcyclohexyl)-4-methoxyphenyl)-2-naphthoic acid), CD437 (6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthoic acid), (chemical structures as shown in Fig. 1; their binding and transactivation activities were shown earlier; Elmazar et al., 1996). The RXR-agonist used (Fig. 1): AGN191701 (CD2425; (2E,4E,6Z,7Z-3-N-propoxy-5,6,7,8-tetrahydro-5,8,8-tetramethylnaphthalen-2-yl)-3-methylcyclohexyloxy)-2,4,6-trienoic acid); their binding is described earlier (Howell et al., 1998; Schulman et al., 1999).

Water for HPLC was purified using a Milli-Q water purification system (Millipore, Eschborn, Germany). Stock solutions of retinoids were prepared in ethanol (or dimethyl sulfoxide) at a theoretical concentration of 0.1 mg/ml, which was subsequently photometrically adjusted. These solutions were kept in glassware at ~20°C, tested for stability, and freshly prepared when necessary. All laboratory manipulations involving the retinoids (preparation of dosing solutions, drug treatment of cells, collection of samples, and analytical procedures) were performed in dark rooms under dim yellow light to prevent photoisomerization.

**Retinoid Analyses.** Retinoids in supernatants and cells were analyzed by a reversed-phase HPLC method with gradient elution following sample enrichment with solid-phase extraction (thoroughly described by Collins et al., 1992). According to this method, samples were treated with isopropanol, and the supernatants were then extracted on solid-phase extraction cartridges prior to introduction into the HPLC system. Cells and medium supernatants were extracted with a 3-fold volume of isopropanol. Further processing was performed as described previously by Collins et al. (1992). UV detection of the HPLC eluate was performed at 340 and 356 nm by use of a two-channel SPD-10AV detector (Shimadzu, Duisburg, Germany).

Recovery and reproducibility of the assay and calibration with an external method were previously reported (Eckhoff et al., 1989; Collins et al., 1992). Identification of HPLC peaks was based on comigration with authentic retinoids and on coincidence of the absorbance ratios (i.e., ratio of peak areas) at the two wavelengths of detection with that of the standard retinoids.

**Metabolism of All-trans-RA in Caco-2 Cells.** Caco-2 cells were cultured for 9 days (maximum of differentiation). All-trans-RA (10 μM) was added to the fresh culture medium and incubated for 3.5 h. A cell pellet and cell medium volume of 150 μl was extracted with a 3-fold volume of isopropanol, followed by short centrifugation and solid-phase extraction (see above).

**Preparation of Caco-2 Cells with RXR- or RAR-Ligands.** All RAR ligands (Am580, CD2019, CD439) and RXR ligands (CD2608, CD3159, and AGN191701) were dissolved in dimethyl sulfoxide. Ligands were incubated at the indicated concentration for 48 h with Caco-2 cells. In metabolism experiments, the substrate all-trans-RA was then added, and after an additional incubation period of 3.5 h at 37°C, the reaction was stopped by the addition of isopropanol. Treated cells were also collected for RNA isolation.

**Preparation of RNA.** Total RNA was prepared from freshly isolated cells according to the method of Chomczynski and Sacchi (1987). RNA concentrations were determined spectrally with a UV-visible spectrophotometer (Perkin Elmer, Rodgau, Germany), and the integrity as well as the concentrations of RNA were checked in an agarose gel using RNA standards.

**RT-PCR Analysis.** Reverse transcription (RT) of 0.1 μg of RNA using oligo(dT)12 was performed for 120 min at 37°C with 200 units of Superscript II reverse transcriptase (Gibco/BRL, Karlsruhe, Germany) in 50 mM Tris, pH 8.3, 75 mM KCl, 3 mM MgCl2, 20 mM dithiothreitol, and 0.2 mM each of dATP, dCTP, dGTP, and dTTP. The polymerase chain reaction was performed on 1 μl of the prepared cDNA. Primers for human CYP26 were nt 87–111 for sense and nt 343–368 for antisense (GenBank accession no. AF005418). Primers for human β-actin were nt 311–330 for sense and nt 740–760 for antisense (GenBank accession no. X00351). PCR was performed with 0.5 units of Taq-polymerase (Qiagen, Germany) in an automatic DNA thermal cycler (Perkin-Elmer/Cetus, Norwalk, CT) by adding 50 μl of a PCR master mixture containing PCR buffer, MgCl2 (to a final concentration of 1.5 mM) and 30 pmol of each primer to the cDNA samples. Thirty cycles (30 s at 94°C, 30 s at 57°C, 30 s at 72°C) followed by an additional 10 min at 72°C were used. In each experiment, water was used as a negative control for contamination. All amplifications were carried out for 30 cycles. Under these conditions, all cDNA fragment amplifications were found to produce single products within a linear range of 28 to 33 cycles (data not shown). The amplified product was cloned by PCR cloning and sequenced. Analysis of the sequence by BLAST (National Center for Biotechnology Information) showed that the product fragment was human CYP26. Furthermore, we checked the length of the product by performing the PCR with plasmid DNA containing the cloned cDNA, which was kindly provided by Prof. M. Petkovich (Department of Biochemistry, Queen’s University, Kingston, Ontario, Canada).

**Statistics.** Values for concentrations and concentration ratios were expressed as means ± S.D. Statistical analysis for comparison of two means using the analysis of variance (ANOVA).

**Results**

**Detection of CYP26 Gene Expression in Human Biopsy Probes.** To show that CYP26 is expressed in human proximal and distal intestine, we collected biopsy probes of the duodenum and colon of healthy patients (each with five patients) and measured the CYP26 gene expression by RT-PCR. As shown in Fig. 2, CYP26 mRNA is...
RAR ligands: Am580 (RAR agonist), CD2608 and CD437 (RAR selective), and CD473 (RAR γ selective). As shown in Fig. 5, the CYP26 gene expression is mainly driven by RARα. Am580 induced CYP26 gene expression the most; 1 μM Am580 induced CYP26 gene expression by the factor of 7. Induction of CYP26 gene expression was also seen after pretreatment of the cells with RARβ and RARγ selective ligands, but they showed only half the induction potency of Am580. In parallel experiments, we measured also an enhanced metabolism of all-trans-RA in Caco-2 cells. The metabolism of all-trans-RA to 4-hydroxy-all-trans-RA was induced by the factor of 5.5 when the cells were pretreated with 100 nM Am580, by the factor of 1.8 when 100 nM CD2019 was used, and by the factor of 2.2 when 100 nM CD437 was used.

Enhanced Induction of CYP26 by Coadministration of RAR and RXR Agonists. Using CV1 cells, it was shown that the RXR-selective agonist CD2608 alone was not able to activate RXR-RAR heterodimers (Schulman et al., 1999). When CD2608 was incubated alone with Caco-2 cells, the CYP26 gene expression was not significantly affected. Coadministration of CD2608 and all-trans-RA resulted in enhanced induction of the CYP26 gene expression (Fig. 6) compared with the treatment with all-trans-RA alone. When the RARα-selective agonist Am580 was coadministered with CD2608, we measured the highest enhancement of the induction of CYP26 gene expression in comparison to the treatment with the RAR agonist Am580 alone.

CD3159 is an RXR agonist that preferably can induce heterodimerization of RXR and RAR. CD3159 does not activate RXR-RXR homodimer (Schulman et al., 1999). Incubation of CD3159 alone had no effect on CYP26 gene expression but in combination with all-trans-RA and Am580 there was an enhancement of the induction of the CYP26 gene expression in Caco-2 cells compared with cells only treated with all-trans-RA (Fig. 7). Coadministration of CD3159 and Am580 enhanced the CYP26 gene expression more than the combination of CD3159 and all-trans-RA.

AGN191701 alone did not affect the CYP26 gene expression at all, but in combination with an RAR agonist (all-trans-RA) we could detect also an enhanced induction of CYP26 gene expression com-

Fig. 2. CYP26 gene expression in the intestinal mucosa.

CYP26 and β-actin gene expression were measured by RT-PCR in human biopsy probes of duodenum and colon. 1 = Caco-2 cells; 2 + 3 = human duodenum; 4 + 5 = human colon; 6 = negative control (no DNA); M = marker. A representative gel is shown.

Fig. 3. Kinetic of CYP26 gene expression in Caco-2 after all-trans-RA treatment measured by RT-PCR.

CYP26 and β-actin gene expression was measured under optimized conditions by RT-PCR after treatment of Caco-2 cells with 5 μM all-trans-RA for different times. M = marker. A representative gel is shown.

Fig. 4. Induction of CYP26 gene expression in Caco-2 by various retinoids.

CYP26 and β-actin gene expression was measured under optimized conditions by RT-PCR after treatment of Caco-2 cells with all-trans-RA, 13-cis-RA, or 9-cis-RA; C = control, 2 = 10 nM all-trans-RA; 3 = 100 nM all-trans-RA; 4 = 3 μM all-trans-RA; 5 = 10 μM all-trans-RA; 6 = 10 nM 13-cis-RA; 7 = 100 nM 13-cis-RA; 8 = 3 μM 13-cis-RA; 9 = 10 μM 13-cis-RA; 10 = 10 nM 9-cis-RA; 11 = 100 nM 9-cis-RA; 12 = 3 μM 9-cis-RA; 13 = 10 μM 9-cis-RA; M = marker. A representative gel is shown.

Fig. 5. Induction of CYP26 gene expression in Caco-2 cells by RXR agonists.

CYP26 and β-actin gene expression was measured under optimized conditions by RT-PCR after treatment of Caco-2 cells with Am580 (RARα agonist), CD2019 (RARβ agonist), or CD473 (RARγ agonist); M = marker. The densitometric analysis of a representative gel is shown.
pared with the induction of CYP26 by all-trans-RA alone. Combination of AGN191701 with Am580 resulted in a higher (synergistic) induction of the CYP26 gene expression compared with the coadministration of AGN191701 and all-trans-RA (Fig. 8).

Enhanced Induction of RA Metabolism by All-trans-RA and Selective RAR and RXR agonists. When the RXR agonist CD2608 was incubated alone with Caco-2 cells, the all-trans-RA metabolism was only marginally affected (Fig. 9, A and B). All-trans-RA (100 nM) alone induced its own metabolism. Coadministration of CD2608 and all-trans-RA did not result in significant enhancement of the metabolism to polar metabolites (Fig. 9A). When the synthetic RARα-selective agonist Am580 was coadministered with the RXRα agonist CD2608, there was a significant enhancement of the induction to all polar metabolites of the RA metabolism compared with the treatment with all-trans-RA alone (Fig. 9B).

Discussion

The existence of interactions between retinoids and CYP enzymes is well established; retinoids both affect and are metabolized by these enzymes. We show here for the first time that CYP26 mRNA is expressed in proximal and distal parts of the human intestinal mucosa. Using human intestinal Caco-2 cells as a model for RA metabolism and CYP26 gene expression, we show here also that CYP26 was rapidly inducible by all-trans-RA and RAR-selective agonists in Caco-2 cells. Although 13-cis-RA and 9-cis-RA also induced CYP26 gene expression, it is not clear whether that is due to an isomerization or a direct induction by these compounds. In Caco-2 cells the RARα ligand Am580 showed the highest inducing effect on CYP26 gene expression compared with RARβ or RARγ ligands. The induction of CYP26 gene expression was enhanced when RAR agonists were
Coadministered with RXR agonists. The RA metabolism was enhanced also by coadministration of RAR/RXR agonists but to a smaller degree.

Recently, we have shown that all-trans-RA is metabolized to polar metabolites using human small intestinal microsomes and human Caco-2 cells. In addition to CYP1A1 and CYP3A, CYP26 was identified as the main CYP enzyme responsible for the metabolism in Caco-2 cells (Lampen et al., 2000). Therefore, it is probable that CYP26 also plays a major role in the human intestinal retinoid pathway. In the adult mouse, CYP26 was expressed only in liver (Fujii et al., 1997). Human CYP26 was found to be expressed in liver, brain, and the placenta (Ray et al., 1997) and also in human colon cancer cells (Sonneveld et al., 1998). According to the preparation method used in our study, it is clear that CYP26 mRNA is expressed in the mucosa cells of the proximal and distal intestine. Further investigations using immunohistochemistry or in situ hybridization may show the exact intracellular localization of CYP26.

Retinoids are compounds that bind to and activate one or more of the known nuclear retinoid receptor subtypes (RXRs, RARs), to modulate gene expression. Studies at the molecular and cellular levels imply that heterodimerization is required for efficient DNA binding and activation of responsive target genes, and in most cases RAR-RXR heterodimers may be the physiologically active forms (Pfahl, 1993). Using mouse F9 cells, Abu-Abed et al. (1998) have shown that RARα and RARγ are involved in CYP26 gene expression. Stable transfection of RARα and RARγ and to a lesser extent RARβ into human HCT116 cells showed that CYP26 induction by retinoic acid is dependent on these retinoic acid nuclear receptors (Sonneveld et al., 1998).

Fig. 8. Effects of AGN191701 and all-trans-RA or Am580 on CYP26 gene expression in Caco-2 cells measured by RT-PCR.

Fig. 9. A, effect of CD2608 and all-trans-RA on the metabolism of RA; B, additive effect of CD2608 and Am580 on the metabolism of RA.

A, Caco-2 cells were pretreated with vehicle (control), CD2608, or all-trans-RA alone and in combination for 48 h, and incubated with 10 µM all-trans-RA for an additional 3 h. Metabolites were extracted and analyzed by HPLC. Values represent means from four determinations ± S.D. *, significant difference (p < 0.05; ANOVA) compared with control; +, no significant difference (p > 0.05; ANOVA) compared with all-trans-RA treatment. T-4-O-RA = all-trans-4-oxo-RA; T-4-OH-RA = all-trans-4-hydroxy-RA; 13C-4-OH-RA = 13-cis-4-hydroxy-RA. B, additive effect of CD2608 and Am580 on the metabolism of RA. Caco-2 cells were pretreated with vehicle (control), CD2608, or Am580 alone and in combination for 48 h, and incubated with 10 µM all-trans-RA for an additional 3 h. Metabolites were extracted and analyzed by HPLC. Values represent means from four determinations ± S.D. *+, significant difference (p < 0.001; ANOVA) compared with control; +, significant difference (p < 0.05; ANOVA) compared with all-trans-RA treatment. T-4-O-RA = all-trans-4-oxo-RA; T-4-OH-RA = all-trans-4-hydroxy-RA; 13C-4-OH-RA = 13-cis-4-hydroxy-RA.
The RA metabolism was also induced. We showed here that all-trans-RA induced CYP26 gene expression in a time-dependent manner. Our results, using specific RARα (Am580), RARβ (CD2608), and RARγ (CD347) agonists, demonstrate that the activation of RARα may play a major role in the induction of CYP26 in the intestinal cells.

To further dissect the role of the RXR and the complex pattern of retinoid-induced effects on CYP26 gene expression and RA metabolism, we have coadministered RXR-selective agonists (CD2608, CD319, AGN191701) alone and together with an RARα-selective agonist (Am580). Our results showed that RXR ligands alone had no effect on CYP26 gene expression, indicating that RXR-RXR heterodimers do not play important functions in retinoid-induced CYP26 gene expression and RA metabolism. But when RAR agonists were coadministered with RAR agonists (natural all-trans-RA or synthetic Am580), there were enhanced effects on CYP26 gene expression (Figs. 6–8). These results showed that heterodimerization may be a very important step in CYP26 gene expression regulation and that RXR agonists have an impact on CYP26 gene expression. The results are in agreement with other studies showing that the combination of an RXR agonist with an RAR agonist has a stronger effect than the treatment of an RAR agonist alone (Apfel et al., 1995; Roy et al., 1995; Chen et al., 1996). The importance of RAR-RXR heterodimers was also supported by in vivo teratogenicity studies (Elmazar et al., 1997; Elmazar and Nau, 1998; Nau and Elmazar, 1999). A number of malformations were synergistically induced by coadministration of RAR and RXR agonists during early (spina bifida, exencephaly) or late (limb defects, cleft palate) organogenesis.

The Effects of RXR and RAR Agonists on the Intestinal RA Metabolism Were Smaller. All-trans-RA induced its own metabolism to polar metabolites. Selective RAR agonists induced also the metabolism of all-trans-RA. RXR agonists alone had no effect on the metabolism of all-trans-RA. The RXR-selective agonist CD2608 had only effects on the metabolism of all-trans-RA in combination with the synthetic RARα-selective agonist (Am580). It enhanced the induction of the retinoic acid metabolism to polar metabolites compared with the induction by the RARα agonist Am580 alone (Fig. 9B). However, the coadministration of CD2608 and all-trans-RA (activates all three RARs) was not able to enhance the metabolism. We conclude that other additional factors may have an impact on the metabolism of all-trans-RA. One reason could be the influence of other CYP enzymes besides CYP26, for example CYP3A and CYP1A, which are not induced by retinoids in intestinal cells, but contribute to the retinoic acid metabolism because of some basal activity (Lampen et al., 2000). Nevertheless, we speculate that in addition to the synthetic RAR agonists used in this study, natural RAR agonists [for example fatty acids (Steiniger et al., 1998)], which are food constituents, may also have an effect, in combination with RAR agonists, on intestinal CYP26 gene expression and probably RA metabolism resulting in physiological alterations. Further investigations are needed to prove, whether these findings have also an impact on CYP26 gene expression, all-trans-RA metabolism, and retinoid function in vivo. In conclusion, we found that CYP26 is constitutively expressed in the human duodenum and colon. In human Caco-2 cells, CYP26 mRNA is rapidly inducible by all-trans-RA. RAR agonists have the highest effect on CYP26 gene expression and all-trans-RA metabolism; heterodimerization with RXR probably plays a role here. Because of such heterodimerization, RXR agonists when coadministered with RAR agonists enhanced CYP26 gene expression.

References