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Orphan Nuclear Receptor Binding Site in the Human Inducible Nitric Oxide Synthase Promoter Mediates Responsiveness to Steroid and Xenobiotic Ligands

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Abstract Constitutive androstane receptor (CAR) and pregnane X receptor (PXR) are members of the nuclear receptor superfamily that regulate target gene transcription in a ligand-dependent manner. CAR and PXR have a rather broad, overlapping set of ligands that range from natural steroids to xenobiotics and also recognize similar DNA binding sites, referred to as response elements (REs), primarily in promoter regions of cytochrome *P450* (CYP) genes. In this study, a CAR and PXR RE, composed of a direct repeat of two GGTTCA motifs in a distance of 4 nucleotides (DR4), was identified in the promoter of the human inducible nitric oxide (NO) synthase (*iNOS*) gene, which is the first nuclear receptor binding site reported for this promoter. In a heterologous promoter context, the DR4-type sequence also acts as a functional RE for the nuclear receptors for 1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$) and 3,5,3′-triiodothyronine (T_3), VDR and T_3R. However, in a direct competition of CAR, PXR, VDR, and T_3R, the PXR-retinoid X receptor (RXR) complex appears to be the dominant regulator on the iNOS DR4-type RE. In the natural iNOS promoter context, the DR4-type RE specifically mediates downregulation of promoter activity by the testosterone metabolite androstanol through CAR-RXR heterodimers and upregulation by the xenobiotic drug clotrimazole through PXR–RXR heterodimers. These results were confirmed on the level of mRNA expression. Since an iNOS-induced production of NO is known to influence inflammation and apoptosis, a CAR- and PXR-regulated iNOS activity may explain a modulatory effect of steroids and xenobiotics on these cellular processes. J. Cell. Biochem. 85: 72–82, 2002.

Key words: gene regulation; orphan nuclear receptors; xenobiotics; steroids; CAR; PXR; nitric oxide

Organisms encounter a wide range of foreign compounds, referred to as xenobiotics, with potential harmful consequences. CYP enzymes metabolize xenobiotics and thus are a primary defense against these compounds. Increased expression of specific CYP genes, such as CYP1A, 2B and 3A in response to particular xenobiotics is a central component of this defense, although such induction can also increase production of toxic metabolites. Two orphan members of the nuclear receptor superfamily of ligand-activated transcription factors, CAR (NR1I3) and PXR (NR1I2) [Nuclear-Receptor-Committee, 1999] have recently been implicated in mediating the effects of some natural steroids as well as of xenobiotics on the expression of different members of the CYP gene family [Waxman, 1999; Honkakoski and Negishi, 2000]. The expression pattern of CAR and PXR overlaps in tissues, such as liver and intestine [Baes et al., 1994; Kliewer et al., 1998], that are exposed to rather high concentrations of

Abbreviations used: 1α,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; androstanol, 5α-androstan-3α-ol; CAR, constitutive androstane receptor; CYP, cytochrome P450; DR4, direct repeat spaced by 4 nucleotides; iNOS, inducible NO synthase; LBD, ligand binding domain; NO, nitric oxide; PCN, pregnenolone 16α-carbonitrile; pregnanedione, 5β-pregnane-3,20-dione; PXR, pregnane X receptor; RE, response element; RXR, retinoid X receptor; T_3, 3,5,3′-triiodothyronine; T_3R, T_3 receptor; VDR, 1α,25$(OH)_2D_3$ receptor.

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xenobiotics and/or steroid metabolites. CAR and PXR collectively regulate CYP2B and CYP3A genes [Honkakoski and Negishi, 2000], such as CYP2B6 and CYP2B10 by CAR [Honkakoski et al., 1998; Sueyoshi et al., 1999], CYP3A4 and CYP3A23 by PXR [Bertilsson et al., 1998; Lehmann et al., 1998] and CYP3A11 by both CAR and PXR [Xie et al., 2000]. This provides both receptors with the role of xenobiotic sensors [Blumberg et al., 1998].

CAR and PXR are closely related to each other as well as to another nuclear receptor superfamily member, VDR (NR1I1), since they share approximately 40% of amino acid identity of their ligand binding domain (LBD). Interestingly, VDR is bound with high affinity (K_d in the order of 0.1 nM) by its natural ligand $1\alpha,25(OH)_2D_3$ [Herdick et al., 2000], whereas CAR and PXR are bound by an overlapping set of natural and synthetic ligands with rather low affinity (K_d in the order of 1 μM) [Moore et al., 2000]. However, CAR differs from most other nuclear receptors by displaying in the absence of ligand already a relatively high constitutive activity, which can be reduced by the binding of the inverse agonist 5α-androstan-3α-ol (androstanol) [Tzameli and Moore, 2001]. CAR and PXR each form a heterodimer with RXR on DR4-type REs [Sueyoshi et al., 1999], but recognize also other RE types. Similarly, classical VDR binding sites have only three spacing nucleotides between the receptor binding motifs (DR3-type), but VDR-RXR heterodimers show even higher affinity for DR4-type REs [Quack and Carlberg, 2000; Toell et al., 2000]. According to the 3-4-5 rule [Umesono et al., 1991], the typical DR4-type RE-binding nuclear receptor complex are T_3R-RXR heterodimers, but it becomes now clear that a broad variety of nuclear receptors can bind to DR4-type REs.

The enzyme iNOS (also called NOS II) is the main producer of NO in the immune system and is necessary for killing of intracellular bacteria in macrophages [Wei et al., 1995]. Although this is protective in normal inflammatory response, iNOS expression can be harmful when leading to excessive NO production. Septic shock, for example, is associated with a major systemic NO production and high local NO concentrations obtained at sites of inflammation may induce apoptosis. Therefore, NO production via iNOS requires tight control. The expression of the *iNOS* gene is regulated mainly on the level of transcription and mRNA stability [Förstermann and Kleinert, 1995]. The human iNOS promoter contains several binding sites for the transcription factors NF-κB, AP-1, IRF-1, STAT1a, and members of the POU-domain family [Förstermann and Kleinert, 1995]. These transcription factors are activated by pro-inflammatory cytokines (TNFα, IL-1β, IFNγ) and/or bacterial products (e.g., lipopolysaccharide) through membrane receptors and subsequent phosphorylation cascades. Therefore, most cells require a set of cytokines for achieving basal activity of the iNOS promoter, which is then modulated by other inducers, such as Janus protein kinases 2 (JAK-2) [Kleinert et al., 1998]. In addition, iNOS promoter activity and transcription also can be modulated by signals that do not involve membrane receptors and cytoplasmatic signal transduction cascades, such as a downregulation by retinoic acid via the nuclear receptors retinoic acid receptor (RAR)α [Sirsjö et al., 2000] or peroxisome proliferator-activated receptor (PPAR)γ and its co-factors [Li et al., 2000]. However, these effects appear not to be mediated by a direct binding of these receptors to the iNOS promoter, but through DNA-independent protein–protein interactions with NF-κB and AP-1, i.e., no nuclear receptor RE has been reported yet for this gene.

In this report, it was hypothesized that nuclear receptors and their ligands directly regulate the *iNOS* gene. A DR4-type RE was identified in the human iNOS promoter, which acts in a heterologous promoter context as a functional RE for CAR, PXR, VDR, and T_3R. However, PXR-RXR heterodimers appear to be the dominant on this RE. In the natural promoter context, the DR4-type RE specifically mediates downregulation of promoter activity by the CAR-ligand androstanol and upregulation by the PXR-ligand clotrimazole. This suggests that steroids and xenobiotics may have via direct regulation of iNOS expression a modulatory effect on inflammation and apoptosis, and even induce some hepato-protective effects.

MATERIALS AND METHODS

Compounds

The steroids androstanol and 5β-pregnane-3,20-dione (pregnanedione) were from Steraloids (Newport, RI), the synthetic steroid pregnenolone 16α-carbonitrile (PCN), the imidazole antimycotic clotrimazole, the antibiotic

rifampicin, the antiepileptic phenobarbital, and the thyroid hormone 3,5,3′-triiodothyronine (T_3) were from Sigma (Deisenhofen, Germany) and $1\alpha,25(OH)_2D_3$ was kindly provided by L. Binderup (Leo Pharmaceutical Products, Ballerup, Denmark). The VDR agonist was dissolved in isopropanol and diluted in ethanol, whereas all other compounds were dissolved and diluted in DMSO.

DNA Constructs

Nuclear receptor expression vectors. The cDNAs for human CAR [Baes et al., 1994], human PXR [Bertilsson et al., 1998], human VDR [Baker et al., 1988], chicken $T_3R\alpha$ [Sap et al., 1986], and human RXRα [Mangelsdorf et al., 1990] were subcloned into the T_7/SV40 promoter-driven pSG5 expression vector (Stratagene, Heidelberg, Germany).

Reporter gene constructs. Four copies of the DR4-type RE from the human iNOS promoter (for core sequence see Fig. 1) were fused with the thymidine kinase (*tk*) minimal promoter driving the luciferase reporter gene as schematically depicted in Figure 2. The iNOS promoter reporter construct ($pGL3_{neo}$-3600) was generated by subcloning a 3,686 bp Kpn I/Eag I fragment ($-3{,}653$ to $+33$) of the human iNOS promoter into $pGL3_{neo}$. The construct $pGL3_{neo}$-3600mut was generated by point mutagenesis of the DR4-type RE in $pGL3_{neo}$-3600 according to the sequence of 5′-mut (Fig. 1). Mutagenesis was performed of a 326 bp Eco RV fragment of the promoter as recommended by the supplier (Stratagene), the fragment was then recloned into $pGL3_{neo}$-3600 and verified by sequencing.

In Vitro Translation and Gel Shift Assays

In vitro translated CAR, PXR, VDR, T_3R, and RXR proteins were generated by transcribing their linearized pSG5-based cDNA expression vectors with T_7 RNA polymerase and translating these RNAs in vitro using rabbit reticulocyte lysate as recommended by the supplier (Promega, Mannheim, Germany). By test-translation in the presence of [^{35}S]-methionine and taking the individual numbers of methionine residues per receptor into account, the specific concentration of the receptor proteins was adjusted to approximately 4 ng/μl. Equal amounts of in vitro translated CAR, PXR, VDR, T_3R, and RXR proteins ($\sim$10 ng each) were mixed and incubated for 15 min at room temperature in a total volume of 20 μl binding buffer (10 mM HEPES [pH 7.9], 1 mM DTT, 0.2 μg/μl poly-[d(I-C)], 5% glycerol). The buffer was adjusted to 150 mM of monovalent cations by addition of KCl. Double-stranded oligonucleotides carrying the RE core sequences (Fig. 1) were labeled by fill-in reactions using [α-^{32}P]-dCTP and the Klenow fragment of DNA polymerase I (Promega). Approximately 1 ng of labeled RE probe (50,000 cpm) was added to the receptor-ligand mixture and incubation was continued for 20 min. Protein-DNA complexes were resolved through 8% non-denaturing polyacrylamide gels (at room temperature) in 0.5× TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA [pH 8.3]) and exposed to a Fuji MP2040S imager screen. The ratio of protein-complexed probe to free probe was quantified with the use of a Fuji FLA2000 reader (Tokyo, Japan) using Image Gauge software (Fuji).

Transfection and Luciferase Reporter Gene Assays

Human epithelial-like colon carcinoma DLD-1 cells were seeded into 6-well plates (10^5 cells/ml) and grown overnight in phenol red-free DMEM supplemented with 10% charcoal-treated fetal bovine serum (FBS). Liposomes were formed by incubating 1 μg of the reporter plasmid and each 1 μg of the pSG5-based receptor expression vectors for CAR, PXR, VDR, T_3R, and RXR with 15 μg *N*-[1-(2,3-Dioleoyloxy)-propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate (DOTAP, Roth, Karlsruhe, Germany) for 15 min at room temperature in a total volume of 100 μl. After dilution with 900 μl phenol red-free DMEM, the liposomes were added to the cells. Phenol red-free DMEM supplemented with 30% charcoal-treated FBS (500 μl) was added 4 h after transfection. At this time, nuclear receptor ligands were also added. The cells were lysed 16 h after onset of stimulation using the reporter gene lysis buffer (Roche Diagnostics, Mannheim, Germany) and the constant light signal luciferase reporter gene assay was performed as recommended by the supplier (Canberra-Packard, Dreieich, Germany). Transfection efficiency in DLD-1 was controlled by co-transfection of pCH110 (Amersham-Pharmacia, Freiburg, Germany) and β-galactosidase assay (Roche Diagnostics, Mannheim). The luciferase activities were normalized with respect to protein concentration and induction factors were calculated as the ratio of

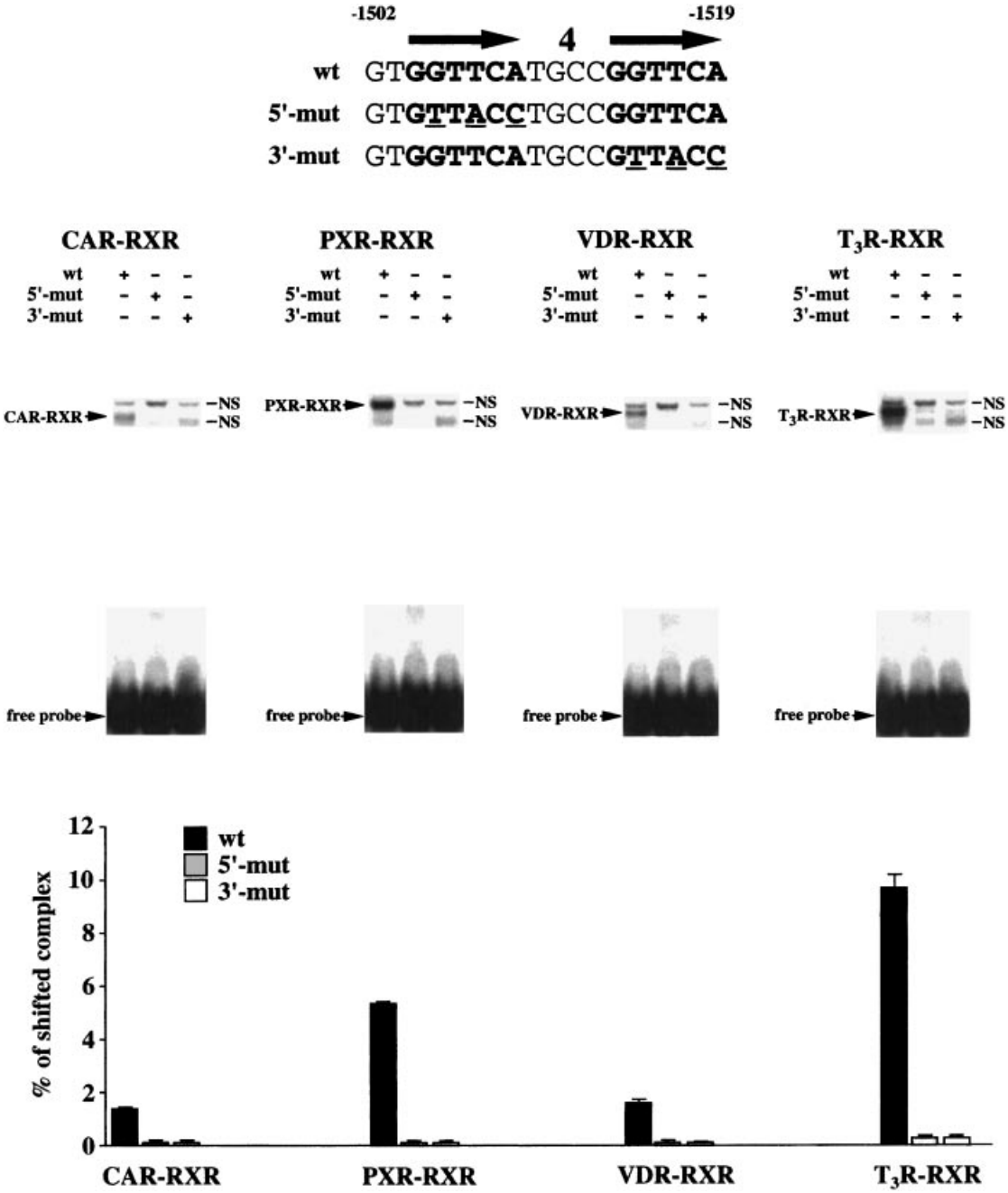

Fig. 1. Nuclear receptor complex formation on the DR4-type RE of the human iNOS promoter. Gel shift experiments were performed with in vitro translated CAR-RXR, PXR-RXR, VDR-RXR, and T_3R-RXR heterodimers and [^{32}P]-labeled DR4-type REs. On the top, the core sequences of the wild type (wt) RE and its 5′- and 3′-binding motif mutants (5′- and 3′-mut) are shown. Protein-DNA complexes were separated from free probe through 8% non-denaturing polyacrylamide gels. Representative experiments are shown. The amount of heterodimer complex formation was quantified in relation to free probe. Columns represent the mean of triplicates and bars indicate standard deviation. NS indicates unspecific complexes.

luciferase activity of ligand-stimulated cells to that of solvent controls.

RT–PCR

First strand cDNA synthesis was performed on total RNA (2 μg) using MMLV reverse transcriptase (Life Technologies, Eggenstein, Germany) and oligo dT primer. This cDNA (2.5% of total) served as a template in a PCR reaction with a profile of 1 min at 94°C, 1 min at 65°C (58°C for GAPDH), and 1 min at 72°C for 32 cycles. PCR reactions were performed with

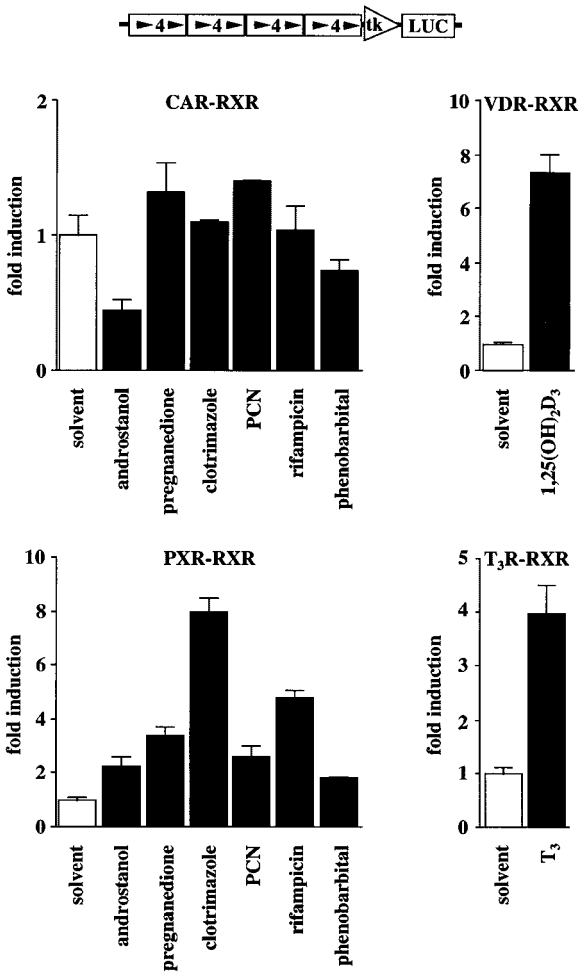

Fig. 2. Nuclear receptor ligand responsiveness of the human iNOS DR4-type RE in a heterologous promoter context. *Luciferase* reporter gene assays were performed with extracts from DLD-1 cells, which were transiently transfected with 1 μg of a *luciferase* reporter gene construct driven by four copies of the iNOS DR4-type RE (schematically shown above) and 1 μg of an expression vectors for CAR, PXR, VDR, or T_3R (each in combination with 1 μg of expression vector for RXR). Cells were treated for 16 h with the indicated compounds (at 10 μM, with the exception of $1\alpha,25(OH)_2D_3$ and T_3; both at 100 nM). Stimulation of normalized luciferase activity was calculated in comparison to solvent-induced controls. Columns represent the mean of triplicates and the bars indicate standard deviation.

1 μM of gene-specific primer pairs with the sequence listed below, that were 5′-end-labeled using [γ-^{32}P]-ATP and T_4 polynucleotide kinase (Promega): iNOS$^+$ GGAATTCACTCA-GCTGTGCATCG; iNOS$^-$ GTTTCCAGGCC-CATTCTCCTGC; GAPDH$^+$ CGTCTTCACCA-CCATGGAGAA; GAPDH$^-$ TCTTACTCCTTG-GAGGCCATG. Amplified PCR products were separated from unincorporated primers on 5% non-denaturing polyacrylamide gels in 0.5 × TBE buffer. The ratio of specific PCR products to unincorporated primer were quantified on a Fuji FLA-2000 reader and normalized to the GAPDH housekeeping gene PCR product/primer ratio, thus providing a value for relative iNOS mRNA expression. The statistical significance of the change of mRNA expression in ligand-treated samples in reference to solvent controls was analyzed by Student's *t*-test.

RESULTS

The 8,296 bp sequence of the human iNOS promoter [Chu et al., 1998] were screened in silico for the hexameric nuclear receptor binding motif RGKTSA (R = A or G, K = G or T, S = C or G) [Carlberg, 1995]. This resulted in 13 motifs in sense orientation and 21 motifs in antisense orientation, of which only two motifs (located between positions −1,521 and −1,504) are in a sufficiently close vicinity to each other to form a nuclear receptor RE. This putative RE is composed of a direct repeat of two GGTTCA motifs that are spaced by four nucleotides (Fig. 1). According to the 3-4-5 rule [Umesono et al., 1991], such a DR4-type RE should preferentially be bound by T_3R-RXR heterodimers, but a couple of other nuclear receptors, such as CAR-RXR, PXR-RXR and even VDR-RXR heterodimers [Quack and Carlberg, 2000], are also known to bind to this type of RE. Therefore, gel shift assays were performed using equal amounts of in vitro translated CAR-RXR, PXR-RXR, VDR-RXR, and T_3R-RXR heterodimers and a [^{32}P]-labeled, double-stranded oligonucleotide probe containing the putative DR4-type RE (Fig. 1). The oligonucleotide probes 5′-mut and 3′-mut (Fig. 1) represent variations of the DR4-type RE, in which the 5′- and the 3′-motif, respectively, were inactivated through the indicated base pair exchanges at the critical positions 2, 4, and 6 of the hexameric motif. These probes were used as negative controls and did not allow complex formation of the four tested heterodimers, whereas specifically all four types of heterodimers bound the wild type RE. A quantification of the relative amount of complex formation of the equal quantity of the four heterodimers on the DR4-type RE indicated that T_3R-RXR heterodimers have a higher affinity for the RE than PXR-RXR heterodimers, which in turn bind stronger than CAR-RXR and VDR-RXR heterodimers (Fig. 1). The binding affinity of VDR-RXR heterodimers

on this DR4-type RE showed to be approximately 40% of that of the DR4-type RE of the rat *Pit-1* gene (data not shown). The latter RE is known to be the strongest of the presently known $1\alpha,25(OH)_2D_3$ REs, which ranks the here identified RE still within the best natural $1\alpha,25(OH)_2D_3$ REs [Quack and Carlberg, 2000; Toell et al., 2000].

The functionality of the identified DR4-type RE was tested in luciferase reporter gene assays, in which human epithelial-like colon carcinoma DLD-1 cells were transiently transfected with a reporter gene construct that contained four copies of the DR4-type RE in a heterologous promoter context (*tk* promoter) driving the firefly luciferase reporter gene (Fig. 2). DLD-1 cells are known to express endogenously the *iNOS* gene, so they are assumed to contain all necessary gene-specific transcription factors. However, some nuclear receptors (in particular CAR) are known to be drastically downregulated, when cells are taken into culture [Pascussi et al., 2000a,b]. This is also the case in DLD-1 cells (data not shown). This problem was avoided by overexpressing the receptors CAR, PXR, VDR, T_3R, and RXR in DLD-1 cells through co-transfection of equal amounts of the respective expression vectors. VDR-RXR and T_3R-RXR overexpressing cells were stimulated with saturating concentrations (100 nM) of the respective natural ligands, $1\alpha,25(OH)_2D_3$ and T_3. This resulted in a 7- and 4-fold induction, respectively, of reporter gene activity (Fig. 2). CAR and PXR are known to share some ligands and activators [Moore et al., 2000], so that both CAR-RXR and PXR-RXR overexpressing cells were stimulated with the same set of compounds. This set included androstanol, pregnanedione, clotrimazole, PCN, rifampicin, and phenobarbital (all at a concentration of 10 μM). From this selection of compounds only androstanol showed a significant effect on CAR-mediated reporter gene activity (a more than 2-fold downregulation), whereas all of the six compounds induced a 2- to 8-fold induction of PXR-triggered transactivation (indicating clotrimazole to be most effective, see Fig. 2). Pregnanedione has been reported to be an activator of human CAR, whereas clotrimazole should be a deactivator of CAR [Moore et al., 2000], which could not be confirmed in the experimental system used here. However, the activation pattern of human PXR is in accordance with a previous report [Moore et al., 2000]. Taken together, the overexpression of individual heterodimer pairs suggests that the newly identified DR4-type RE is capable to mediate functional activity of all four tested heterodimer pairs.

Most cells simultaneously express several members of the nuclear receptor superfamily, which may cause competition between those receptors that have a similar RE preference. In order to simulate more closer the in vivo condition, reporter gene assays were performed in DLD-1 cells, in which equal amounts of CAR, PXR, VDR, T_3R, and RXR were coexpressed at the same time and were tested for their ligand-dependent action on the multimer of the DR4-type RE (Fig. 3). Stimulation was performed with the same set and concentration of compounds than in the previous experiment (Fig. 2). Interestingly, under these conditions $1\alpha,25(OH)_2D_3$ and T_3 provided only marginal induction of reporter gene activity, a stimulation with androstanol resulted in a 2-fold activation and clotrimazole was most potent

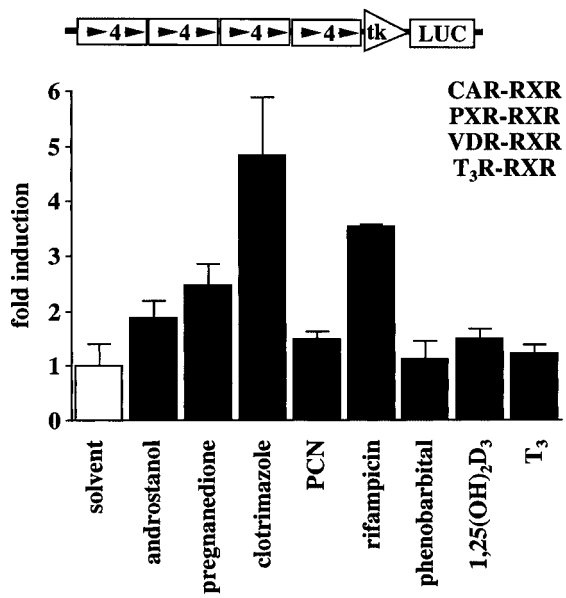

Fig. 3. Competition of nuclear receptors on the human iNOS DR4-type RE. *Luciferase* reporter gene assays were performed with extracts from DLD-1 cells, which were transiently transfected with 1 μg of a *luciferase* reporter gene construct driven by four copies of the iNOS DR4-type RE (schematically shown above) and each 1 μg of expression vectors for CAR, PXR, VDR, T_3R, and RXR. Cells were treated for 16 h with the indicated compounds (at 10 μM with the exception of $1\alpha,25(OH)_2D_3$ and T_3; both at 100 nM). Stimulation of normalized luciferase activity was calculated in comparison to solvent-induced controls. Columns represent the mean of triplicates and the bars indicate standard deviation.

providing a 5-fold induction. Moreover, pregnanedione and rifampicin treatment resulted in a 2.4- and 3.5-fold induction of reporter gene activity, respectively. Taken together, the activation pattern resembled mostly that of the PXR-RXR heterodimers (Fig. 2). In particular, the fact that androstanol activates the receptor mixture, suggests that PXR-RXR heterodimers are the dominant nuclear receptor complex on the DR4-type RE of the iNOS promoter, i.e., that they transactivate from this RE more effective than CAR-RXR, VDR-RXR, and T_3R-RXR heterodimers. This is similar to the DR4-type RE of the *CYP3A4* gene promoter that is also dominantly regulated by PXR even in the molar excess of CAR [Moore et al., 2000].

The functional profile of a RE in a heterologous promoter context does not always reflect the activity of the element in its natural promoter context. Therefore, a 3,653 bp fragment of the proximal iNOS promoter was tested in transiently transfected DLD-1 cells for reporter gene activity (Fig. 4). For comparison, a mutation of this construct was tested, in which the 5′-motif of the DR4-type RE was changed to a non-receptor-binding form (compare 5′-mut in Fig. 1). In this case, CAR-RXR, PXR-RXR, VDR-RXR, and T_3R-RXR heterodimers were overexpressed individually and only the ligands androstanol, clotrimazole, $1\alpha,25(OH)_2D_3$, and T_3, respectively, were used. Interestingly, on the natural promoter androstanol provided with a 4-fold reduction of CAR-mediated reporter gene activity a more drastic effect than on the heterologous promoter construct. Moreover, a co-stimulation of androstanol and clotrimazol resulted in a nearly 2-fold stimulation compared to the reduced promoter activity by treatment

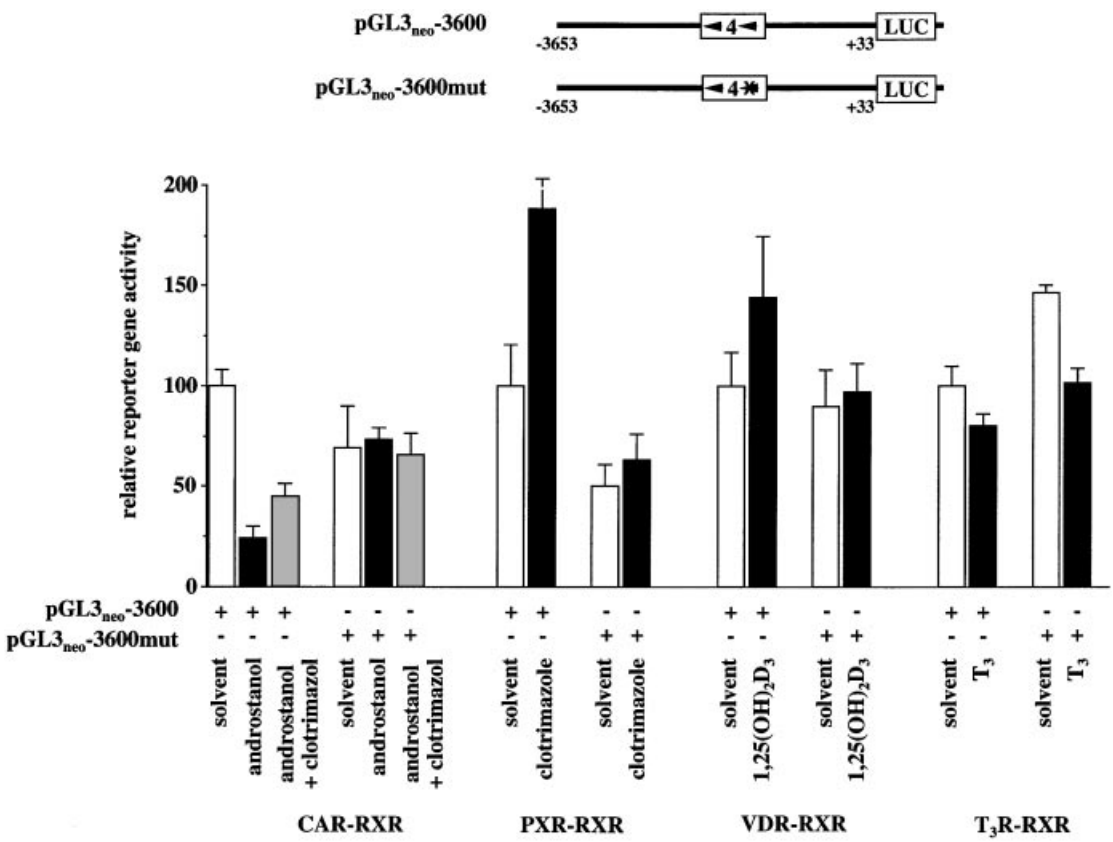

Fig. 4. Nuclear receptor ligand responsiveness of the human iNOS promoter. *Luciferase* reporter gene assays were performed with extracts from DLD-1 cells, which were transiently transfected with 1 μg of iNOS promoter reporter constructs and 1 μg of expression vectors for CAR, PXR, VDR, or T_3R (each in combination with RXR). The reporter gene constructs contained 3,653 bp of the proximal promoter either in its wild type form or mutated in the 5′-motif of the DR4-type RE (as schematically shown above, compare also Fig. 1). Cells were treated for 16 h with 10 μM androstanol and 10 μM clotrimazole (alone or in combination), 100 nM $1\alpha,25(OH)_2D_3$ or 100 nM T_3. Stimulation of normalized luciferase activity was calculated in comparison to solvent-induced activity of the wild type construct. Columns represent the mean of triplicates and the bars indicate standard deviation.

with androstanol alone. The approximately 2-fold induction of PXR-mediated transactivation by clotrimazol was found to be weaker on the natural promoter than on the heterologous promoter construct. The VDR ligand $1\alpha,25(OH)_2D_3$ again showed only marginal stimulation of reporter gene activity and T_3 even provided a slight reduction. On the point-mutated promoter all ligand effects disappeared (with the exception that the reduction by T_3 was even a little bit more prominent). Moreover, in case of CAR-RXR and PXR-RXR overexpressing cells, the mutation resulted in a reduction of basal promoter activity, whereas it showed in case of T_3R-RXR heterodimers a slight increase of basal promoter activity and with VDR-RXR heterodimers no effect was observed. Taken together, the DR4-type sequence acts also in the natural promoter context as a RE, but it seems to mediate only ligand-dependent transactivation by CAR and PXR, but not by VDR and T_3R.

In order to demonstrate that the results on iNOS promoter activity (Fig. 4) can be transferred to the level of iNOS mRNA expression, DLD-1 cells were transiently overexpressed with CAR and RXR or PXR and RXR, and stimulated with androstanol and clotrimazol, respectively. Total RNA was extracted from these cells and semi-quantitative RT–PCR was performed on respective cDNAs using gene-specific primers for iNOS and GAPDH as a "housekeeping" reference (Fig. 5). Since DLD-1 cells show a relatively high basal iNOS mRNA expression, these values were subtracted. In CAR-overexpressing cells androstanol treatment resulted in a downregulation of iNOS mRNA expression, whereas in PXR-overexpressing cells a stimulation with clotrimazol provided a clear up-regulation of iNOS transcription. This confirmed the assumed linearity between promoter activity and transcriptional activity.

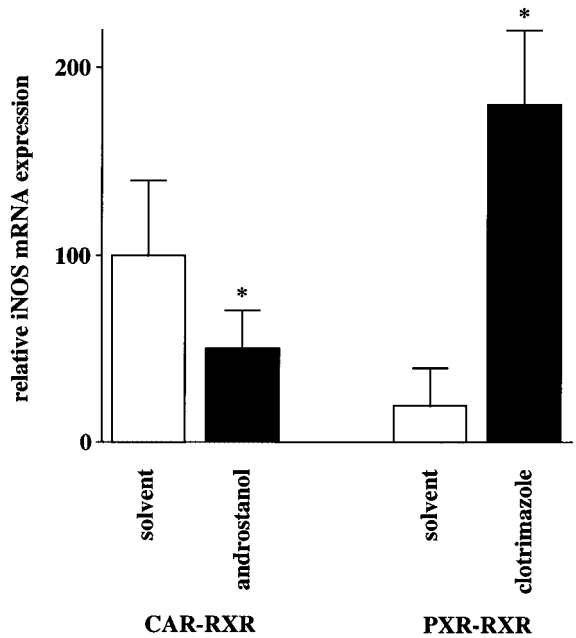

Fig. 5. Expression of iNOS mRNA in response to androstanol and clotrimazol. DLD-1 cells were transiently transfected with expression vectors for CAR or PXR (each in combination with RXR) and treated for 16 h with 10 μM androstanol or 10 μM clotrimazole, respectively. Total RNA was isolated and RT–PCR was performed with primer pairs that were specific for the *iNOS* gene. The mRNA expression was normalized for *GAPDH* ("housekeeping") gene mRNA expression and basal iNOS mRNA expression of DLD-1 cells was subtracted. Columns represent mean values of three to six PCR; the bars indicate standard deviation. The statistical significance is indicated by asterisks ($P < 0.05$).

DISCUSSION

The orphan nuclear receptors CAR and PXR regulate the expression of those *CYP* genes that are involved in the oxidative metabolism of natural steroids as well as xenobiotics, CYP2B10 and CYP3A4, respectively. It appears that CAR and PXR are components of a regulatory network that governs endocrine hormone homeostasis and is also of central importance in the regulation of drug metabolism. In this study, a new aspect to the function of CAR and PXR was added by the observation that the human *iNOS* gene is also a primary responding gene for both orphan receptors. It is assumed that CAR and PXR each may have hundreds of low-affinity ligands that are mainly of xenobiotic origin, so that the main ligands of this study, androstanol and clotrimazole, should only be considered as representatives for ligand classes. This suggests that there are probably much more xenobiotic compounds that are direct regulators of iNOS expression than presented here. One example is the HMG-CoA reductase inhibitor lovastatin, which has recently been reported to up-regulate iNOS mRNA and protein [Hausding et al., 2000]. This effect was suggested to be mediated indirectly through the inhibition of the farnesylation of the small G protein Ras, which then enhances the cytokine-mediated induction of iNOS. Interestingly, lovastatin is also known as activator of CYP3A4 expression through PXR [Moore and Kliewer,

2000], which would also provide a more straightforward explanation for the upregulation of iNOS by this statin.

The co-regulation of *CYP* gene family members and iNOS may simply be a side effect of some CAR- and PXR-activating xenobiotics. This would then suggest that the two types of enzymes are not functionally linked, but it would still be important for understanding the effects of some xenobiotics on a molecular level. However, effects of high-output NO synthesis through iNOS are complex and often contradictory, since NO can act as a powerful inducer of cellular apoptosis or necrosis, but in other cases, it can also protect cells from a toxic insult. NO is an antioxidant but prolonged exposure to NO will shift the cellular redox state to a more oxidized state, e.g. via oxidation of thiols such as glutathione. As NO is known to terminate lipid peroxidation reactions by scavenging lipid peroxyl radicals [Kröncke et al., 2000], iNOS-derived NO may protect cells from xenobiotic-induced oxidative stress or from oxygen-radicals generated during the metabolism of xenobiotics. Alternatively, iNOS derived NO may serve as a relatively unspecific but broad signal for cells to switch after *S*-nitrosation reactions and disruption of various zinc sulfur structures into another state [Kröncke and Carlberg, 2000]. Interestingly, NO has been found to inhibit CYP enzymes via binding to their heme group [Khatsenko et al., 1993], so that iNOS activity could be considered as a counter-regulator of CYP enzyme activity in liver, where both enzymes are coexpressed [Li and Billiar, 1999]. Therefore, iNOS activity could function as a sensor for to elevated metabolic activity by CYPs that may affect also the level of other natural and synthetic CYP substrates.

Interestingly, under certain cellular stress situations, such septic shock and intoxication, iNOS-mediated NO production was shown to have a liver-protective effect [Ou et al., 1997; Muriel, 1998]. Moreover, the *iNOS* gene appears to be critical for liver regeneration [Rai et al., 1998]. This parallels with the observation that PXR acts as a sensor for toxic bile acids, such as lithocholic acid, and has an essential role in the detoxification of these compounds [Staudinger et al., 2001; Xie et al., 2001]. Taken together, this suggests that PXR may mediate its hepato-protective effects via the induction of iNOS activity.

The iNOS DR4-type RE contains two perfect GGTTCA motifs, which is relatively rare for natural REs, but still is about three-times less potent than the DR4-type RE from the rat *Pit-1* gene [Quack and Carlberg, 2000] (data not shown). This may explain the relative weak activation of the iNOS promoter by CAR, PXR, and their ligands and could suggest that, like the *CYP3A* gene promoter [Goodwin et al., 1999], the iNOS promoter may contain further REs in the regions upstream of the 8 kB fragment that was screened in this study. However, an in silico screening of a fragment of human chromosome 17 that contains 140 kB 5′-flanking sequence of the *iNOS* gene (accession no. AC005697) did not provide evidence of a CAR and PXR RE with comparable perfect half site motifs than the element at position −1.5 kB (data not shown).

In conclusion, the first demonstration of a nuclear receptor binding site in the promoter of the human *iNOS* gene provides an interesting link between xenobiotics, iNOS activity and *CYP* genes and may explain some hepatoprotective effects of NO.

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