Vol. 215, No. 1, 1995
October 4, 1995

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 338-345

ANTAGONISM OF COUP-TF AND PPARα/RXRα ON THE ACTIVATION OF THE MALIC ENZYME GENE PROMOTER: MODULATION BY 9-CIS RA

Myriam Baes[1], Hilde Castelein, Lieve Desmet and Peter E. Declercq

Laboratory for Clinical Chemistry, Catholic University of Leuven, Faculty of Pharmaceutical Sciences, Onderwijs en Navorsing, Herestraat 49, B- 3000 Leuven, Belgium

Received August 14, 1995

We previously demonstrated that heterodimers of the Peroxisome Proliferator Activated Receptor α (PPARα) and the Retinoid X Receptor α (RXRα) stimulate malic enzyme gene transcription through a regulatory element in the promoter region (ME-PPRE). In this report, we show that the orphan nuclear receptor COUP-TF also displays affinity for the ME-PPRE and competes with PPARα/RXRα for binding to this element. In transient transfections of a reporter driven by the ME-PPRE in a heterologous or in the homologous promoter context, COUP-TF strongly antagonizes the transactivation by PPARαRXRα in the absence of exogenously added ligands. Although 9-cis RA did not further enhance the transcriptional effects of the heterodimers activated by ciprofibrate, it greatly impaired the suppressive effects of COUP-TF on the ciprofibrate activated PPARα/RXRα. We conclude that the antagonism by COUP-TF uncovers differential activation states of PPARα/RXRα heterodimers in the absence and in the presence of 9-cis RA.

We recently demonstrated (1) that peroxisome proliferators, a diverse class of xenobiotic amphipathic carboxylates, induce malic enzyme gene transcription through the action of heterodimers of the Peroxisome Proliferator Activated Receptorα (PPARα) and Retinoid X Receptorα (RXRα), both members of the thyroid receptor subclass of the nuclear receptor gene family, on a regulatory element in the promoter region of the malic enzyme gene (PPRE). Similar to the PPREs characterized in other genes, the malic enzyme PPRE consists of a direct repeat of the consensus hexamer for this receptor family (AGGTCA) with an interspacing of 1 base pair (DR1). It is generally accepted that the spacing between the hexamers is an important discriminating factor for response element recognition between the different members of the nuclear receptors of the thyroid subfamily (2).

[1] To whom correspondence should be addressed. FAX. +3216347281.

ABBREVIATIONS: ARP-1, Apolipoprotein AI-Regulatory Protein-1; CAT, Cloramphenicol Acetyl Transferase; COUP-TF, Chicken Ovalbumin Upstream Promoter Transcription Factor; EMSA, Electrophoretic Mobility Shift Assay; Ear3, Erb A Related protein 3; ER, Estrogen Receptor; HNF-4, Hepatocyte Nuclear Factor 4; RAR, Retinoic Acid Receptor; RXR, Retinoic X Receptor; TR, Thyroid hormone Receptor; PPAR, Peroxisome Proliferator Activated Receptor; PPRE, Peroxisome Proliferator Responsive Element; VDR, Vitamin D Receptor.

0006-291X/95 $12.00

However, the DR1 configuration is not exclusive for PPAR/RXR heterodimers but also recognizes homodimers of RXR (2), of the orphan receptors COUP-TF (3) and its homologues and of HNF-4 (4).

COUP-TF (3) was originally isolated as a transcription factor activating the Chicken Ovalbumin Promoter through a DR1 regulatory element. However, the suppressive effects of COUP-TF on gene transcription are presently better documented. Antagonism of the transcriptional activities of VDR/RXR (5), RAR/RXR (5,6), TR/RXR (5), HNF-4 (7,8) and ER (9) have been described. COUP-TF was also shown to inhibit the activation by PPAR/RXR of the peroxisomal hydratase/dehydrogenase gene involved in β-oxidation (10). Different mechanisms for these suppressive effects have been documented (11) including competitive binding of COUP-TF homodimers to the regulatory elements, active silencing on gene transcription and formation of transcriptionally inactive COUP/RXR, TR/COUP and RAR/COUP complexes.

In the present study we found that COUP-TF represses the activity of PPARα/RXRα on malic enzyme gene transcription by competitive occupation of the DR1 PPRE, either as a homodimer or as a heterodimer with RXR. However, transactivation experiments demonstrate that this antagonism is modulated by the RXR ligand 9-cis RA.

MATERIALS AND METHODS

Plasmids The pMECAT and pME-PPRE2-CAT reporter vectors have been used in previous studies (1) as well as the eukaryotic expression vectors pCDM (parental), pCDM-RXRα (encoding human RXRα) and pCDM-PPARα (encoding mouse PPARα). pCDM-COUP (encoding hamster COUP-TF) as well as the bacterial expression vectors pT7*myc*RXRα, pT7*myc*PPARα and pT7*myc*COUP were generously provided by D.D.Moore and T. Gulick (Department of Molecular Biology, Massachusetts General Hospital, Boston) (12). In these vectors, the coding regions of the receptors are preceded by sequences encoding the 9E10 c-*myc* epitope (13).

Protein production and gel shift analysis Bacterial expression and partial purification of the receptors and electrophoretic mobility shift assays (EMSA) were done as previously dexcribed (1). The antisera used in the EMSA were kindly provided by Dr.S. Karathanasis, American Cyanamid Company, New York (antiserum raised to ARP-1 and crossreacting with COUP-TF) and by R. Evans, The Salk Institute, San Diego (anti RXR).

Cell culture and transfections COS-M6 cells were transfected using calcium phosphate precipitation as previously described (1). After shocking, cells were treated with 100 μM ciprofibrate (Winthrop Laboratories, Brussels, Belgium), 1 μM 9-cis retinoic acid (RA) (Ligand Pharmaceuticals, La Jolla, CA), 100 μM decanoic acid (Sigma) or a combination of these ligands. Each ligand was dissolved in dimethyl sulfoxide and diluted a thousand fold in the culture medium.

RESULTS

COUP-TF and PPARα/RXRα compete for binding to the ME-PPRE

To test the possibility that COUP-TF interacts with the ME-PPRE, a mobility shift assay (EMSA) was performed. As shown in Fig. 1, bacterially expressed *myc*

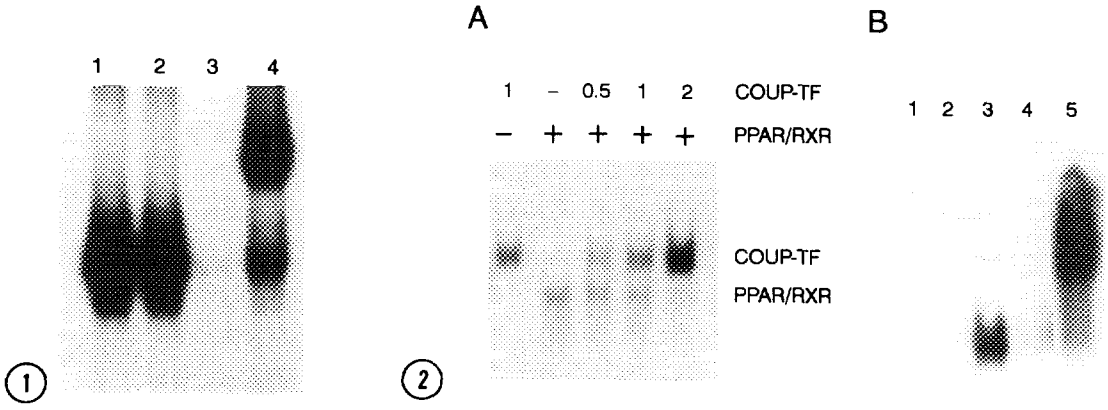

Figure 1. Binding of *myc*COUP to the ME-PPRE. EMSA of the bacterially expressed fusion protein *myc*COUP with the [^{32}P] endlabeled ME-PPRE probe (lane1). The specificity of the retarded complex was shown by coincubation with a 100 fold excess of a nonrelated oligonucleotide (lane 2) and with a 100 fold excess of the ME-PPRE (lane 3). Coincubation with the monoclonal anti *myc* antibody gave rise to a supershift (lane 4).

Figure 2. Competition of COUP-TF and PPARα/RXRα for binding to the ME-PPRE. A) Bacterially expressed *myc*COUP-TF and/or *myc*PPARa/*myc*RXRα were used as indicated in an EMSA with the ME-PPRE as a probe. Arbitrary units used for COUP-TF are indicated above the respective lanes. Proteins were preincubated at room temperature for 15 min prior to addition of the other components of the binding reaction. The concentration of the radiolabeled probe was reduced from 50000 cpm/ reaction (Fig 1) to 7500 cpm/reaction in order to better visualize competitive binding.
B) *myc*COUP and *myc*RXRα were incubated either separately (lane 1,2) or in combination (lane 4,5,6) with the ME-PPRE. The presence of *myc*COUP and *myc*RXRα in the retarded complex was demonstrated by coincubation with antiRXR (lane 4) or antiCOUP (lane 5) polyclonal antisera.

epitope tagged COUP-TF formed a specific retarded complex with the labeled probe. Since both PPARα/RXRα and COUP-TF bind to the ME-PPRE, we investigated whether these transcription factors interact in a competitive fashion with this element. Binding experiments were set up in which the amount of PPARα/RXRα was kept constant, whereas the COUP-TF concentration was gradually increased. Coincubation of a small amount of COUP-TF with PPARα/RXRα resulted in the appearance of two discrete bands in the gel (Fig 2A, lane 3). The mobility of these bands corresponded with those of COUP-TF homodimers and PPARα/RXRα heterodimers. Upon increasing the COUP-TF concentration, the COUP-TF band became more prominent whereas the PPARα/RXRα band faded and eventually disappeared (lanes 4,5).
Although no bands with intermediate mobility were observed, these results do not exclude that alternative receptor heterodimer combinations were formed. Therefore more binding assays were performed in which COUP-TF was combined with either PPARα or RXRα. As shown in fig.2B, COUP-TF and RXRα bound synergistically to

the ME-PPRE in a complex with the same mobility as COUP-TF homodimers (not shown in figure). In contrast, no evidence for heterodimerization of PPARα and COUP-TF on the ME-PPRE was obtained. These data suggest that the binding competition of COUP-TF with PPARα/RXRα may either occur through COUP-TF homodimers or through COUP-TF/RXRα heterodimers.

Antagonism of the PPARα/RXRα induced transactivation of the ME-PPRE by COUP-TF

In order to investigate whether the binding of COUP-TF to the ME-PPRE can affect malic enzyme gene transcription, cotransfection experiments were performed. First, we examined the effects of COUP-TF on a tandem copy of the ME-PPRE cloned upstream of the TK promoter in a CAT reporter vector (pME-PPRE2-CAT). Cotransfection with 3 μg of COUP-TF alone reduced the activity of this reporter by 28 ± 6 % (n=3, not shown) using the parental expression vector as a control. This effect was mediated by the ME-PPRE since COUP-TF did not influence transcriptional activity of the parental reporter. As shown previously (1), PPARα/RXRα induced a 10 fold increase of the activity of the ME-PPRE2-CAT reporter, which was further enhanced when the cells were incubated with ciprofibrate. Coincubation with the RXR ligand 9-cis RA only marginally improved the stimulation induced by ciprofibrate (Fig 3A). When COUP-TF was cotransfected

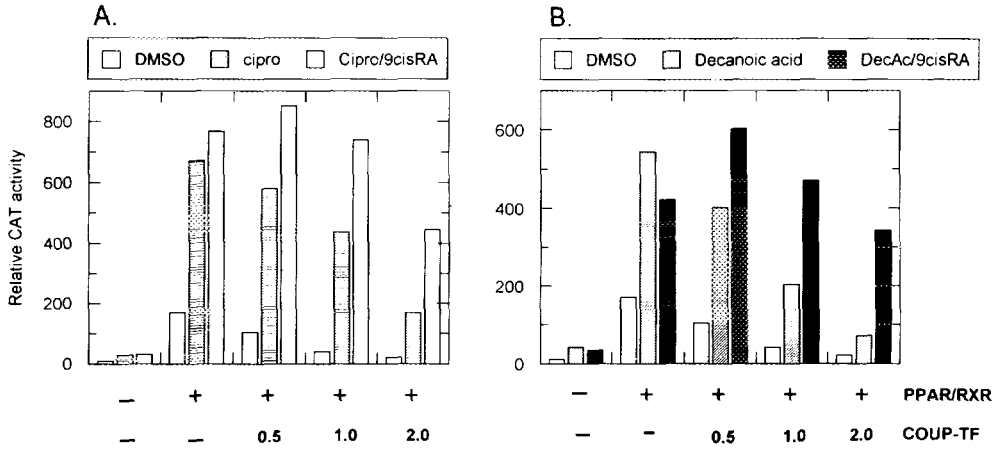

Figure 3. **Antagonism of COUP-TF and PPARα/RXRα on the transactivation of the ME-PPRE.** The ME-PPRE was cloned as a tandem repeat in the pUTKAT reporter and used in cotransfection experiments with receptor expression plasmids in COS cells. A) The indicated amounts of a COUP-TF expressing plasmid were cotransfected with a constant amount of PPARα/RXRα expression plasmids (1 μg each). The parental expression vector CDM was used either as a control or to equalize the total amount of expression vector to 4ug/60 mm plate. The cells were treated with either vehicle (DMSO), a PPAR activating ligand (ciprofibrate, 100 μM) or the combination of ciprofibrate and the RXR ligand, 9-cis RA (1 μM). A representative experiment out of 3 with similar results is shown. B) Decanoic acid (100 μM) was used as a PPARα activating ligand in an analogous experiment.

with PPARα/RXRα, a dose dependent repression of the activation by nonliganded PPARα/RXRα was observed. At the maximum dose of COUP-TF tested, the activation by PPARα/RXRα was completely abolished (95 % inhibition). However, the ciprofibrate activated PPARα/RXRα appeared to be less sensitive to the inhibitory effects of COUP-TF: maximal inhibition reached 80%. More dramatically, when PPARα/RXRα was activated by the cognate activating ligands for both receptors, the suppressive effect of high doses of COUP-TF was limited to approximately 45%. Unexpectedly, we repeatedly observed that low doses of COUP-TF enhanced the stimulation by the ciprofibrate and 9-cis RA activated PPARα/RXRα. In order to test whether the differential sensitivity of PPARα/RXRα to inhibition by COUP-TF would also be observed with other PPARα activators, we repeated the experiment using decanoic acid, a medium chain fatty acid (Fig 3B). Essentially the same results were obtained as with ciprofibrate: the PPARα/RXRα activated by both decanoic acid and 9-cis RA was the least sensitive to inhibition by COUP-TF. Taken together, these results suggest that PPARα/RXRα can only overcome the suppressive effects of COUP-TF when activated by both a peroxisome proliferator and 9-cis RA.

To confirm the differential effect of COUP-TF on the ciprofibrate activated PPARα/RXRα, in the presence and absence of 9-cis RA, these cotransfection studies were repeated with the homologous malic enzyme gene promoter. (pMECAT -490/+31). Cotransfection with 3 μg of COUP-TF expression plasmid alone reduced the activity of this reporter by 37 ± 13% (n=3) using the parental expression vector as control (not shown). PPARα/RXRα stimulated CAT activity of the pMECAT to almost the same extent, regardless of the absence or presence of their respective ligands (Fig. 4). However, cotransfection with COUP-TF expressing plasmids revealed that this apparent unresponsiveness of PPARα/RXRα to ligand activation masks different activation states of the heterodimer. In agreement with the findings with the ME-PPRE2-TKCAT, COUP-TF dose dependently suppressed CAT activation by the nonliganded PPARα/RXRα and, to a lesser extent, by the ciprofibrate activated receptors. However, COUP-TF was completely unable to counteract PPARα/RXRα activated by ciprofibrate and 9-cis RA. Moreover, the paradoxical increase of the response to PPARα/RXRα in the presence of low doses of COUP-TF was more pronounced than in the experiment with the isolated ME-PPRE (see fig. 3). Based on these transactivation studies, it appears that the competitive interaction of COUP-TF and peroxisome proliferator activated PPARα/RXRα depends on the presence of 9-cis RA. Furthermore, as a result of the differential sensitivity to COUP-TF of unliganded and liganded PPARα/RXRα, the degree of induction of transcription by peroxisome proliferators in combination with

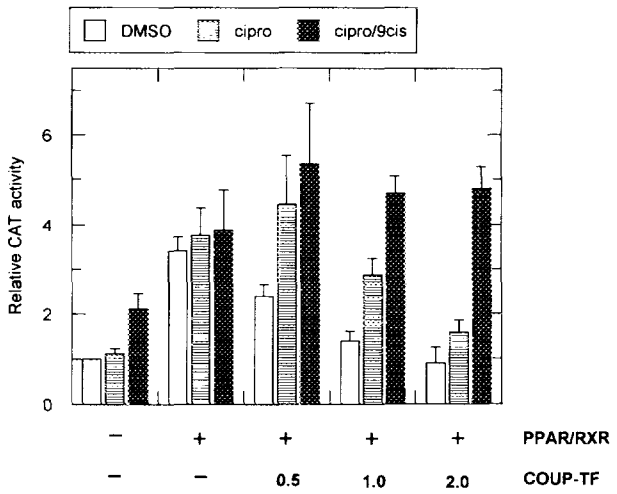

Figure 4. Antagonism of COUP-TF and PPARα/RXRα on the transactivation of the ME promoter. The -490/+31 malic enzyme promoter sequence was inserted in the pOCAT2 reporter and used in cotransfection experiments with receptor expressing plasmids in COS cells. Increasing amounts of COUP-TF expressing plasmid were cotransfected with a constant amount of PPARα/RXRα expressing vectors as in Fig 3. The cells were treated with either DMSO, ciprofibrate (100 μM) or the combination of ciprofibrate and 9-cis RA (1 μM). The mean ± SEM of three independent experiments is shown.

9-cis RA is greatly enhanced in the presence of COUP-TF e.g. the activation of the isolated ME-PPRE by ciprofibrate and 9-cis RA was increased from 4.5 fold in the absence of COUP-TF to 20.5 fold in its presence (fig. 3).

DISCUSSION

In the present study we demonstrated that the orphan nuclear receptor COUP-TF counteracts the effects of PPARα/RXRα heterodimers on the ME-PPRE, a DR1 regulatory element that confers responsiveness of the malic enzyme promoter to peroxisome proliferators. We demonstrated that COUP-TF competes with PPARα/RXRα for binding to this element as a homodimer or as a heterodimer with RXRα.

We further demonstrated that the inhibitory potential of COUP-TF depends on the activation state of the PPARα/RXRα heterodimer. In the presence of a peroxisome proliferator, the RXR ligand, 9-cis RA greatly impaired the antagonistic effect of COUP-TF on PPARα/RXRα. The role of 9-cis RA in the transactivation by RXR containing heterodimers is controversial: it has been been shown for VDR/RXR (14) and TR/RXR (15) that addition of 9-cis RA promotes the formation of RXR homodimers and thus reduces the transactivation potential of the heterodimers. However, Rosen et al. (16) found synergistic effects of 9-cis RA and T3 on

palindromic thyroid receptor response elements. The effect of 9-cis RA on PPAR/RXR heterodimers bound to DR1 regulatory elements can be expected to be even more complex since the RXR homodimers that may be formed are themselves activators of DR1 elements (2). Cotransfection experiments of PPAR and RXR with a multimerized acyl CoA oxidase PPRE-TK-CAT reporter revealed additive effects of fibrates and 9-cis RA (17). The present data show that 9-cis RA plays an essential modulatory role in the competitive interaction of PPARα/RXRα and the inhibitory orphan receptor COUP-TF.

From the present results it can be speculated that COUP-TF may provide an environment that allows greater activation by fibrates and fatty acids in the presence of 9-cis RA. Indeed, the fold induction by the combined addition of 9-cis RA with either ciprofibrate or decanoic acid of a reporter driven by the ME-PPRE was greatly enhanced in the presence of COUP-TF. This sensitization by COUP-TF of the ME promoter to respond to peroxisome proliferators and 9-cis RA is reminiscent of the sensitization by COUP-TF of the ApoA1 promoter for activation by 9-cis RA (18).

In conclusion, the orphan receptor COUP-TF may play an essential role in modulating the responsiveness of the malic enzyme gene promoter to exogenously administered peroxisome proliferators and to PPARα/RXRα activators of endogenous origin.

ACKNOWLEDGMENTS

The authors wish to thank Drs.D.D.Moore and T. Gulick for providing expression plasmids and Drs. S. Karathanasis and R. Evans for providing antisera.

REFERENCES

1. Castelein, H., Gulick, T., Declercq, P.E., Mannaerts, G.P., Moore, D.D., and Baes, M.I. (1994) J. Biol. Chem.269, 26754-26758.
2. Glass, C.K. (1994) Endocr. Rev.15, 391-407.
3. Wang, L.H., Tsai, S.Y., Cook, R.G., Beattie, W.G., Tsai, M.-J., and O'Malley, B.W. (1989) Nature340, 163-166.
4. Sladek, F.M., Zhong, W., Lai, E., and Darnell, J.E. (1990) Gen. Dev.4, 2353-2365.
5. Cooney, A.J., Tsai, S.Y., O'Malley, B.W., and Tsai, M.-J. (1992) Mol. Cel. Biology12, 4153-4163.
6. Tran, P., Zhang, X.-K., Salbert, G., Hermann, T., Lehmann, J.M., and Pfahl, M. (1992) Mol. Cell Biol.12, 4666-4676.
7. Kimura, A., Nishiyori, A., Murakami, T., Tsukamoto, T., Hata, S., Osumi, T., Okamura, R., Mori, M., and Takiguchi, M. (1993) J. Biol. Chem.1993, 11125-11133.
8. Mietus-Snyder, M., Sladek, F.M., Ginsburg, G.S.K., C.F., Ladias, J.A.A., Darnell, J.E.J., and Karathanasis, S.K. (1992) Mol. Cell. Biol.12, 1708-1718.
9. Burbach, J.P.H., Lopes da Silva, S., Cox, J.J., Adan, R.A.H., Cooney, A.J., Tsai, M.-J., and Tsai, S.Y. (1994) J. Biol. Chem.269, 15046-15053.
10. Miyata, K.S., Zhang, B., Marcus, S.L., Capone, J.P., and Rachubinski, R.A. (1993) J. Biol. Chem.268, 19169-19172.
11. Cooney, A.J., Leng, X., Tsai, S.Y., O'Malley, B.W., and Tsai, M.J. (1993) The Journal of Biological Chemistry268, 4152-4160.

12. Carter, M.E., Gulick, T., Moore, D.D., and Kelly, D.P. (1994) Molecular and Cellular Biology14, 4360-4372.
13. Evan, G.I., Lewis, G.K., Ramsay, G., and Bishop, J.M. (1985) Mol. Cell. Biol.5, 3610-3616.
14. Cheskis, B. and Freedman, L.P. (1994) Molecular and Cellular Biology14, 3329-3338.
15. Lehmann, J.M., Zhang, X., Graupner, G., Lee, M., Hermann, T., Hoffmann, B., and Pfahl, M. (1993) Molecular and Cellular Biology13, 7698-7707.
16. Rosen, E.D., O'Donnell, A.L., and Koenig, R.J. (1992) J. Biol. Chem.267, 22010-22013.
17. Kliewer, S.A., Umesono, K., Noonan, D.J., Heyman, R.A., and Evans, R.M. (1992) Nature358, 771-774.
18. Widom, R.L., Rhee, M., and Karathanasis, S.K. (1992) Mol. Cell. Biol.12, 3380-3389.