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Transcriptional suppression of human microsomal triglyceride transfer protein by hypolipidemic insulin sensitizers

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Abstract

Microsomal triglyceride transfer protein (MTP) catalyzes the assembly and secretion of liver triglyceride-rich lipoproteins. The human MTP (hMTP) promoter activity is reported here to be suppressed by HNF-4α ligand antagonists (e.g., Medica analogs) or by PPARγ ligand agonists (e.g., thiazolidinediones), thus accounting for their hypolipidemic activity in humans. Suppression of liver hMTP by Medica analogs or by thiazolidinediones was mediated by the TAAA sequence that serves as non-canonical TATA box of the hMTP core promoter. MTP suppression was evident in the specific context of the wild type hMTP core promoter, but not in the context of the mutated rodent-conforming hMTP core promoter governed by a canonical TATA box conjoined with its proximal (−50/−38)DR-1 element. hMTP suppression by Medica analogs or thiazolidinediones mediated by hMTP TAAA was independent of HNF-4α or PPARγ. hMTP suppression by Medica analogs, but not by thiazolidinediones, was further complemented by inhibition of HNF-4α transcriptional activity transduced by the distal (−83/−70)DR-1 element of hMTP promoter. hMTP promoter activity was unaffected by PPARα activation. Furthermore, in contrast to hMTP, the promoter activity of the rodent-conforming hMTP was robustly activated by Wy-14,643-activated PPARα or by thiazolidinedione-activated PPARγ. Transcriptional activation by PPARα or PPARγ of the rodent-conforming, but not the wild type hMTP gene promoter, resulted from the species-specific context of the respective proximal DR-1 elements. Hence, suppression of hMTP transcription by hypolipidemic insulin sensitizers requires the specific context of hMTP core promoter. In light of the species-specific context of MTP core promoters, the rodent MTP promoter may not substitute for the human promoter when searching for hypolipidemic MTP suppressors.

Keywords: MTP; HNF-4α; PPARα; PPARγ; Thiazolidinediones; Medica

1. Introduction

Microsomal triglyceride transfer protein (MTP) plays a pivotal role in the assembly and secretion of hepatic and intestinal triglyceride-rich, apo B-containing lipoproteins (e.g., liver VLDL and intestinal chylomicrons) (reviewed in Refs. [1,2]). Lack of functional MTP results in blocking lipoprotein assembly, ubiquitination of nascent apo B and its subsequent proteolysis, thus leading to abetalipoproteinemia with concomitant hypolipidemia at the expense of hepatic steatosis [3–6]. Overexpression of MTP results in lipidation of nascent apo B and its incorporation into plasma apo B-containing triglyceride-rich lipoproteins, leading to hyperlipidemia [7]. MTP gene transcription is driven by the highly conserved 5′-flanking 200 bp sequence of the MTP gene promoter [8]. Both, the human and rodent proximal promoters consist of consensus sequences for SREBP ((−124/−116)hMTP), insulin ((−122/−111)hMTP), AP-1 ((−109/−104)hMTP) and HNF-1 ((−103/−98)hMTP), together with a distal ((−83/−70)hMTP) and proximal ((−50/−38)hMTP) DR-1 elements. The two respective DR-1 elements act in concert in mediating hMTP transactivation by HNF-4α or its transuppression by COUP-TF [9].

In light of MTP pivotal role in catalyzing the assembly and production of liver and intestinal triglyceride-rich lipoproteins, inhibition of MTP activity and/or suppression of its expression could serve as targets for lipid-lowering

Abbreviations: AOX, peroxisomal acyl-CoA oxidase; Apo B, apolipoprotein B; CHIP, chromatin immunoprecipitation; Cl-DICA, α,α′-tetrachloro-tetradecanedioic acid; DR-1, direct repeat 1; hMTP/rMTP, human/rat MTP; HNF-1α, hepatocyte nuclear factor-1α; HNF-4α, hepatocyte nuclear factor-4α; Medica 16 (M16), β,β′-tetramethyl-hexadecanedioic acid; Medica 18 (M18), β,β′-tetramethyl-octadecanedioic acid; MTP, microsomal triglyceride transfer protein; P-DR1/D-DR1, proximal/distal DR-1; P-mut/D-mut, mutated P-DR1/D-DR1; PPARα, peroxisome proliferator activated receptor α; PPARγ, peroxisome proliferator activated receptor γ; PUFA, polyunsaturated fatty acids; TZD, thiazolidinediones

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drugs. Indeed, inhibitors of MTP activity proved effective in lowering plasma triglycerides and cholesterol in normolipemic or hyperlipemic animal models as well as in humans, with concomitant amelioration of atherosclerotic plaques [10–12]. Similarly, in line with the role played by HNF-4α in transactivating MTP transcription [9], HNF-4α ligand antagonists (e.g., (*n* − 3)PUFA, Medica analogs [13,14]) inhibit the assembly and production of liver triglyceride-rich lipoproteins resulting in hypolipidemia [15,16]. However, suppression of MTP expression by HNF-4α ligand antagonists [9] still remains to be investigated in the detailed context of the MTP promoter.

PPARα agonists/fibrates as well as PPARγ agonists/thiazolidinediones (TZD) have been repeatedly reported to induce hypotriglyceridemia in human and rodents [17–22]. Thus, Rosiglitazone decreases MTP mRNA and its protein level in hepatocytes derived from insulin-resistant fructose-fed hamsters, in line with decreasing their VLDL production in vivo [23]. However, the TZD effect in fructose-fed hamsters was not studied in the context of the MTP gene promoter. Moreover, Rosiglitazone has been reported to be ineffective in modulating MTP expression in primary mouse hepatocytes [24], indicating that its MTP lowering activity in fructose-fed hamsters could perhaps reflect an extrahepatic insulin-sensitizing activity or hepatic sensitization to insulin converging onto the insulin response element, rather than PPAR-responsive DR-1 elements, of the MTP gene promoter.

In contrast to hypolipidemic PPARγ agonists/TZD, a representative hypolipidemic PPARα agonist (e.g., Wy-14,643) has recently been reported to surprisingly increase liver MTP expression and activity in rodents due to transactivation of the proximal (−45/−33)DR-1 element of the rMTP gene promoter by activated PPARα [24]. Also, the proximal DR-1 element of the rodent MTP gene promoter has been reported to be transactivated by RXR as well as by its 9-*cis* retinoic acid agonist [25]. Transactivation of MTP transcription by PPARα agonists [24] may apparently imply that their hypolipidemic activity was in spite of, rather than due to their modulation of MTP expression by activated PPARα. The hypolipidemic activity of PPARα agonists may however be accounted for by suppression of apo C-III transcription, resulting in activation of clearance of plasma triglycerides-rich lipoproteins [14,26].

Modulation of MTP expression by HNF-4α ligand antagonists has previously been verified in the context of the human MTP promoter [9] while transcriptional modulation of MTP expression by Wy-14,643 or TZD has been verified in the context of the rodent MTP promoter [24]. However, MTP core promoter sequences implicated in modulating MTP expression by PPARα agonists/Wy-14,643, PPARγ agonists/TZD or HNF-4α antagonists/Medica analogs, differ between human and rodents [8], thus questioning the projection to human of hypolipidemic modes of action derived from the rodent case and vice versa. This study has been initiated to verify the mode of modulation of MTP transcription by hypolipidemic HNF-4α antagonists and PPAR agonists in the specific context of the human MTP promoter. The findings reported here may indicate that the mode of modulating MTP expression by the respective hypolipidemic insulin sensitizers is species-specific as a result of species-specific context of the MTP core promoter. These findings may further imply that the rodent MTP promoter may not substitute for the human promoter when designing hypolipidemic MTP suppressors.

2. Materials and methods

2.1. hMTP promoter constructs

Wild type (−611/+87)hMTP-CAT, wild type (−74/+87)hMTP-CAT, (−611/+87)hMTP(P-mut)-CAT and (−611/+87)hMTP(P-mut/D-mut)-CAT promoter fragments were generated as previously described [9]. (−611/+87)hMTP(TATA)-CAT and (−74/+87)hMTP(TATA)-CAT were generated by in vitro mutagenesis (QuikChange Site Directed Mutagenesis Kit (Stratagene)) of wild type (−611/+87)hMTP-CAT and wild type (−74/+87)hMTP-CAT, respectively. The TATA primers consisted of forward 5′-GGAGTCTGACCTTTCCCCTATAGATAAA-CATGATTGTTGC-3′ and reverse 5′-GCAACAATCAT-GTTTATCTATAGGGGAAAGGTCAGACTCC-3′ sequences, respectively. (−74/+87)hMTP(P-mut/TATA) was generated by in vitro mutagenesis of (−74/+87)hMTP(TATA)-CAT. Forward and reverse primers were: 5′-GGAGTTTGGAGTCTGTGCTTTCCCCTATAGATAAA-C-3′ and 5′-GTTTATCTATAGGGGAAAGCACAGACTC-CAAACTCC-3′, respectively. Oligonucleotide primers for PCR were prepared by IDT (Coralville, IA). All promoter constructs were confirmed by sequencing.

2.2. Expression plasmids

pSG5-HNF-4α was constructed as previously described [26]. pSG5-mPPARα was from Issemann and Green [27]. pSV-SPORT-mPPARγ2 was from Spiegelman and coworkers [28].

2.3. Cell cultures and transfection assays

HepG2 cells grown in MEM EAGLE containing 10% fetal calf serum were transfected for 6 h by calcium phosphate precipitation with 5.0 μg of respective hMTP promoter-CAT reporter plasmids, together with expression vectors as indicated, washed and further incubated for 40 h with or without added ligands as indicated. HeLa cells grown in DMEM containing 10% fetal calf serum were transfected overnight by calcium phosphate precipitation with 5.0 μg of (−611/+87)hMTP-CAT reporter plasmid, together with expression vectors as indicated, followed by

glycerol shock and further incubation for 24 h with or without added ligands as indicated. Medica 16 (M16), Medica 18 (M18) or Bezafibrate were added as sodium salt. Wy-14,643 and TZD were added as ×1000 stock in dimethyl sulfoxide. When cotransfecting expression vectors, total amount of DNA was kept constant by supplementing with the empty vector. CAT values were normalized to β-galactosidase activity [26].

2.4. hMTP mRNA levels

Total RNA was prepared from HepG2 cells, using EZ-RNA Kit (Biological Industries, Beit Haemek). hMTP mRNA levels were determined by semi-quantitative RT-PCR. First strand cDNA used as template was synthesized by reverse transcription using oligo(dT) as primer and M-MLV-RT (Invitrogen). PCR was performed in a total volume of 25 μl containing 1 μl of the RT reaction as template, 10 mM Tris–HCL (pH 9 at 25 °C), 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 0.2 mM dNTP's, 1 μCi [^{32}P]dCTP, 0.5 μM of forward and reverse hMTP primers, 0.05 μM of forward and reverse β-actin primers and 1.25 units of taq DNA polymerase (Promega). PCR conditions consisted of denaturation (94 °C, 1 min), annealing (56 °C, 1 min) and extension (72 °C, 1 min). Fifteen to 18 cycles of this program were within the linear range of amplification for both MTP and β-actin. The following primers were used: MTP forward 5′-TGTGTCAGAATGAAGGCTGC-3′ and reverse 5′-AAGGTCTTCTTCACCTCATC-3′ primers; β-actin forward 5′-TCACCAACTGG-GACGACTAG-3′ and reverse 5′-GTACAGGGATAGCACAGCCT-3′ primers. Product sizes were 256 bp and 200 bp for MTP and β-actin, respectively. The products were separated by electrophoresis in 10% polyacrylamide gel and quantified by PhosphorImager. Results were expressed as a ratio of MTP to β-actin.

2.5. Chromatin immunoprecipitation (CHIP) analysis

HepG2 cells were incubated for 48 h, in the absence or presence of Pioglitazone or M16. Chromatin immunoprecipitation analysis was carried out as previously described [9]. The purified DNA isolated by immunoprecipitation was analyzed by PCR under conditions of cycling linearity using the forward 5′-CTGGTTTGGTTTAGCTCTC-3′ and reverse 5′-GACCCTCTTCAGAACCTG-3′ primers for the (−211/−1)hMTP gene promoter. The amplified DNA was labeled with SYBER Green I (Molecular Probes), separated by electrophoresis using 1.8% agarose gel and analyzed by Flour-S^{TM} Multimager (BioRad).

2.6. Gel electrophoretic mobility shift assay

Double-stranded oligonucleotides were used as radiolabeled probes and/or unlabeled competitors. Labeled oligonucleotides were prepared by the Klenow fragment of DNA polymerase I and [^{32}P]dCTP. The following double-stranded oligonucleotides were used for wild type (−61/−27)hMTP:

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5′-AGTTTGGAGTCTGACCTTTCCCCAAAGATA
       CCTCAGACTGGAAAGGGGTTTCTATTTGTA;
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The following double-stranded oligonucleotides were used for hMTP(TATA):

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5′-AGTTTGGAGTCTGACCTTTCCCCTATAGATA
       CCTCAGACTGGAAAGGGGATATCTATTTGTA.
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Nuclear extracts were prepared from COS7 cells overexpressing HNF-4α, PPARα or PPARγ as previously described [9]. Nuclear extracts were incubated with or without added unlabeled competitor oligonucleotides as indicated, in a total volume of 20 μl containing 10 mM Hepes (pH 7.8), 50 mM KCl, 2.5 mM $MgCl_2$, 10% glycerol, 1 mM DTT and 3.0 μg of poly(dI–dC). Following 30 min incubation on ice the respective radiolabeled oligonucleotide was added, and the reaction mixture was further incubated for 15 min at room temperature. Protein–DNA complexes were separated by 5% polyacrylamide gel and analyzed by PhosphorImager.

2.7. Materials

Medica analogs were prepared as previously described [29]. Thiazolidinediones were kindly supplied by K. Motojima, Meiji Pharmaceutical University, Kiyose, Japan.

3. Results

3.1. Suppression of hMTP promoter activity by PPARγ and HNF-4α ligands

The effect of HNF-4α antagonists/Medica analogs, PPARγ agonists/TZD and PPARα agonists/Cl-DICA/fibrates on human MTP mRNA levels was evaluated in HepG2 cells cultured in the presence of the respective drugs. Cl-DICA was used as specific PPARα agonist since it specifically activates PPARα while being inactive in modulating HNF-4α [14]. As shown in Fig. 1, TZD represented by Pioglitazone, Rosiglitazone or Troglitazone as well as Medica analogs represented by β,β′-tetramethyl-hexadecanedioic acid (M16) and β,β′-tetramethyl-octadecanedioic acid (M18), were effective in suppressing MTP mRNA, while Bezafibrate or Cl-DICA were ineffective. Hence, hMTP gene expression is specifically affected by prototypes of the three respective hypolipidemic drug classes.

Suppression of hMTP gene transcription by TZD and Medica analogs has been verified in HepG2 cells transfected with (−611/+87)hMTP upstream of CAT reporter plasmid. As shown in Fig. 2A, both TZD and M16 suppressed hMTP promoter activity by 50–70% whereas Cl-DICA was essentially ineffective. The (−611/+87)hMTP

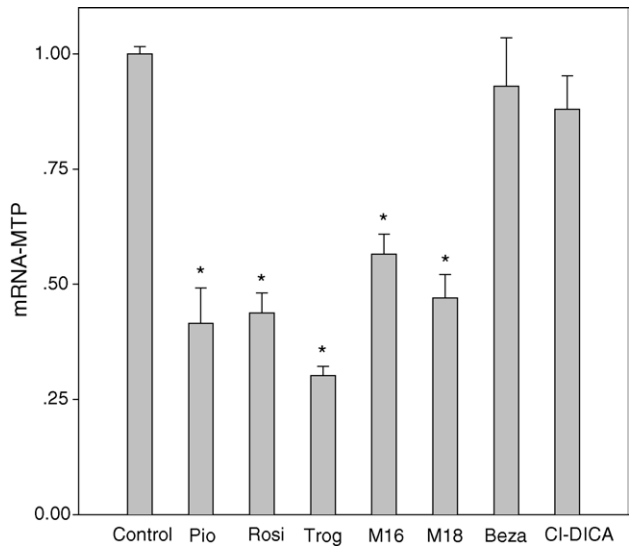

Fig. 1. Effect of TZD, fibrates and Medica analogs on hMTP mRNA levels. HepG2 cells were incubated for 48 h in MEM EAGLE containing 10% FCS in the absence or presence of Pioglitazone (100 μM), Rosiglitazone (100 μM), Troglitazone (100 μM), Medica 16 (M16) (250 μM), Medica 18 (M18) (250 μM), Bezafibrate (250 μM) or Cl-DICA (250 μM). Relative hMTP mRNA levels were determined by semi-quantitative RT-PCR and normalized to β-actin mRNA levels as described in Section 2. hMTP mRNA level of non-treated cells is defined as 1.0. Mean ± S.D. for three to five independent experiments for each ligand. *Significantly differs from the non-treated value ($p < 0.05$).

promoter activity was similarly suppressed by TZD and Medica analogs, but not by Cl-DICA, under conditions of being synergistically transactivated in HeLa cells by overexpressed HNF-4α together with HNF-1α [9] (Fig. 2B), or by overexpressed HNF-4α together with P300 (not shown). Hence, suppression of hMTP mRNA levels by prototype ligands of PPARγ and HNF-4α (Fig. 1) was specific and accounted for by suppression of hMTP transcription.

Suppression of hMTP promoter activity by TZD and Medica analogs was further evaluated in HepG2 cells transfected with the (−74/+87)hMTP promoter upstream of CAT reporter plasmid. The truncated (−74/+87)hMTP promoter consists of the proximal (−50/−38)DR-1 element (P-DR1) but lacks the distal (−83/−70)DR-1 element (D-DR1) of the hMTP promoter [9]. As shown in Fig. 3A, the truncated hMTP promoter activity was suppressed by TZD to an extent similar to TZD suppression of the (−611/+87)hMTP promoter activity (Fig. 2A), implying that the distal DR-1 element of the hMTP gene promoter was not involved in hMTP suppression by TZD, thus restricting the TZD effect to the hMTP core promoter. In contrast to TZD, suppression of the truncated (−74/+87)hMTP promoter activity by Medica 16 (Fig. 3A) was significantly less pronounced than suppression of the extended (−611/+87)hMTP gene promoter (Fig. 2A), in concordance with the role played by HNF-4α in transactivating the distal DR-1 element [9], and in line with the specific suppression of HNF-4α transcriptional activity by Medica analogs [14]. Hence, hMTP suppression by Medica analogs is accounted

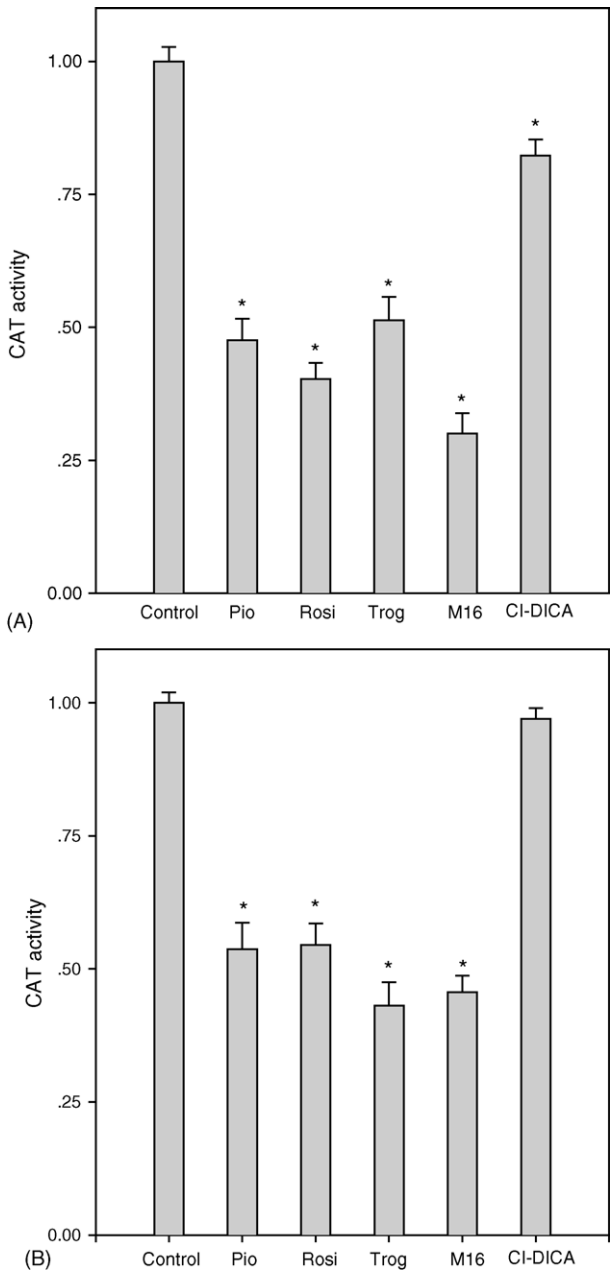

Fig. 2. Suppression of hMTP transcription by TZD and Medica 16. (A) HepG2 cells were transfected with (−611/+87)hMTP-CAT reporter plasmid as described in Section 2, and further cultured for 40 h in MEM EAGLE containing 10% FCS supplemented with Pioglitazone (50 μM), Rosiglitazone (100 μM), Troglitazone (50 μM), M16 (200 μM), Cl-DICA (200 μM) or vehicle. CAT activities normalized to β-galactosidase are presented as fold induction relative to CAT activity in the absence of added ligand, defined as 1.0. Mean ± S.E. for three to six independent experiments for each ligand. *Significantly differs from the non-treated value ($p < 0.05$). (B) HeLa cells were cotransfected with (−611/+87)hMTP-CAT reporter plasmid and with expression vectors for HNF-4α (0.05 μg) and HNF-1α (0.07 μg) as described in Section 2. Cells were then cultured for 40 h in DMEM containing 10% FCS supplemented with Pioglitazone (50 μM), Rosiglitazone (50 μM), Troglitazone (50 μM), M16 (200 μM) Cl-DICA (200 μM) or vehicle. CAT activities normalized to β-galactosidase are presented as fold induction relative to CAT activity in the absence of added ligand, defined as 1.0 (representing 1250-fold induction relative to CAT activity in cells transfected with empty pSG5). Mean ± S.E. for four independent experiments for each ligand. *Significantly differs from the non-treated value ($p < 0.05$).

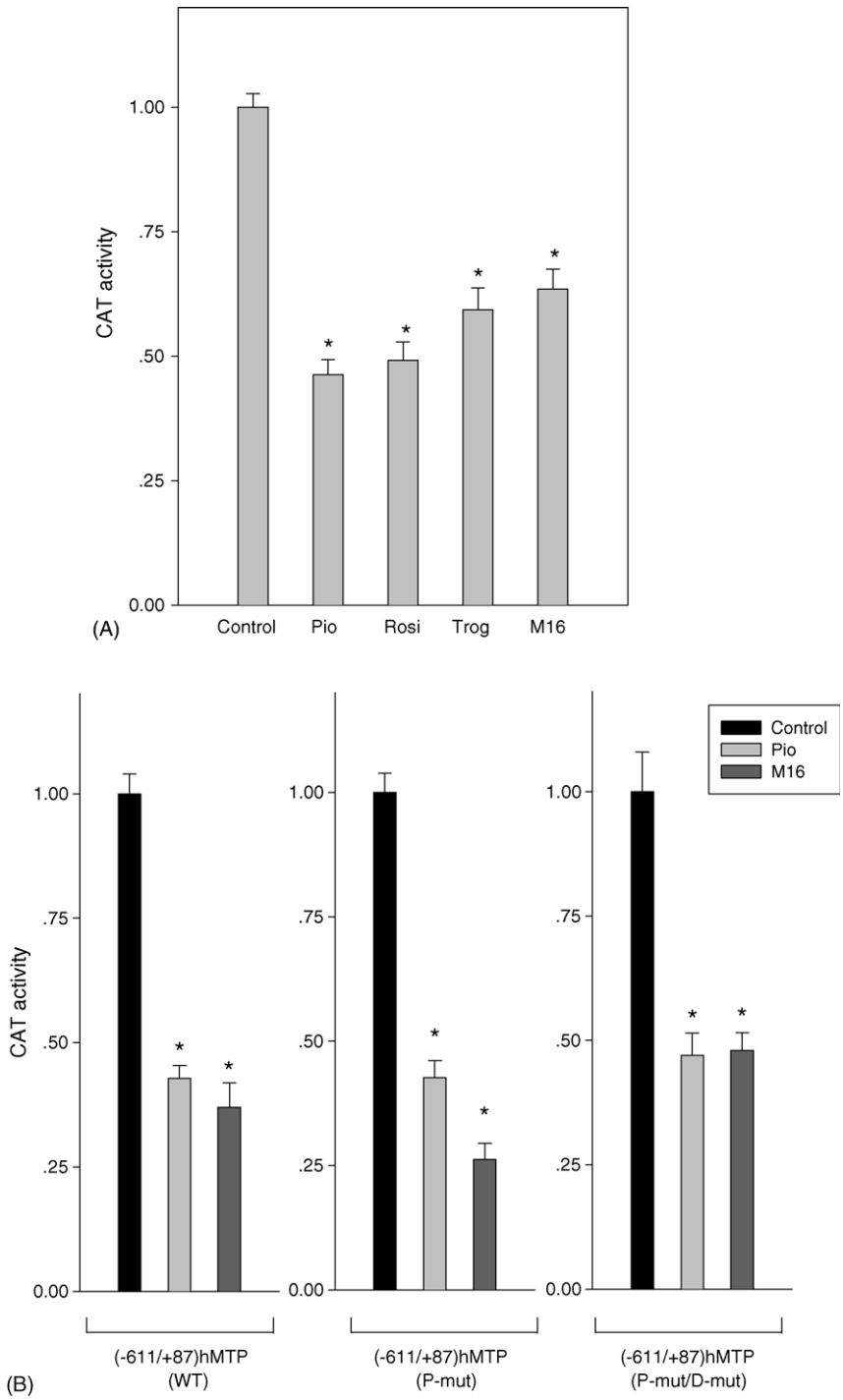

Fig. 3. hMTP promoter elements involved in hMTP suppression by TZD and Medica analogs. (A) Deletion analysis—HepG2 cells were transfected with (−74/+87)hMTP-CAT reporter plasmid and further cultured for 40 h in MEM EAGLE containing 10% FCS supplemented with Pioglitazone (50 μM), Rosiglitazone (50 μM), Troglitazone (50 μM), M16 (200 μM) or vehicle. CAT activities normalized to β-galactosidase are presented as fold induction relative to CAT activity in the absence of added ligand, defined as 1.0. Mean ± S.E. for three to five independent experiments for each ligand. *Significantly differs from the non-treated value ($p < 0.05$). (B) Mutation analysis—HepG2 Cells were transfected with wild type, P-mut or P-mut/D-mut (−611/+87)hMTP-CAT reporter plasmids as indicated. Cells were then cultured for 40 h in MEM EAGLE containing 10% FCS supplemented with Pioglitazone (50 μM), Rosiglitazone (100 μM), Troglitazone (50 μM), M16 (200 μM) or vehicle. The promoter activity of (−611/+87)hMTP(P-mut)-CAT and (−611/+87)hMTP(P-mut/D-mut)-CAT reporter plasmids was reduced by 76% and 99%, respectively, compared with wild type (−611/+87)hMTP(WT)-CAT [9]. CAT activities normalized to β-galactosidase are presented as fold induction relative to CAT activity in the absence of added ligand, defined as 1.0. Mean ± S.E. for three to five independent experiments for each ligand. *Significantly differs from the respective non-treated value ($p < 0.05$).

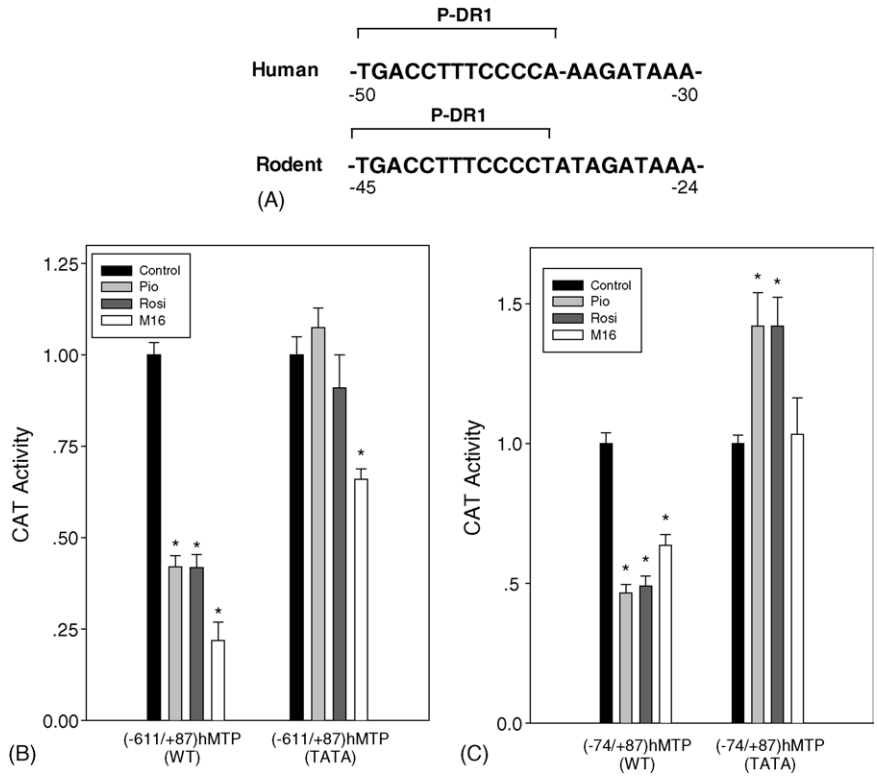

Fig. 4. Suppression of hMTP and the rodent-conforming MTP by TZD and Medica analogs. (A) Nucleotide sequences of the human and rodent (hamster, rat, mice [25]) MTP core promoters. (B and C) HepG2 cells were transfected with wild type (−611/+87)hMTP-CAT or with the (−611/+87)hMTP(TATA)-CAT reporter plasmids as indicated (B), or were transfected with the truncated wild type (−74/+87)hMTP-CAT or with the (−74/+87)hMTP(TATA)-CAT reporter plasmids as indicated (C). Cells were then cultured for 40 h in MEM EAGLE containing 10% FCS supplemented with Pioglitazone (50 μM), Rosiglitazone (100 μM) (B) or (50 μM) (C), M16 (200 μM) or vehicle as indicated. Promoter activities of (−611/+87)hMTP(TATA)-CAT and (−74/+87)hMTP(TATA)-CAT in non-treated cells were 0.9- and 1.5-fold activated, respectively, as compared with the respective wild type promoters. CAT activities normalized to β-galactosidase are presented as fold induction relative to CAT activity in the absence of added ligand, defined as 1.0. Mean ± S.E. for three to five independent experiments for each ligand. *Significantly differs from the respective non-treated value ($p < 0.05$).

for by both, suppression of HNF-4α transactivation of the distal DR-1 element of the hMTP promoter as well as suppression of the hMTP core promoter activity.

The putative role played by the proximal DR-1 element in mediating suppression of the hMTP core promoter by TZD or Medica analogs could not be further verified by mutating the proximal DR-1 element in the context of the truncated (−74/+87)hMTP promoter, due to nil promoter activity of the mutated truncated construct [9]. Instead, the proximal DR-1 element was mutated in the context of the (−611/+87)hMTP promoter. As shown in Fig. 3B, suppression of hMTP promoter activity by TZD or by Medica analogs still prevailed in the context of the (−611/+87)hMTP promoter mutated in its proximal DR-1 element, implying that suppression of the truncated (−74/+87)hMTP promoter by TZD or Medica analogs (Fig. 3A) was not transduced by the proximal (−50/−38)hMTP DR-1. Moreover, suppression of hMTP by TZD or Medica analogs prevailed as well in the context of the (−611/+87)hMTP promoter doubly mutated in its proximal and distal DR-1 elements, thus implicating promoter sequences downstream of hMTP P-DR1.

The proximal DR-1 element of the hMTP core promoter conjoins with a degenerate TATA box, being separated by 4 bp from its TAAA sequence previously reported to serve as genuine hMTP TATA [8]. That in contrast to the rodent MTP core promoter that consists of its proximal DR-1 in immediate conjunction with a canonical TATA (Fig. 4A). To evaluate the role of the distinctive TATA contexts of the human and rodent MTP core promoters in mediating hMTP suppression by TZD and Medica analogs, the degenerate TATA box of hMTP core promoter was mutated and replaced by a canonical TATA in immediate conjunction with the proximal DR-1 element. The rodent-conforming core TATA was introduced in the context of the (−611/+87)hMTP promoter (defined as (−611/+87)hMTP(TATA)) as well as the (−74/+87)hMTP promoter (defined as (−74/+87)hMTP(TATA)) (Fig. 4A). As shown in Fig. 4B and C, replacing the degenerate by a canonical TATA box resulted in eliminating altogether suppression of hMTP promoter activity by TZD, both in the context of (−611/+87)hMTP(TATA) as well as the (−74/+87)hMTP(TATA) promoters. These results may imply that TZD suppression of hMTP is accounted for by the distinctive constitution/configuration of the wild type hMTP core promoter lacking a canonical TATA box conjoined with its proximal DR-1 element. Similarly to TZD, suppression by Medica analogs was eliminated in the (−74/+87)hMTP(TATA) promoter

context, indicating the specific requirement for the distinctive wild type hMTP core promoter for MTP inhibition by Medica analogs. However, in contrast to TZD, suppression by Medica 16 still prevailed in the context of the (−611/+87)hMTP(TATA) promoter, in line with its additional inhibitory effect transduced by the distal DR-1 element.

3.2. Involvement of HNF-4α and PPARs in hMTP suppression by respective ligands

The role played by HNF-4α in mediating suppression of hMTP promoter activity by Medica analogs in HepG2 cells in vivo was verified by CHIP analysis of the (−211/−1)hMTP promoter sequence, previously shown to associate with endogenous HNF-4α [9,30]. HNF-4α association with the hMTP proximal promoter was robustly suppressed by M16 (Fig. 5), indicating that suppression of hMTP by Medica analogs may be partly accounted for by interfering with HNF-4α transactivation of the hMTP proximal promoter. In contrast to Medica analogs, suppression of hMTP promoter activity by TZD was not mediated by modulating HNF-4α interaction with the hMTP proximal promoter in vivo (Fig. 5).

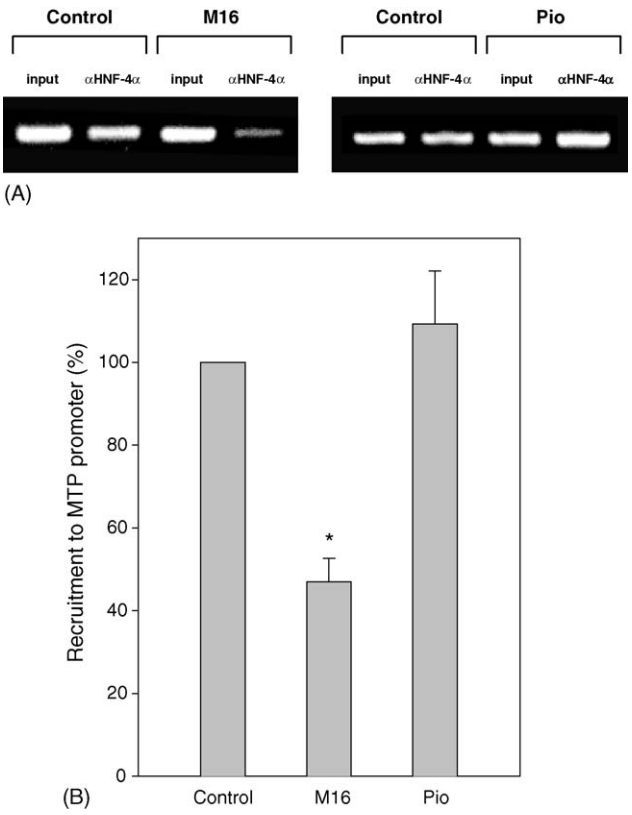

Fig. 5. Chromatin immunoprecipitation (CHIP) analysis of hMTP promoter. Soluble chromatin, prepared from HepG2 cells cultured for 48 h in the absence or presence of Pioglitazone (50 μM) or M16 (200 μM), was immunoprecipitated with antiHNF-4α antibody or with control IgG as previously described [9]. The immunoprecipitated DNA was amplified using primers flanking the (−211/−1)hMTP gene promoter as described in Section 2. Data presented as percentage of HNF-4α-immunoprecipitated DNA to input DNA. HNF-4α recruitment to the MTP promoter in non-treated cells is defined as 100%. Mean ± S.E. for five and three independent experiments for M16 and Pioglitazone, respectively. *Significantly differs from the non-treated value ($p < 0.05$).

The role played by PPARs and their respective agonists in modulating the distinctive core promoters of the human and rodent MTP genes has been evaluated by verifying the promoter activity of (−74/+87)hMTP(TATA) as compared with wild type (−74/+87)hMTP in response to overexpressed PPARs. As shown in Fig. 6A, wild type (−74/+87)hMTP promoter was slightly activated by overexpressed PPARγ, and added TZD was suppressive, both in the absence or presence of overexpressed PPARγ, indicating that TZD suppression of hMTP promoter activity was not transduced by TZD-activated PPARγ. Moreover, in contrast to wild type (−74/+87)hMTP, the (−74/+87)hMTP(TATA) promoter was significantly transactivated by overexpressed PPARγ and further activated by TZD (Fig. 6A). Similarly, the (−74/+87)hMTP(TATA) promoter was significantly transactivated by overexpressed PPARα and further activated by Wy-14,643 (Fig. 6B). That is in contrast to wild type (−74/+87)hMTP promoter where PPARα was ineffective, and where Wy-14,643 was inactive in the absence or presence of overexpressed PPARα (Fig. 6B). Activation of the (−74/+87)hMTP(TATA) (Fig. 6A and B) and the (−611/+87)hMTP(TATA) (not shown) promoters by Wy-14,643-activated PPARα or by TZD-activated PPARγ may imply that modulation of the rodent-conforming core promoter activity by the concerned drugs is mediated by their respective nuclear receptors. That is in contrast to wild type hMTP promoter being modulated by the concerned drugs independently of, and oppositely to, the effect exerted by their cognate nuclear receptors. In contrast to PPARs and PPAR agonists, activation of MTP promoter activity by overexpressed HNF-4α, and suppression of HNF-4α-activated MTP by HNF-4α ligand antagonists/Medica analogs, prevailed in the context of both, the wild type (−74/+87)hMTP or the mutated (−74/+87)hMTP(TATA) promoters (Fig. 6C).

The specific involvement of PPARs and HNF-4α in mediating the effects of their respective ligands in the specific context of (−74/+87)hMTP(TATA) was corroborated by mutating the (−50/−38)DR-1 element of (−74/+87)hMTP(TATA). The promoter activity of the mutated (−74/+87)hMTP(P-mut/TATA) was reduced by 65%, with concomitant elimination of transactivation by all three nuclear receptors, indicating that modulation of the rodent-conforming core promoter by the concerned drugs, as mediated by their respective nuclear receptors, is transduced by its proximal DR-1 (data not shown).

The role played by PPARs in mediating the effects of their respective ligands in the context of (−74/+87)hMTP(TATA), but not the wild type (−74/+87)hMTP promoter context, was further evaluated by analyzing the binding of PPARα and PPARγ to the two respective promoter constructs by gel shift analysis. PPARα

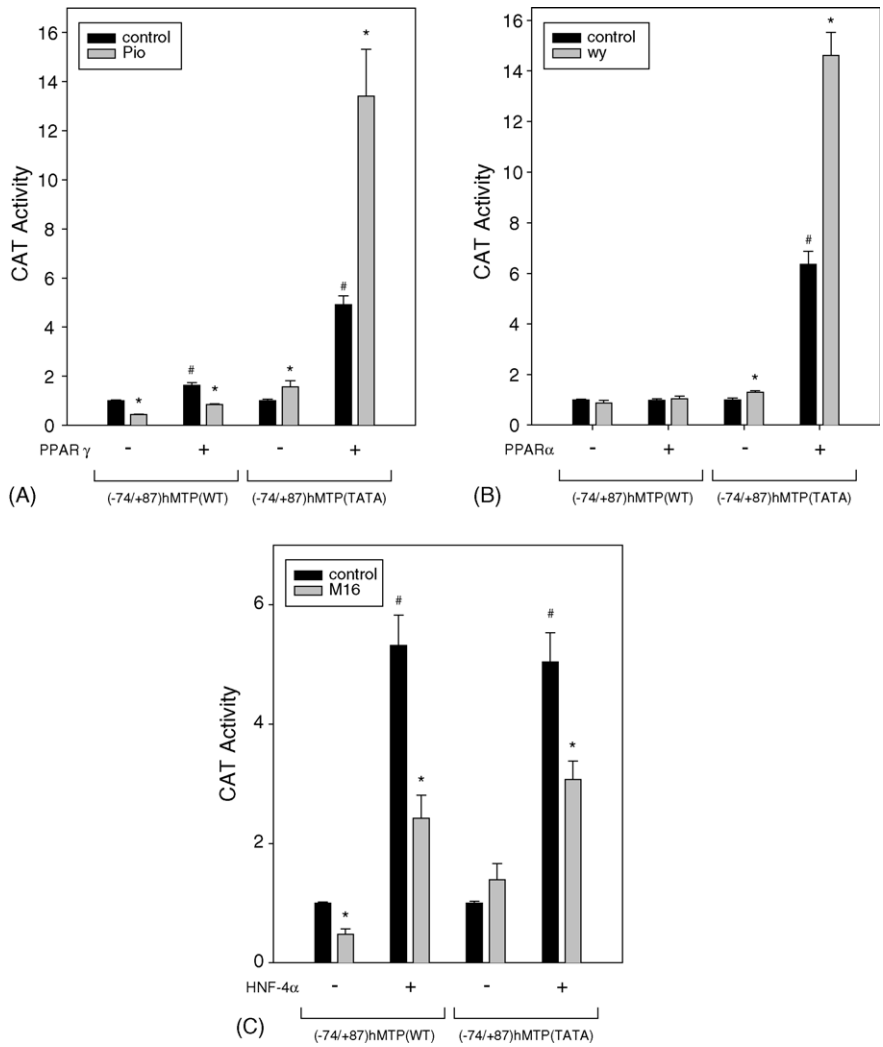

Fig. 6. Transactivation of wild type hMTP and the rodent-conforming MTP by DR-1 nuclear receptors. HepG2 cells were cotransfected with the truncated wild type (−74/+87)hMTP-CAT or (−74/+87)hMTP(TATA) reporter plasmids and the indicated expression vectors for PPARγ (0.1 μg) (A), PPARα (0.025 μg) (B), HNF-4α (0.05 μg) (C) or the empty vector as described in Section 2. Cells were then cultured for 40 h in MEM EAGLE containing 10% FCS in the absence or presence of added Pioglitazone (50 μM), Wy-14,643 (50 μM) or Medica 16 (200 μM) as indicated. CAT activities normalized to β-galactosidase are presented as fold induction relative to CAT activity of cells transfected with pSG5, defined as 1.0. Mean ± S.E. for three independent experiments. #Significantly differs from respective pSG5 values ($p < 0.05$). *Significantly differs from the respective non-treated value ($p < 0.05$).

(Fig. 7A) or PPARγ (Fig. 7B) bound to labeled hMTP(TATA) were effectively competed out by non-labeled hMTP(TATA), but not by wild type hMTP. Hence, the thymine 3′-terminal base of the rodent-conforming hMTP promoter accounted for its high binding affinity to PPARα and PPARγ, and its replacement by adenine (Fig. 4A) resulted in their robustly decreased binding affinity to wild type hMTP core promoter. In contrast to PPARs, binding of HNF-4α to labeled hMTP(TATA) promoter construct was effectively competed out by both, non-labeled hMTP(TATA) as well as (albeit less effectively) by wild type hMTP (Fig. 7C), indicating that both, the hMTP as well as rMTP core promoters could be transactivated by HNF-4α.

4. Discussion

4.1. Human MTP promoter

In light of its pivotal role in catalyzing the assembly and production of triglyceride-rich lipoproteins, MTP suppression could serve as target for lipid-lowering drugs. This study has been initiated in order to verify the mode of action of three hypolipidemic drug prototypes, namely Medica analogs/HNF-4α antagonists, TZD/PPARγ agonists, and Cl-DICA/Wy-14,643/PPARα agonists in modulating MTP transcription. The mode of action of the three drug prototypes appears to be specific and determined by the MTP promoter context.

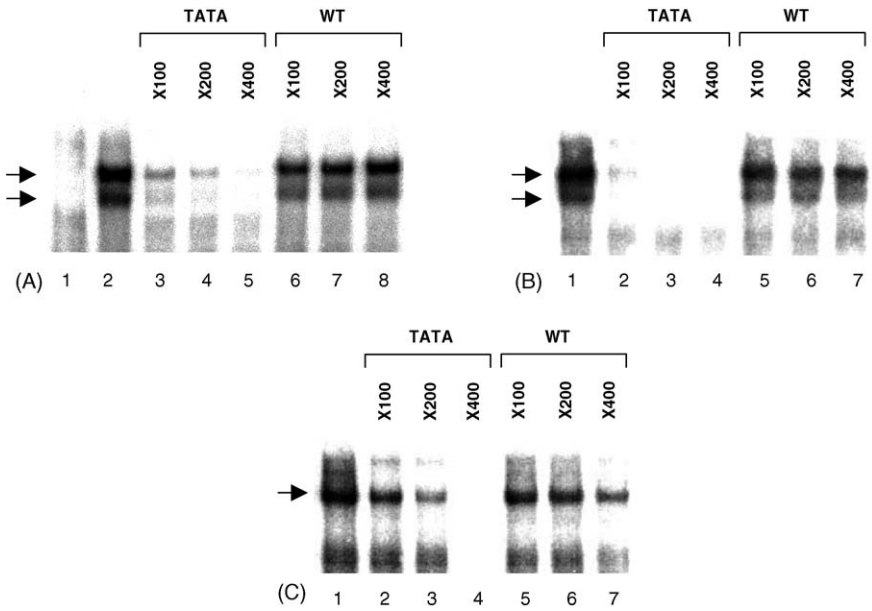

Fig. 7. Binding of DR-1 nuclear receptors to hMTP and hMTP(TATA) P-DR1. Gel electrophoretic mobility shift assays were carried out as described in Section 2. Protein–DNA complexes are indicated by arrows. (A) Nuclear extracts of COS7 cells lacking (lane 1) or overexpressing PPARα (lanes 2–8) were incubated with double-stranded [^{32}P]-end-labeled hMTP(TATA) oligonucleotide in the absence (lane 2) or presence (lanes 3–8) of excess unlabeled self-competitor or wild type hMTP oligonucleotides as indicated. (B) Nuclear extracts of COS7 cells overexpressing PPARγ were incubated with double-stranded [^{32}P]-end-labeled hMTP(TATA) oligonucleotide in the absence (lane 1) or presence (lanes 2–7) of excess unlabeled self-competitor or wild type hMTP oligonucleotides as indicated. (C) Nuclear extracts of COS7 cells overexpressing HNF-4α were incubated with double-stranded [^{32}P]-end-labeled hMTP(TATA) oligonucleotide in the absence (lane 1) or presence (lanes 2–7) of excess unlabeled self-competitor or wild type hMTP oligonucleotides as indicated.

Suppression of hMTP expression by Medica analogs (Fig. 1) is accounted for by suppressing its promoter activity (Fig. 2). Suppression by Medica analogs results partly from inhibiting HNF-4α association with the hMTP promoter in vivo (Fig. 5), in line with the established function of Medica analogs in suppressing HNF-4α transcriptional activity [14]. hMTP suppression by Medica analogs due to inhibition of HNF-4α transcriptional activity is transduced by the distal DR-1 (D-DR1) element of hMTP promoter that serves as response element for HNF-4α [9]. Thus, inhibition of the (−611/+87)hMTP promoter activity consisting of the distal element (70–80% inhibition, Figs. 2 and 4) was significantly more pronounced than inhibition of the truncated (−74/+87)hMTP that lacks the distal DR-1 element (40% inhibition, Figs. 3 and 4). In addition to Medica inhibition of HNF-4α transcriptional activity transduced by hMTP D-DR1, Medica analogs may inhibit hMTP transcription by interfering with the function of the TAAA sequence that serves as TATA box of hMTP gene promoter [8]. Thus, (a) inhibition by Medica analogs persisted in the context of the truncated (−74/+87)hMTP (Fig. 3A) as well as in the context of the P-mut/D-mut doubly mutated (−611/+87)hMTP promoter (Fig. 3B), indicating a putative site of action proximal to P-DR1 and (b) inhibition by Medica analogs was essentially eliminated in the context of the (−74/+87)hMTP(TATA) promoter, namely under conditions of overtaking the TAAA function by the canonical rodent-conforming TATA conjoined with the proximal DR-1 (P-DR1). Medica interference with hMTP TAAA but not with the rodent-conforming canonical TATA conjoined with P-DR1 still remains to be investigated in terms of specific components of the pretranscriptional initiation complex (PIC) recruited to each of the respective TAAA/TATA boxes. It is noteworthy however, that the (−611/+87)hMTP(P-mut/D-mut) promoter doubly mutated in its proximal and distal DR-1 elements is suppressed by HNF-4α antagonists (Fig. 3B), but remains unaffected by overexpressed HNF-4α [9], indicating that Medica interference with human TAAA is independent of HNF-4α. Hence, inhibition of hMTP transcription by Medica analogs may be ascribed to (a) inhibition of transactivation by HNF-4α transduced by D-DR1 of the hMTP promoter and (b) specific interference with the TAAA function of hMTP core promoter that serves as TATA box of wild type hMTP.

Similarly to Medica analogs, TZD inhibit hMTP expression by suppressing its promoter activity (Figs. 1 and 2). Also, similarly to Medica analogs, suppression of hMTP transcription by TZD is mediated by interfering with the hMTP TAAA box, independently of PPARγ. Thus, (a) suppression by TZD persisted in the context of the truncated (−74/+87)hMTP promoter lacking the D-DR1 element as well as in the context of the (−611/+87)hMTP promoter doubly mutated in its D-DR1 and P-DR1, thus indicating that the TZD effect was exclusively mediated by core promoter sequences proximal to P-DR1, (b) suppression of hMTP by TZD was eliminated in the context of the rodent-conforming TATA conjoined with P-DR-1, (c) inhibition by TZD was not

mimicked by overexpressed PPARγ, and still persisted under conditions of PPARγ overexpression (Fig. 6A) and (d) the binding affinity of PPARγ to the proximal DR-1 element of hMTP was robustly decreased as compared with the rodent-conforming P-DR1 (Fig. 7). Hence, both TZD and Medica analogs may interfere with the hMTP TAAA function, independently of their cognate receptors, sharing a mode of action that still remains to be investigated in terms of PIC components recruited by the respective TAAA/TATA boxes.

Suppression of hMTP expression by Medica analogs and TZD may offer a molecular basis for their hypolipidemic activity in human in addition to previously reported modes of action concerned with activation of plasma lipoproteins clearance due to suppression of apo C-III expression [26,31] or the induction of adipose lipoprotein lipase [32,33]. Suppression of hMTP expression by Medica analogs and TZD may also account for decreased VLDL production rates independently of the availability of free fatty acids generated by adipose lipolysis.

In contrast to Medica analogs or TZD, PPARα agonists are ineffective in suppressing hMTP transcription, both in the absence or presence of overexpressed PPARα (Figs. 1, 2 and 6), in concordance with the robustly decreased affinity of PPARα to the proximal DR-1 element of hMTP (Fig. 7). Hence, the hypolipidemic effect of PPARα agonists/fibrates in human may not be ascribed to suppression of hMTP transcription by fibrate-activated PPARα. Moreover, in light of the questionable responsiveness of the human liver to hPPARα (reviewed in Refs. [34,35]), the hypolipidemic activity of fibrates in human must be independent of liver hPPARα [36].

4.2. Rodent MTP promoter

The human and rodent MTP promoters share similar response elements 5′ to P-DR1, while differing in their respective core promoters in two main aspects, (a) PIC recruitment by the rodent MTP promoter is driven by its canonical TATA conjoined with its P-DR1, whereas the respective degenerate human sequence is overtaken by a non-canonical TAAA box 4 bp downstream the P-DR1 element (Fig. 4A), with concomitant 3′-shift in transcription initiation [8]. (b) The P-DR1 sequence of the rodent core promoter differs in its 3′-terminal bp from the respective human promoter sequence, resulting in robustly decreased binding affinity of PPARα and PPARγ to hMTP P-DR1. These distinctive features of the respective core promoters may result in specificity in PIC recruitment and its amenability to interference by the concerned drug prototypes, together with differences in binding affinities of the concerned nuclear receptors to their cognate P-DR1. These characteristics may determine a promoter-specific mode of action of the concerned nuclear receptors and their respective drug prototypes. Based on the close homology between the rodent-conforming hMTP(TATA) promoter and the rodent MTP promoter, characteristics verified in the context of the hMTP(TATA) promoter may be projected to the rodent promoter and may offer a molecular basis for effects of fibrates and TZD previously verified in rodents and in the context of rMTP.

Thus, in contrast to the wild type hMTP promoter where PPARγ is essentially inactive and TZD is inhibitory due to interfering with hMTP TAAA, the rodent-conforming hMTP(TATA) promoter is activated by overexpressed PPARγ and further by TZD-activated PPARγ (Fig. 6). Activation by PPARγ is transduced by the rodent-conforming P-DR1 as verified by the effect exerted by overexpressed PPARγ and by TZD-activated PPARγ in the context of the truncated (−74/+87)hMTP(TATA) (Fig. 6A), in line with the high binding affinity of hMTP(TATA) core promoter for PPARγ (Fig. 7B). This conclusion is further corroborated by lack of MTP activation by PPARγ in the context of the (−74/+87)hMTP(P-mut/TATA) promoter mutated in its P-DR1. Hence, lack of TZD interference with the rodent-conforming TATA box, together with P-DR1 transactivation by TZD-activated PPARγ, may result in overall activation of rMTP expression under conditions of PPARγ availability. These results, if confirmed in the genuine context of the rodent MTP promoter, may indicate that the previously reported suppression of MTP expression by TZD in insulin-resistant fructose-fed hamsters [23] may not be ascribed to rMTP suppression transduced by TZD-activated PPARγ. The TZD effect may however be accounted for by the insulin-sensitizing effect of TZD transduced by the insulin response element of the rMTP gene promoter, 5′ to P-DR1 [8].

In contrast to the wild type human MTP promoter where PPARα and its agonists are essentially inactive, the hMTP(TATA) promoter is activated by PPARα and further by Wy-14,643-activated PPARα. Similarly to activation of hMTP(TATA) by PPARγ, activation by PPARα and by Wy-14,643 is transduced by rMTP P-DR1 as verified by (a) the activation exerted by PPARα and by Wy-14,643-activated PPARα in the context of the truncated (−74/+87)hMTP(TATA) (Fig. 6B), (b) the high binding affinity of hMTP(TATA) core promoter for PPARα (Fig. 7A) and (c) loss of activation by PPARα of the (−74/+87)hMTP(TATA) promoter mutated in its P-DR1. Hence, similarly to TZD, lack of interference of PPARα agonists with the rodent-conforming TATA box, together with transactivation by PPARα transduced by the rodent-conforming P-DR1, may result in overall activation of rMTP expression under conditions of PPARα availability. These results are corroborated by and may offer a molecular basis for the recently reported activation of liver rMTP expression in rodents in vivo and in liver cells treated with PPARα or RXRα agonist (e.g., Wy-14,643, 9-*cis* retinoic acid, respectively), being transduced by the proximal DR-1 of rMTP [24,25]. These results may indicate that characteristics of hypolipidemic drug prototypes verified in the

context of hMTP(TATA) may indeed be projected onto the genuine rodent promoter. Also, these results may imply that the hypolipidemic activity of fibrates in rodents is in spite of, rather than due to, modulating MTP expression.

The findings reported here may indicate that modulation of MTP expression by hypolipidemic insulin sensitizers is the resultant of the species-specific context of respective TATA boxes and P-DR1 elements of MTP core promoters, the respective nuclear receptors involved (e.g., HNF-4α, PPARγ, PPARα), their modulation by their respective ligands/drugs, as well as drug effects mediated independently of the respective cognate nuclear receptors (Fig. 8). It is worth noting that the species-specific context of the proximal DR-1 elements of respective MTP core promoters may account for the non-responsiveness of hMTP to PPARα or PPARγ, in line with the previously reported species-specific context of peroxisomal AOX DR-1 claimed to account for the non-responsiveness of human AOX to peroxisome proliferators/PPARα agonists [37,38]. These comparative results may further imply that the rodent MTP promoter may not substitute for the human promoter when searching for hypolipidemic insulin sensitizers based on suppression of MTP transcription. Similarly, since MTP is implicated in regulating CD-1d antigen presentation of CD-1d bearing cell types (e.g., hepatocytes, intestinal epithelial cells) [39], searching for MTP suppressors designed to protect from CD-1d-mediated hepatitis and/or colitis should be based on verifying their effects in the specific context of the human MTP promoter.

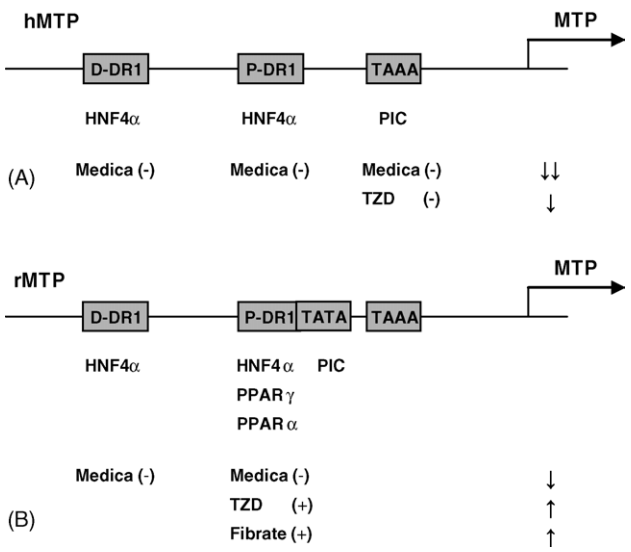

Fig. 8. Transcriptional modulation of hMTP (A) and the rodent-conforming hMTP (B) promoters by hypolipidemic insulin sensitizers. Respective promoter boxes reflect the concerned response elements. Transcription factors (HNF-4α, PPARγ or PPARα) that may transactivate the MTP gene promoter are denoted in concordance with their respective response elements. Hypolipidemic insulin sensitizers (HNF-4α antagonists/Medica drugs, PPARγ agonists/TZD, PPARα agonists/fibrates) that may either activate (+) or suppress (−) MTP gene expression are denoted in concordance with their respective transcription factors and response elements. The resultant effects of hypolipidemic insulin sensitizers on hMTP and the rodent-conforming hMTP expression are denoted by upward or downward arrows.

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