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Clofibric acid down-regulation of metallothionein IIA in HepG2 human hepatoma cells

Arnaud Bianchi, Philippe Bécuwe*, Philippe Collet, Jean Marie Keller, Lionel Domenjoud, Michel Dauça

Laboratoire de Biologie Cellulaire du Development, Ea 3446, Proliferateurs de Peroxysomes, Faculte des Sciences, Universite Henri Poincare Nancy I, B.P. 239, 54506 Vandoeuvre-les-Nancy, France

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Abstract

Among the different hypotheses advanced to explain the peroxisome proliferator (PP)-induced hepatocarcinogenicity in rodents, one is based on the development of an oxidative stress due to an imbalance in the production of reactive oxygen species that leads to DNA damages and lipid peroxidation. On the other hand, human cells appear to be nonresponsive to PPs. As metallothionein proteins play an important antioxidant role, the aim of the present study was to investigate the expression of metallothionein IA (MTIA) and IIA (MTIIA) in HepG2 human hepatoma cells exposed to clofibric acid. When HepG2 cells were treated for 24 hr with 0.50 or 0.75 mM CA, a significant decrease was observed in MT protein-level determined by Western blotting and in the MTIIA mRNA content analyzed by RT-PCR and Northern blotting. No significant change was observed in the MTIA mRNA amount whatever the CA concentration and the duration of treatment. The decrease in MTIIA mRNA-level was not mediated *via* peroxisome proliferator-activated receptor alpha as attested by our data from gel mobility shift DNA binding assays, Dot blotting and cotransfection experiments with MTIIA promoter-driven luciferase reporter gene and PPARα expression vector. These results provide new insights about the pleiotropic effects of PPs on human cells.

Keywords: Metallothionein; Peroxisome proliferators; Clofibric acid; PPAR; Human hepatoma cells; Gene expression

1. Introduction

The PPs represent a class of structurally diverse compounds, including the fibrate hypolipidaemic drugs such as clofibric acid (CA), which increase in some rodents the number of peroxisomes and the activities of several peroxisomal enzymes involved in the long chain fatty acid degradation [1]. The control of PP-inducible genes is regulated *via* peroxisome proliferator-activated receptors (PPARs) which interact with specific response elements (PPREs) located upstream of PPAR target genes [2].

Chronic administration of PPs in rats and mice results in the development of hepatocellular carcinomas [3–6]. The basic mechanisms by which this class of chemicals induces tumor formation in rodents are not yet understood. Several hypotheses have been proposed. One of them is based on the fact that PPs act rather as promoting factors of tumor formation than as initiating agents. A convergence of PPs with growth factor pathways, in particular the mitogen-activated protein kinase, has been reported by Rokos and Ledwith [7]. Suppression of apoptosis and sustained stimulation of cell growth may be sufficient to induce carcinogenesis [8,9]. An alternative mechanism is that PPs induce an oxidative stress due to an imbalance in the production and degradation of hydrogen peroxide (H_2O_2) and to a decrease in the activities of antioxidant defense enzymes [10–12].

In contrast to rodent cells, no peroxisome proliferation is observed in human PP-treated cells. Nevertheless, the activity of the H_2O_2-producing peroxisomal fatty acyl-CoA oxidase (ACO) is stimulated by fibrates in human

* Corresponding author. Tel.: +33-383-91-23-18; fax: +33-383-91-23-19.
E-mail address: becuwe@scbiol.u-nancy.fr (P. Bécuwe).

Abbreviations: ACO, acyl-CoA oxidase; CA, clofibric acid; DMEM, Dulbecco minimum essential medium; DMSO, dimethyl sulfoxide; G3PDH, glyceraldehyde 3-phosphate dehydrogenase; MT, metallothionein; MTIA, metallothionein IA; MTIIA, metallothionein IIA; PP, peroxisome proliferator; PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator-response element; RT-PCR, reverse transcriptase-polymerase chain reaction; SOD, superoxide dismutase.

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HepG2 hepatoma cells [13,14] but the increased H_2O_2-production does not result in the development of an oxidative stress [15]. This could be partly explained by the fact that the fibrate drug increases the expression of the gene encoding the antioxidant enzyme manganese superoxide dismutase (SOD) [15].

In order to provide new insights about the pleiotropic effects of PPs in human cells which could explain the putative differences in the response of rodent and human cells to these chemicals, we have investigated the expression of the MTIA and MTIIA in HepG2 cells treated with CA. Metallothioneins (MTs) are ubiquitous proteins enriched in cysteine residues which play a major role in the regulation of Zn and Cu homeostasis, in the detoxification of heavy metals [16] and participate actively to the maintenance of the intracellular redox status. MTs which are induced by reactive oxygen species are considered as protective cellular stress proteins [17,18]. Seven functional members of the human MT family have been identified [16]. The present work was focalized on the effects of CA on the expression of MTIA and MTIIA as these two proteins are the most expressed among the MT members in hepatic cells. We demonstrate that CA decreases the MT protein amount and the MTIIA mRNA level in human HepG2 cells and that this down-regulation is not mediated *via* PPAR.

2. Materials and methods

2.1. Cell culture conditions and clofibric acid treatment

HepG2 cells [19] were seeded at 2×10^4 cells/cm^2 and grown in Dulbecco's minimum essential medium (DMEM, Eurobio) supplemented with 10% (v/v) fetal calf serum (Eurobio), at 37° in a water-saturated atmosphere with 5% CO_2/95% air. Two days later, the medium was replaced by the one containing CA [2-(*p*-chlorophenoxy)-2-methyl propionic acid] (Sigma) at a final concentration of 0.10, 0.25, 0.50 or 0.75 mM in 0.1% (v/v) dimethyl sulfoxide (DMSO, Merck). Cells were exposed to CA for 12 or 24 hr of treatment. As a negative control, cells were cultured in a medium supplemented with only 0.1% DMSO. For each cell culture set, the viability of CA-treated and untreated cells was assayed using the trypan blue exclusion test.

2.2. Production of anti-MTIA and IIA polyclonal antibody

A mixture containing both MTIA and IIA provided by Sigma was coupled to keyhole limpet hemocyanin (Sigma) used as a carrier according to the glutaraldehyde method [20]. Immunogens (200 μg/100 μL of saline/injection) were mixed with an equal volume of complete Freund's adjuvant for the primary series of injections and with incomplete Freund's adjuvant for the following ones. Polyclonal antibody was raised by multiple subcutaneous injections into rabbits using standard procedures. They were characterized by Western blotting.

2.3. Western blot analysis

The HepG2 cells treated or not with CA or with H_2O_2 were harvested and lysed in a 10 mM Tris–HCl buffer, pH 7.4, containing 5 mM EDTA and 1% Triton X-100, at 4° for 30 min. After centrifugation at 17,000 *g* for 20 min at 4°, the supernatants were used for protein assay [21] with bovine serum albumin as a standard. MT proteins (2 μg) used for immunization and protein samples (50 μg) were run through SDS-polyacrylamide gels (15%) according to Laemmli [22] and transferred onto polyvinyldifluoride membranes (NEN) as previously described [23]. MT proteins were detected using the produced polyclonal antibody (dilution 1:500 in TBS). Manganese (Mn) and copper/zinc (Cu/Zn) SODs were also detected using specific antibodies from Janssen Pharmaceutica and Biodesign, respectively. Mn SOD was used as a positive control as its content increases in CA-treated HepG2 cells meanwhile Cu/Zn SOD was the normalization control for protein loading as its protein level does not change in those treated cells [15]. The protein bands were visualized using appropriate IgG polyclonal antibodies conjugated to peroxidase exposed to luminol, a chemiluminescent substrate (Roche). The intensities of the MT band were quantified by densitometry with a computerized image processing system (Biocom 200). Results were expressed as percentages of control values.

2.4. RNA extraction and RT-PCR analysis

Total RNA was isolated by the guanidium isothiocyanate–phenol–chloroform (Appligene) method [24] and used for RT-PCR [25]. Five micrograms of total RNA were reverse-transcribed for 50 min at 42° in 20 μL of PCR buffer provided by Life Technologies with 2.5 mM dNTPs, 5 μM random hexamer primers (Life Technologies), 1.5 mM $MgCl_2$ and 200 U SuperScript II reverse transcriptase (Life Technologies).

The primers used were selected from published nucleotide sequences in the open reading frames of the human genes encoding MTIA [26], MTIIA [27] and G3PDH [28]. Sense primers were 5′-ACTGGTGGCTCCTGCACCTG-CACT-3′ and 5′-GCCGGTGACTCCTGCACCTGCG-3′ for MTIA and MTIIA, respectively. Antisense primers were 5′-ACAGCAGCTGCACTTCTCTGAT-3′ and 5′-GCAG-CAGCTGCACTTGTCCGAC-3′ for MTIA and MTIIA, respectively. The following sense and antisense primers, 5′-TGGGGAAGGTGAAGGTCGGA-3′ and 5′-GGGATC-TCGCTGCTGGAAGA-3′, were used to amplify G3PDH as an internal control. Each primer was added at a final concentration of 0.50 μM in 50 μL reaction mixture in PCR buffer containing 1 μL cDNA, 0.25 mM of each dNTP, 1.5 mM $MgCl_2$, and 2.5 U Taq polymerase (Life

Technologies). An initial denaturation was carried out for 3.5 min at 94° and 30 cycles were performed with the following PCR program: 1 min at 94°, 1 min at 59° for MTIA or 61° for MTIIA or 56° for G3PDH, 1 min at 72°. This program was completed with a final extension for 3 min at 72°. Preliminary assays have shown that for 30 cycles amplification was in the linear phase. DNA fragments with expected sizes of 175 bp for both MTIA and MTIIA and 220 bp for G3PDH were purified with the Prep A gene DNA purification matrix kit (Bio-Rad) and sequenced according to the dideoxy chain-termination method [29]. These sequences were found identical to those previously published. After staining with ethidium bromide, the fluorescence intensities of the bands were quantified using a Gel Doc 1000 system (Bio-Rad). Values were expressed as the ratio of the MTIA or MTIIA signals relatively to that of G3PDH from CA-treated cells, and figured as percentages of control values from untreated cells.

2.5. Northern blot analysis

Total RNA (15 μg) was denatured with glyoxal-DMSO, then subjected to electrophoresis on 1.5% agarose gels. The latter were then blotted onto nylon membranes (Amersham). The MTIA and MTIIA probes produced by RT-PCR as described above were labeled with [α-^{32}P]dCTP (Amersham) by random priming and further purified by Sephadex G50 column chromatography (Pharmacia). After prehybridization, MTIA and IIA mRNA were hybridized with the [α-^{32}P]dCTP-labeled cDNA probes. Membranes were washed four times (10 min each wash) at room temperature in 2× SSC (30 mM NaCl, 3 mM trisodium citrate) containing 0.1% SDS and twice (15 min each wash) at 60° in 0.2× SSC with 0.1% SDS. After autoradiography, the membranes were reprobed with the [α-^{32}P]dCTP-labeled G3PDH cDNA (Clontech) used as an internal control for load and integrity of the RNA. Autoradiograms were quantified with a densitometer (GelDoc 1000). Values were expressed as the ratio of the MTIA or IIA signal relatively to that of G3PDH from CA-treated cells, and figured as percentages of control values from untreated cells.

2.6. Gel mobility shift DNA binding assay

As the maximal down-regulation was observed with HepG2 cells treated with 0.50 mM CA for 24 hr, cells were used for the extraction of nuclear proteins prepared as previously described [30], with the following modifications. The nuclei were resuspended in 20 mM HEPES, pH 7.9, containing 450 mM NaCl, 0.20 mM EDTA, 0.15 mM $MgCl_2$, 25% (v/v) glycerol, 0.5 mM dithiothreitol and 0.5 mM phenylmethylsulfonyl fluoride, then homogenized with Dounce and incubated for 30 min at 4° after addition of 0.25% (v/v) Nonidet P-40. Nuclear extracts were cleared by centrifugation at 15,000 *g* for 20 min at 4°, and the resulting supernatants were immediately frozen in liquid nitrogen and stored at −80° until used for gel retardation assays. In order to investigate whether the PP-induced down-regulation of the human *MTIIA* gene was or not mediated by peroxisome proliferator-activated receptor alpha (PPARα), we looked for PP-response elements in the promoter of this gene. Two putative PPREs (P1 and P2) were found (Fig. 1). The P1 binding site (nt: −342, −313) contained a PPAR half site (TGTCCT) located into the glucocorticoid response element. In the P2 binding site (nt: −701, −680), two putative PPAR half sites (GGACCT and TCTCCT) were spaced by 6 nucleotides. However, these sequences differ significantly from the canonic peroxisome proliferator-response element (PPRE) (TGACCTnTGACCT) which is a DR1 sequence. The P1 and P2 DNA fragments were radiolabeled with [α-^{32}P]dCTP (3000 Ci/mmol) by fill-in reactions using Klenow large fragment DNA polymerase (Life Technologies) and used as probes. After preincubation for 15 min with the reaction mixture, radiolabeled probes (1 pmol) were added to 12.5 μg of nuclear proteins in the binding buffer containing 10% glycerol, 50 mM HEPES (pH 7.9), 10 mM KCl, 0.5 mM dithiothreitol, 1.25 mM $MgCl_2$ and 1 μg poly dI-dC. The reaction was carried out at room temperature for 15 min, then analyzed on 4% polyacrylamide gels using 0.5× Tris-borate/EDTA. Electrophoresis was carried out under a constant voltage of 15 V/cm for 1 hr in 0.5× TBE buffer. Gels were dried under vacuum then exposed to X-ray films (Kodak). For competition experiments, 180 pmol of unradiolabeled probes were added to the preincubation

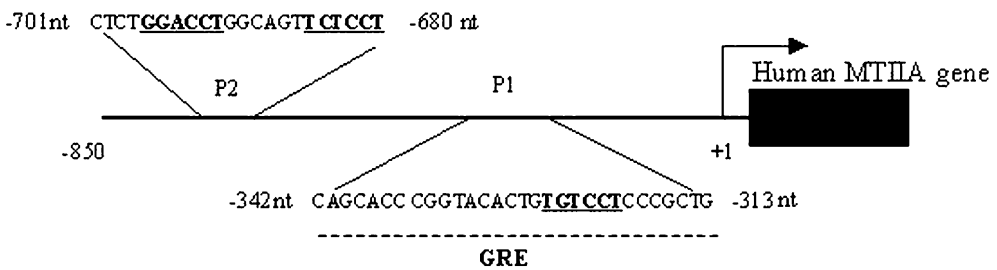

Fig. 1. Schematic representation of the human *MTIIA* gene promoter exhibiting two putative PPAR response elements, P1 and P2. The P1 region contains a putative half site of PPRE (bold underlined characters) located in the glucocorticoid response element (GRE) and the P2 domain possesses two putative half sites of PPRE (bold underlined characters) spaced by six nucleotides.

mixture. As a positive control, gel mobility shift DNA binding assay was done with 1 ng of radiolabeled PPRE of the human peroxisomal *ACO* gene [31,32].

2.7. Immunoprecipitation and Dot blot experiments

We investigated the capacity of PPAR to bind the two probes, P1 and P2. Different amounts (0.6, 1.3 and 2.6 pmol) of each radiolabeled probe were incubated at 37° for 1 hr in the binding buffer containing 12.5 μg of nuclear proteins from CA-treated HepG2 cells and the antibody which recognizes the three PPAR subtypes [33]. The polyclonal antibody was diluted 1/500 in the mixture. After incubation for 1 hr, 5 μL of a protein A-Sepharose solution (50%, v/v) were added to the mixture, and the incubation was carried out for 1 hr.

Immunoselected complexes were collected by centrifugation at 7800 *g* for 10 min at 4°, and washed twice with 100 μL of a buffer containing 150 mM KCl, 10 mM Tris–HCl (pH 7.5), 1 mM EDTA and 0.1% (v/v) Triton X-100. After centrifugation, they were resuspended in 20 μL of TE solution [10 mM Tris–HCl (pH 7.5), 1 mM EDTA] and subjected to Dot blotting. The DNA–protein mixture was filtered through a nylon membrane (Amersham) by gravity flow and the wells were washed twice with TE buffer. As a positive control, different amounts (0.3, 0.6 and 1.2 pmol) of radiolabeled *ACO* gene PPRE [31,32] were loaded. Nylon membranes were dried and DNA was immobilized by UV irradiation for 1 min. The blots were then exposed to X-ray films (Kodak) overnight.

2.8. Cloning and construction of the pGL3/MTIIA-luc plasmid

The full length (0.8 kb) promoter region of the human *MTIIA* gene previously described [27] was amplified (30 cycles for 1 min at 94°, 1 min at 56°, 1 min at 72°) from genomic DNA isolated from cultured HepG2 cells, using the following sense and antisense primers: 5′-GAGGGCC-GTTAGCATCTGCT-3′ and 5′-GAGTCGGGACAGGTT-GCAC-3′, respectively. After agarose gel purification, the amplified DNA fragment was inserted in the *Sma*I site of the pGL3/luc plasmid which contained the luciferase gene reporter yielding the pGL3/MTIIA-luc construct. Identity and orientation of the recombinant plasmid were confirmed by PCR and sequencing analyses.

2.9. Transfections

HepG2 cells were cultured in 6-well plates at 2×10^4 cells/mL. They were transiently cotransfected with a mixture of plasmids which contained the pSV40/SEAP plasmid (Clontech) (0.6 μg) used as control for transfection efficiency and either the pGL3/MTIIA-luc plasmid (1 μg) or the ACO-PPRE luciferase reporter plasmid (1 μg) comprising three copies of PPRE from the promoter of the rat *ACO* gene [34], in addition or not with the pSG5/PPARα expression vector (0.5 μg). Cells were transfected by the lipofection method with Exgen 500 according to the supplier's instructions (Euromedex). After a 2 hr incubation period, cells were washed with PBS, then refed with fresh medium and treated with 0.5 mM CA or 0.1% DMSO. Cells were harvested after a 24 hr incubation. The luciferase (Sigma) and alkaline phosphatase (Clontech) activities were determined in cell extracts and culture medium, respectively, following the manufacturer's instructions. Transfection experiments were performed in quadriplicate.

3. Results

3.1. Effects of CA on MT protein levels

The MTIA and MTIIA protein levels in HepG2 cells treated with different concentrations of CA for 12 or 24 hr were investigated by Western blotting. Treatments did not affect cell growth as attested by trypan blue exclusion. The polyclonal antibody produced for this study recognized both MTIA and MTIIA proteins from the mixture used for immunization (Fig. 2). With protein extracts from HepG2 cells treated or not with H_2O_2, a large band was detected (Fig. 2). Its intensity was increased when the cells were exposed to H_2O_2 as expected for MTs (Fig. 2). The MT protein level did not change significantly when HepG2 cells were treated for 12 hr with different concentrations of CA (Fig. 3). It decreased by 4-fold when the cells were exposed for 24 hr to 0.50 or 0.75 mM CA (Fig. 3). Equal protein amounts were loaded as attested by the absence of changes in the intensity of the immunodetected Cu/Zn SOD, an antioxidant enzyme of which expression is not altered by CA treatment (Fig. 3). On the other hand, the Mn SOD protein-level increased under these experimental conditions as already reported [15].

3.2. Effects of CA on MTIA and MTIIA mRNA levels

The MTIA and MTIIA mRNA levels of CA-treated and control HepG2 cells were analyzed by RT-PCR and North-

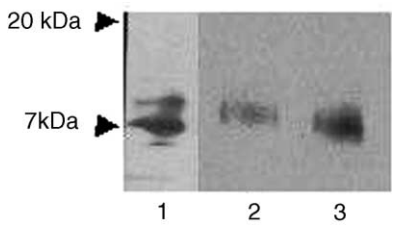

Fig. 2. Characterization by Western blot analysis of the polyclonal antibody raised against both MTIA and MTIIA proteins. Lane 1: mixture containing both MTIA and MTIIA proteins (2 μg) which was used for immunization; lanes 2 and 3: proteins (50 μg) extracted from HepG2 cells treated (lane 3) or not (lane 2) with 0.50 mM H_2O_2 for 6 hr.

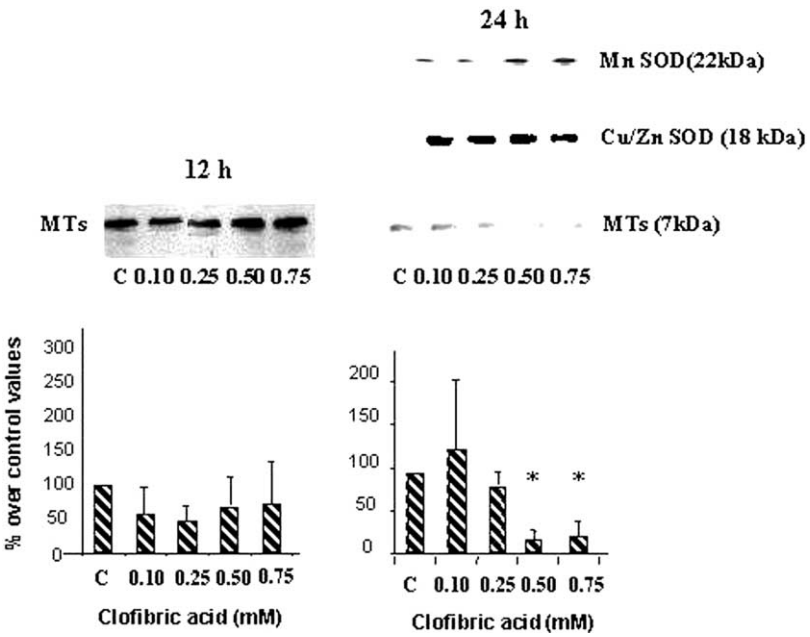

Fig. 3. Effects of CA on the MTIA and MTIIA protein levels in HepG2 cells. Total protein (50 μg) from HepG2 cells treated or not with different concentrations of CA for 12 or 24 hr was subjected to Western blotting using the produced anti-MT antibody. The CA concentrations used were 0.10, 0.25, 0.50 and 0.75 mM. A normalization control for equal amount loading and a positive control for CA induction were performed by detection of the levels of Cu/Zn SOD and Mn SOD, respectively, as described in Section 2. The MT protein band intensities were quantified by densitometry from ECL-immunoblots. Results are means (±SD) from three independent experiments and expressed as percentages of control values. Evaluation of statistical significance was assessed using analysis of variance (ANOVA) and the Fisher protected least significant test (FPLST). C, untreated cells.

ern blotting. As a first step, the sequences of the amplified MTIA, MTIIA and G3PDH DNA fragments were determined by sequencing. They were found identical to those of the previously published sequences [26–28]. Densitometric analyses were performed in three independent experiments and data were internally controlled relatively to the G3PDH signal, then plotted as percentages of data from untreated cells.

RT-PCR analyses showed that the treatment of HepG2 cells with CA did not affect significantly the level of MTIA mRNA whatever the concentration of CA and the duration of treatment (Fig. 4). Similar results were obtained by Northern blotting (data not shown).

The MTIIA mRNA level investigated by RT-PCR was not significantly altered when HepG2 cells were treated for 12 hr whatever the concentration of CA used (Fig. 4). It was decreased by 2-fold for a 24 hr treatment with 0.75 mM CA (Fig. 4). These results were confirmed by Northern blotting with the aid of the specific DNA probe produced (data not shown).

3.3. Binding analysis of PPAR to the MTIIA gene promoter

To further examine the mechanism involved in the down-regulation of the human *MTIIA* gene, gel shift–supershift assays (Fig. 5A) and Dot blotting (Fig. 5B) were carried out using two putative PPAR binding sites, P1 and P2, found in the *MTIIA* gene promoter.

As compared to the radiolabeled probe corresponding to the PPRE of the *ACO* gene and used as a positive control, a shift was obtained when the radiolabeled P1 probe was incubated with nuclear extracts from CA-treated HepG2 cells (Fig. 5A). On the other hand, no gel retardation was obtained with the radiolabeled P2 probe (Fig. 5B). However, when the anti-PPAR polyclonal antibody (which recognizes the different PPAR subtypes) was added to the reaction mixture, no supershift was observed with both probes (data not shown).

No signal was obtained by Dot blotting when CA-treated HepG2 nuclear extracts were incubated with the radiolabeled P1 or P2 probe then immunoprecipitated with the anti-PPAR antibody (Fig. 5B). Under the same conditions, the result with the radiolabeled ACO-PPRE used as a control was positive and the intensity of the signal was proportional to the amount of the probe (Fig. 5B). It was negative when the anti-PPAR antibody was replaced by the preimmune serum (Fig. 5B).

3.4. Analysis of the MTIIA gene promoter activity

To firmly establish whether modulation of the *MTIIA* gene transcription was PPAR-mediated or not, the activity of the full length *MTIIA* gene promoter was investigated in CA-treated and control HepG2 cells using reporter constructs and transfection experiments. In that purpose, the full length (0.8 kb) promoter region of the human *MTIIA* gene was amplified from genomic DNA isolated from

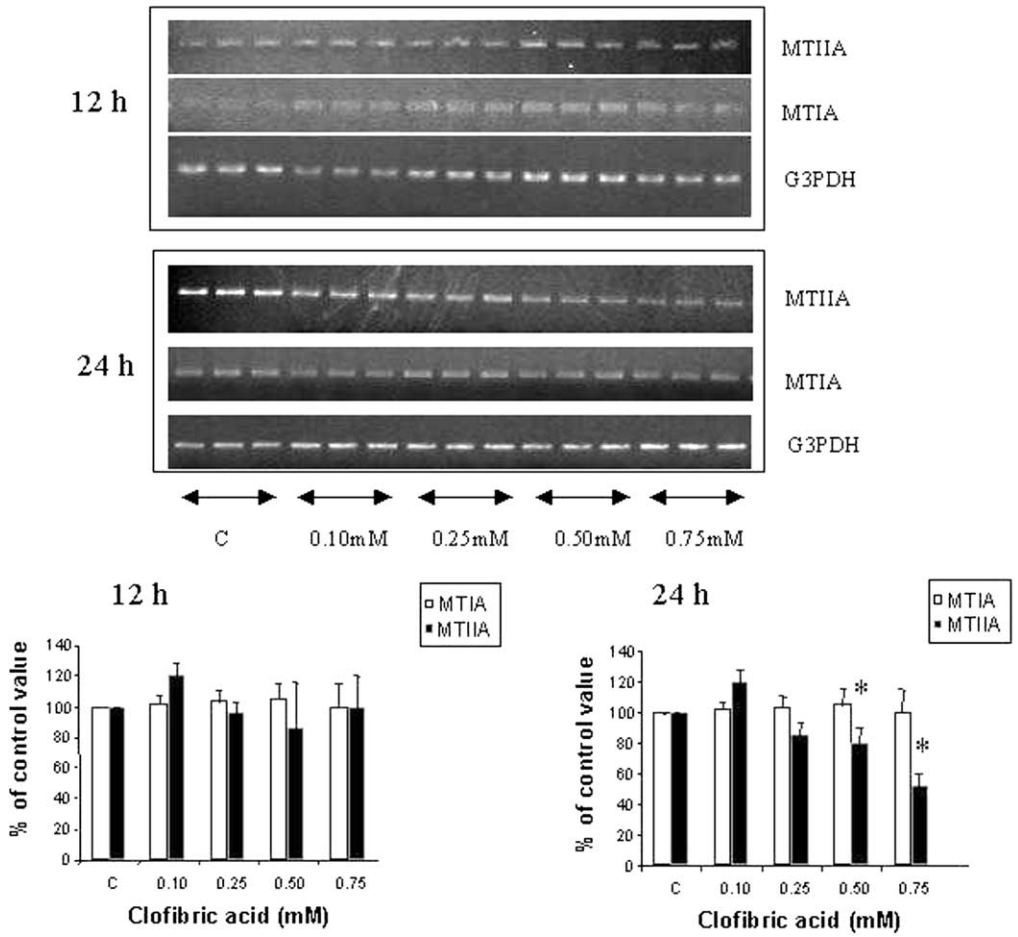

Fig. 4. RT-PCR analysis of the effects of CA on MTIA and MTIIA mRNA levels in HepG2 cells treated or not with different concentrations of CA for 12 and 24 hr. Total RNA (5 μg) was subjected to RT-PCR as described in Section 2. The relative abundances of MTIA, MTIIA and G3PDH transcripts were measured and the values corresponding to the levels of MT mRNAs were normalized to those of G3PDH mRNAs. Final data presented in the lower panel are expressed as mean percentages (±SD) of control (C) values and were obtained from three independent experiments. Statistically significant differences from control are indicated as $^{*}P < 0.05$. C, untreated cells.

HepG2 cells and its identity was confirmed by sequencing. Then it was cloned upstream a luciferase reporter gene. The resulting pGL3/MTIIA-luc construct was transfected in HepG2 cells. When the latter were treated with 0.50 mM CA for 24 hr, a 40% decrease was observed in the luciferase activity (Fig. 6). This decrease was not significantly enhanced in HepG2 cells cotransfected with both pGL3/MTIIA-luc and pSG5/PPARα plasmids. These results indicate that PPARα is not involved in the down-regulation of the luciferase reporter gene driven by the MTIIA

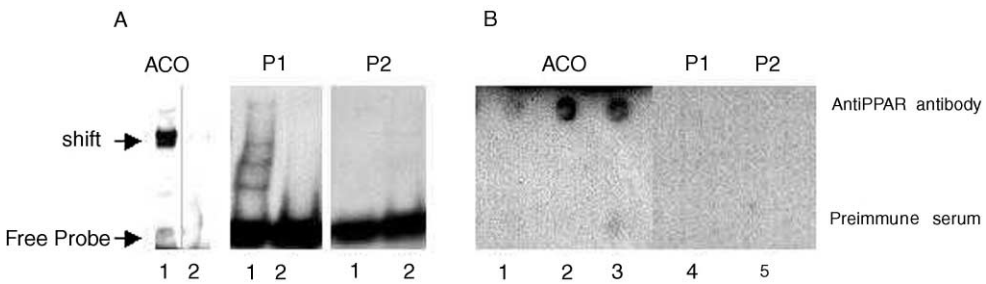

Fig. 5. Gel mobility shift DNA binding (A) and Dot blot (B) analyses. The P1 and P2 probes (see Fig. 1) containing putative PPAR response elements were used in gel mobility shift binding assays using nuclear extracts from HepG2 cells treated with 0.50 mM CA for 24 hr (A). A positive control was performed with the PPRE of the *ACO* gene as probe. Lane 1: radiolabeled probe with nuclear extract; lane 2: competition with cold probe (100×). Dot blot experiments (B) were carried out using either the anti-PPAR antibody or the preimmune serum as described in Section 2. Lanes 1–3: positive (anti-PPAR antibody) and negative (preimmune serum) controls with 0.3, 0.6 or 1.2 pmol of the labeled rat *ACO* gene PPRE, respectively; lanes 4 and 5: labeled P1 and P2 probes. In each assay, 10 μg nuclear protein from CA-treated cells were used.

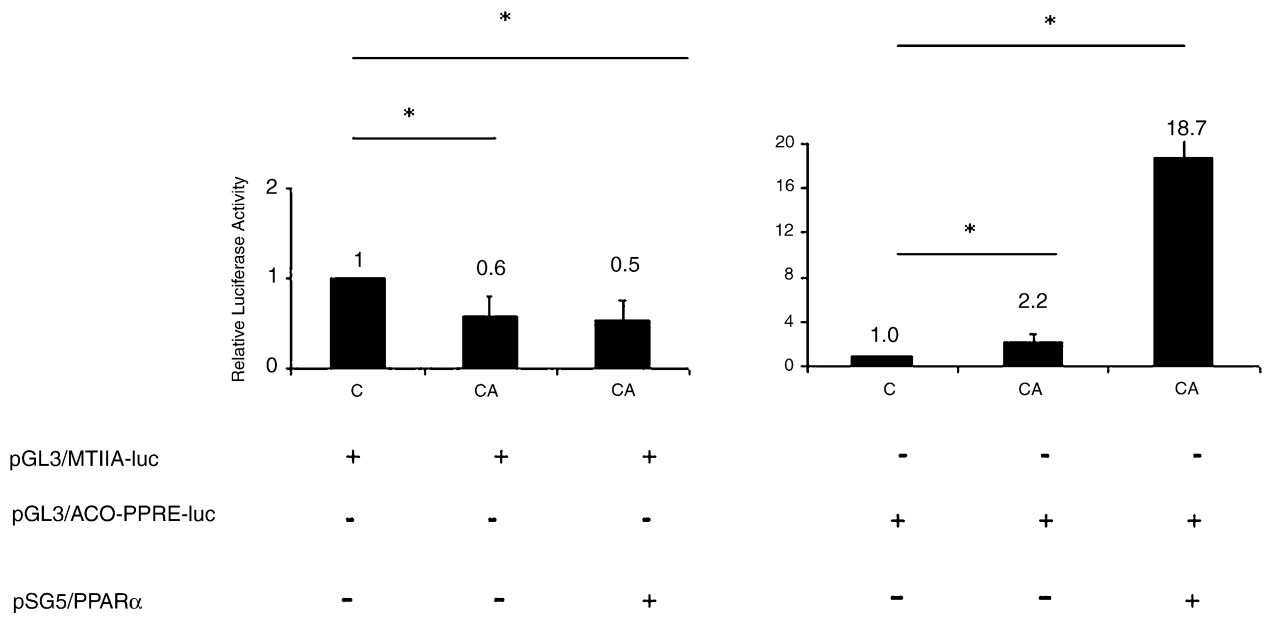

Fig. 6. Transfection analysis of the CA-induced down-regulation of the human *MTIIA* gene promoter activity. HepG2 cells were transfected with the pGL3/MTIIA-luc vector in which the luciferase reporter gene was driven by the full length human *MTIIA* gene promoter, in the presence or not of the pSG5/PPARα expression vector. Then transfected HepG2 cells were incubated with 0.50 mM CA or vehicle (C) for 24 hr. A positive control was carried out with HepG2 cells cotransfected with the pGL3/ACO-PPRE-luc reporter and the pSG5/PPARα plasmid. Results are means (±SD) from four independent experiments and are normalized to the alkaline phosphatase activities used as internal control. Statistically significant differences are indicated as $^{*}P < 0.05$.

promoter. Indeed, the PPARα expression vector was efficient as treatment with CA of HepG2 cells cotransfected with the ACO-PPRE-driven luciferase reporter construct and the pSG5/PPARα plasmid resulted in a 18.7-fold increase in luciferase activity (Fig. 6).

4. Discussion

As CA increases in HepG2 cells the activity of the fatty acyl-CoA oxidase, a H_2O_2-generating peroxisomal enzyme [13,14] which may induce an oxidative stress, we have investigated in these cells the effects of CA on the expression of genes encoding the metallothioneins, MTIA and MTIIA. Indeed, these proteins which represent the predominant MT subtypes expressed in hepatic cells [16] are known to be involved in cell protection against oxidative stress [17,18].

The high structural homology between the MTIA and MTIIA proteins did not allow the polyclonal antibody that we have produced for this study to discriminate them in Western blotting. A significant decrease in the MT protein level occurred after 24 hr of treatment with 0.50 and 0.75 mM CA and was related with the decrease observed in the MTIIA mRNA levels. For a 24 hr treatment, our data suggest that changes in the MT protein level were rather relevant to MTIIA than to MTIA as no significant change was noted in the MTIA mRNA level whatever the CA concentration and the duration of treatment. The down-regulation exerted by CA in HepG2 cells transfected with the human MTIIA promoter-driven luciferase expression vector indicates that CA modulates the MTIIA promoter activity. As major-PP-controlled gene expression is generally mediated *via* PPARs, we look for an eventual participation of these transcription factors in the down-regulation of *MTIIA* gene. We focused this possibility on PPARα because it is more expressed in HepG2 cells [35–37] and that CA is a ligand for PPARα and not for β and γ [38]. Using a polyclonal antibody which recognized the different PPAR subtypes [33], we failed to detect in shift–supershift assays and Dot blotting the binding of these receptors to two putative PPAR response elements found in the promoter of the human *MTIIA* gene. In order to exclude the possibility that PPARα binds to other sites of the MTIIA promoter, we performed cotransfection experiments with a luciferase reporter gene driven by a full length human MTIIA promoter previously described [27] and with a PPARα expression vector. When cotransfected HepG2 cells were treated with CA, the decrease in the luciferase activity was in the same magnitude than that observed with HepG2 cells only transfected with the pGL3/MTIIA promoter-luciferase construct. These data confirm that PPARα is not involved in the control of the MTIIA promoter activity. A number of proteins that bind to transcription start sites of many different genes including *MTIIA* gene have previously been reported, but very few have been shown to function as repressors. Two human MTIIA initiation sequence-binding proteins, RPA [39] and PZ120 [40] have been identified which both possess transcriptional repression activity. Despite

the many exhaustive studies of the human MTIIA promoter, the exact number of different factors capable of regulating the human *MTIIA* gene is unclear at this time. It will be interesting to investigate whether RPA and/or PZ120 modulate the down-regulation exerted by CA.

Among the different hypotheses advanced to explain the PP-induced hepatocarcinogenicity in rodents, one is based on the development of an oxidative stress due to an imbalance in the production of reactive oxygen species that leads to DNA damages and lipid peroxidation [41,42]. MT proteins play a role as antioxidants and are major intracellular heavy metals binding proteins [16]. The data dealing with the effects of PPs on the rodent liver metallothionein expression are somewhat at variance. A down-regulation of the rat metallothionein gene by CA has been reported by Motojima *et al.* [43]. On the other hand, liver metallothionein mRNA did not change in rats fed diets containing clofibrate for 3–60 days, but increased by 2.5-fold in tumors [44]. A rapid induction of the *MT1A* gene occurred in mice treated with the potent PP [4-chloro-6-(2,3-xylidino)-2-pyrimidinyl-thio]acetic acid so called Wy-14,643 [45]. In addition, copper accumulation and changes in copper-related genes expression occurred in hyperplastic liver and tumors induced by PP [44]. According to Eagon *et al.* [44], they may be contributing factors in liver neoplasia in PP-treated rats. For evident reasons, no comparison is possible with human cells concerning the changes in the levels of Cu or other heavy metals due to the difficulty to dispose of human tumors induced by PP. Differences have also been reported in the human and rodent superoxide dismutase expression in response to PP treatment [15]. Taken together, these differences could contribute in part to the fact that humans appear to be nonresponsive to the carcinogenic effect of PPs (recently reviewed in [46,47]).

In summary, the present study extends previous investigations [15] dealing with the effects of PP on the antioxidant defense system of human hepatoma cells. Our results indicate that CA treatment causes a decrease in the human *MTIIA* gene promoter activity which is not regulated by PPARα.

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