

Transcriptional regulation of apolipoprotein A-I expression in Hep G2 cells by phorbol ester

Yves Vandenbrouck, Gilles Lambert, Brigitte Janvier, Delphine Girlich, Gilbert Berezziat, Marise Mangeney-Andreani*

CNRS URA 1283, CHU Saint Antoine, 27 rue Chaligny, 75012 Paris, France

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Abstract The regulation of apolipoprotein A-I (apo A-I) gene expression by 12-*O*-tetradecanoylphorbol 13-acetate (TPA) was investigated in the human hepatoma cell line Hep G2. TPA treatment decreased apo A-I mRNA levels in a time-dependent manner, by up to 50% versus control cells within 24 h. Nuclear run-on transcription assays demonstrated a transcriptional effect of TPA. Using transfection analysis with a plasmid construct containing the -1378/+11 apo A-I promoter fused to the secreted placental alkaline phosphatase (SPAP) reporter gene, we showed that the SPAP activity was decreased to 50% when Hep G2 cells were incubated in the presence of TPA. The inhibitory effect of TPA was still maintained when fragment -253 to -4 of apo A-I promoter was linked to the CAT reporter gene. These data indicate that transcriptional modulation of apolipoprotein A-I gene expression following phorbol ester treatment is transduced by gene elements located between -253 and -4 of the apo A-I promoter.

Key words: Apolipoprotein A-I; Phorbol ester; Gene expression; Hep G2 cell

1. Introduction

Apolipoprotein A-I (Apo A-I) is the major protein component of high density lipoprotein [1] and is synthesized in the liver and intestine [2]. Apo A-I has several roles in lipoprotein metabolism. It acts as a cofactor for catalytic conversion of cholesterol into cholesterol ester by the action of lecithin-cholesterol acyl transferase (LCAT) [3] and has been linked to reverse cholesterol transport [4,5]. Lipid transport is modulated by an altered level of apolipoproteins, in response to physiological conditions [6,7]. Some of the regulation is mediated via apolipoprotein gene expression. Several studies have shown that the amount of apo A-I mRNA in the liver is controlled by hormonal and nutritional factors [8–10] and is developmentally regulated [11]. Human subjects with genetically determined low or high plasma apo AI or high density lipoprotein levels have an increased or decreased risk of developing atherosclerosis [12], respectively, indicating the importance of regulation of apo A-I synthesis.

Phorbol esters have been shown to induce several changes in

lipoprotein metabolism, including changes in the LDL-receptor mediated uptake and lipoprotein lipase activity [13,14]. However, much less is known about the molecular mechanism by which phorbol ester modulates apolipoprotein gene expression. In a recent study, it was shown that apo C-III gene transcription was regulated by a TPA-inducible activity mediated by NF κ B binding sites [15]. In another study, it was shown that transcriptional activation of the apo E gene during TPA-induced macrophage differentiation was associated with induction of AP-1-like proteins [16]. The promoter regions of several inducible genes share a conserved 8 bp motif which is known to bind the AP-1 transcription complex [17]. This complex includes the transcriptional activator proteins c-fos and c-jun which are induced by the activation of protein kinase C [18]. Additionally, for a number of genes, enhanced gene expression after TPA stimulation has also been shown to be transduced by AP-2 and NF κ B binding sites [19,20]. In this paper, we investigate the effect of TPA on apo A-I gene expression in the Hep G2 cell line. This cell line is well characterized and has been used extensively as a model to study hepatic lipoprotein metabolism and apolipoprotein gene expression [8,21–22]. We provide evidence that TPA exerts a negative effect on apo A-I gene expression at the transcriptional level in Hep G2 cells and that this effect is mediated by element(s) located between -253 to -4 in the 5'-flanking region of the apo A-I gene.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), L-glutamine, antibiotics, fetal calf serum (FCS) and Dulbecco's phosphate-buffered saline (PBS) were obtained from Gibco-BRL (European division). [¹⁴C]chloramphenicol, [α -³²P]dCTP, [γ -³²P]ATP, [α -³²P]UTP, Nylon Hybond N⁺, Royal X-Omat films and multi-primer kits were purchased from Amersham International. We obtained β -galactosidase expression vector pCHI10 from Pharmacia. All other products were purchased from Sigma.

2.2. Cell culture

Hep G2 cells were cultured at 37°C in a humidified atmosphere of 5% CO₂, 95% air. Hep G2 cells were grown in DMEM containing 10% fetal calf serum (FCS), 2 mM L-glutamine and antibiotics (penicillin, streptomycin). 24 h prior to TPA treatment, when Hep G2 cells were confluent, the cells were washed twice with PBS (pH 7.4) and further maintained DMEM medium without FCS.

2.3. RNA extraction and analysis

After extraction by the method of Chomczynski and Sacchi [23], total RNA (10 μ g) was analyzed by Northern-blot as previously described [8]. The blots were hybridized with apo A-I cDNA probe labeled with [α -³²P]dCTP by random priming and rehybridized to a 28S rRNA probe labeled with T4 polynucleotide kinase and [γ -³²P]ATP. The apo A-I mRNA signal was quantified by densitometric scanning and was

*Corresponding author. Fax: (33) (19 1) 40 01 14 95.

Abbreviations: apo, apolipoprotein; CAT, chloramphenicol acetyl transferase; FCS, fetal calf serum; PBS, phosphate-buffered saline; SPAP, secreted placental phosphatase alkaline; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

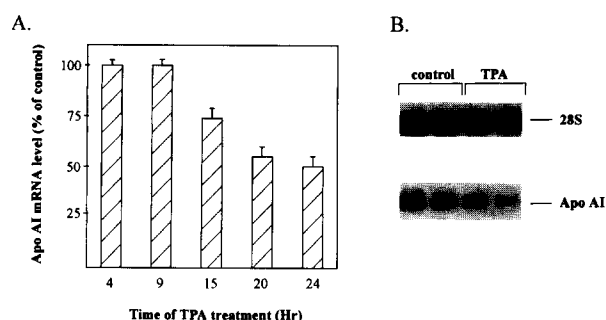


Fig. 1. Time course of TPA effect on apo A-I mRNA level in Hep G2 cells. Hep G2 cells were maintained for 24 h in serum-free medium and were then treated with 100 nM TPA. Total RNA was extracted at the time indicated and analyzed by Northern blotting as described in section 2. (A) Data obtained by densitometric scanning were corrected for differences in RNA loading by using the 28S rRNA signal. Data are expressed as the percentage of the apo A-I signal from control cells. Each value is the mean \pm S.E.M. of data collected from three independent experiments. (B) Representative autoradiogram of Northern-blot performed on Hep G2 cells 24 h after 100 nM TPA treatment.

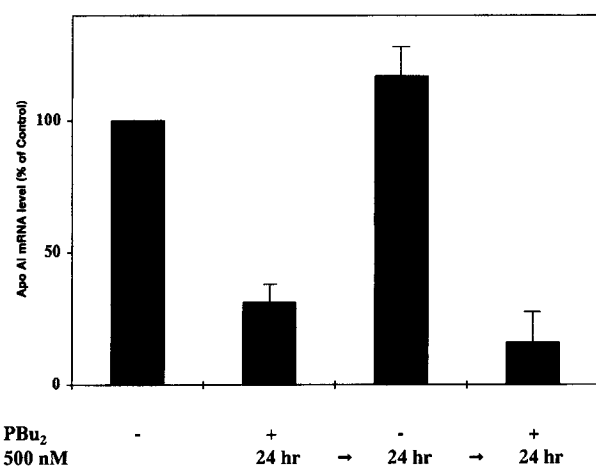


Fig. 2. Relationship between the presence of Pbu2 and the reduction of apo AI mRNA. Cells were incubated with 500 nM PBu₂ for 24 h and then washed three times with DMEM medium, and incubated for 24 h. PBu₂ (500 nM) was added again and cells were reincubated for 24 h. The apo AI mRNA level was normalized to the value of control cell, which was given the arbitrary value of 100. Data represent the mean \pm S.D. from three independent experiments.

corrected for differences in RNA loading by comparison with the signals generated by the 28S rRNA.

2.4. Nuclei isolation and run-on assays

The rate of apo A-I gene transcription was measured by a run-on assay 24 h after TPA treatment. Nuclear isolation and hybridization of nuclear transcripts to immobilized DNA were performed as previously described [8].

2.5. Plasmids, cell transfections, alkaline phosphatase and CAT assays

The plasmid pA-I-SPAP containing the -1378/+11 region of the human apo A-I gene cloned upstream the secreted placental alkaline phosphatase (SPAP) gene was generously donated by Glaxo-France (Les Ulis) and the plasmid pEMBL-8-CAT containing the human apo A-I promoter sequence -253/-4 was generously donated by Dr. Zanini [24]. Transient transfections of Hep G2 cells were performed by the calcium phosphate co-precipitation method. Chloramphenicol acetyl transferase (CAT) activity was determined by acetylation of [¹⁴C]chloramphenicol followed by thin layer chromatography as previously

described [25]. Secreted placental alkaline phosphatase (SPAP) was assayed on the day following transfection. Hep G2 cells were trypsinized, seeded on microwell plates (microdish well-plate 96F). SPAP activity was measured in FCS-free DMEM without phenol red as follows; the reaction was started by incubating 100 μ l of medium with 150 μ l of *p*-nitrophenyl phosphate substrate 1X on a microplate at 37°C for 1 h and stopped by adding 2M NaOH. Absorbance of each microwell plates was determined at 405 nm using a Dynatec MR5000 microplate reader. For total cellular protein determination, cells were washed once in PBS, then fixed by ethanol for 15 min, ethanol was removed and 0.25% Bengal pink was added for 15 min. Microplates were rinsed, dried, eluted with 0.2 M NaOH/DMSO (v/v) and the absorbance was determined at 550 nm using the microplate reader.

3. Results and discussion

Apo AI gene expression has been shown to be regulated by diets and thyroid hormone at a post-transcriptional level [8,9]. Nothing is known about a putative transcriptional regulation of the apo AI gene. Furthermore, the transduction pathways leading to apo AI gene expression in response to extracellular signals have not been elucidated. In this paper, we have demon-

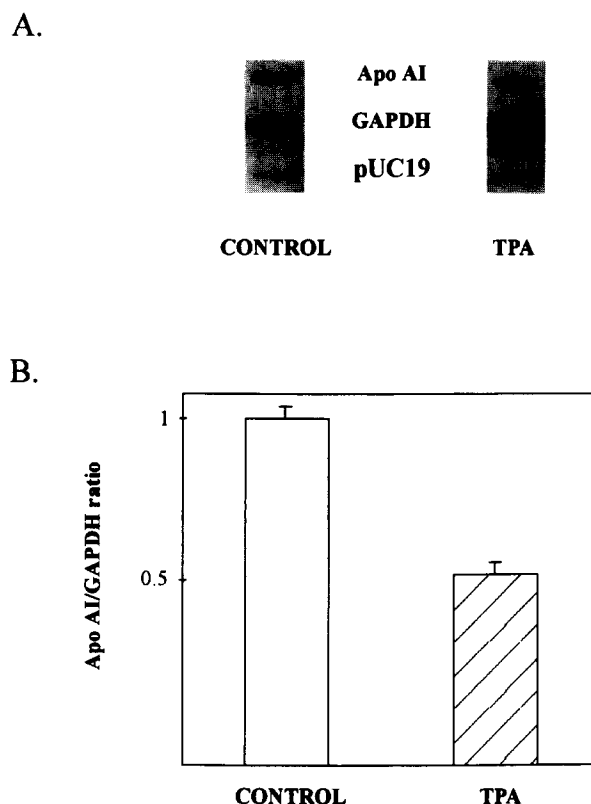


Fig. 3. Nuclear run-on analysis of apo A-I gene transcription after TPA treatment. After 24 h of TPA (100 nM) treatment, nuclei from control and treated Hep G2 cells were isolated and transcription run-on assays were performed as described in section 2. Equal amounts ($50 \cdot 10^6$ cpm) of ³²P-labeled RNA were hybridized to nitrocellulose filters containing 10 μ g of linearized plasmids bearing apo A-I, GAPDH cDNA sequences and pUC19 as a background control: (A) Representative autoradiogram of run-on assays performed on Hep G2 cells after 24 h incubation with (right panel) or without 100 nM TPA (left panel). (B) The amount of each band was analyzed using a videocopy/Densylab system from Bioprobe and the data shown are the results of the apo A-I/GAPDH ratio after subtraction of background radioactivity hybridized to pUC19. The control value is arbitrarily designated as 1.

strated for the first time the inhibition of apo A-I gene expression by phorbol esters which stimulate protein kinase C.

By Northern blot analysis, we examined the apo A-I mRNA levels in Hep G2 cells incubated with 100 nM TPA for various times (Fig. 1A). Data from scanning densitometry showed that TPA had no effect on apo A-I mRNA level before 15 h of treatment, at which time a significant 25% decrease compared to the control value was observed. At 24 h of TPA treatment, apo A-I mRNA level decreased to 50% compared to the control (Fig. 1B). The effect of TPA on apo A-I mRNA level was maximal at 24 h and was still observed at 48 h (not shown). In order to investigate the reversibility of this effect, Hep G2 cells were incubated in the presence of phorbol ester 12-13 dibutyrate (PBU₂) which is rapidly released from its receptor after washing [26]. A 24 h incubation in the presence of PBU₂ also reduced the apo A-I mRNA level (Fig. 2). After washing and incubation for 24 h, the apo A-I mRNA level was restored to the control value. Renewed PBU₂ treatment then decreased the apo A-I mRNA level. The relatively long lag period and slow inhibition kinetics of apo A-I by TPA suggest the involvement of a long-lived protein intermediate, as suggested in other cell systems [27]. In order to determine whether the changes in the steady-state level of apo A-I mRNA were due to changes in the rate of transcription initiation or elongation, run-on assays were performed on Hep G2 cells using nuclei from either control cells or cells treated with TPA for 24 h. Quantification of the autoradiographic data (Fig. 3A) by scanning densitometry after correction for glyceraldehyde-3-phosphate dehydroge-

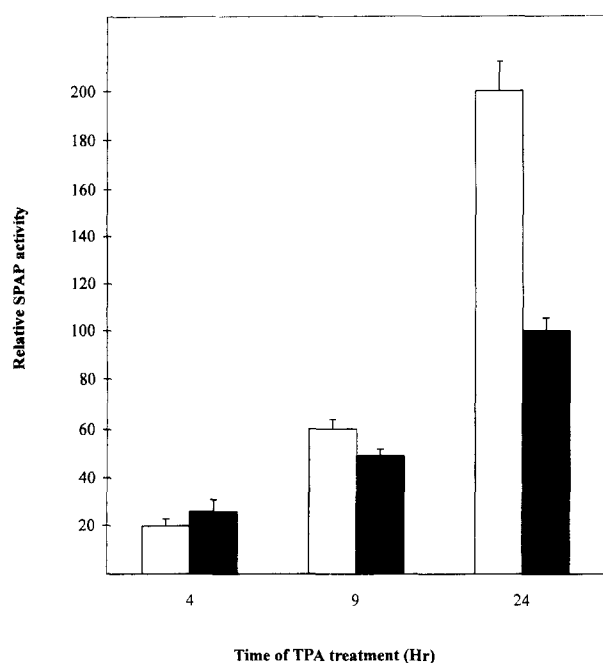
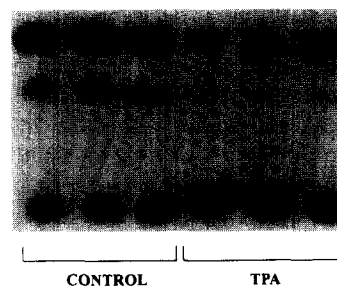


Fig. 4. Time course of TPA action on SPAP activity in Hep G2 cells transfected with apo A-I promoter (–1378/+11)-SPAP reporter gene construct. Hep G2 cells transiently transfected with the apo A-I (–1378/+13)-SPAP construct were trypsinized and seeded in microwell plate. The following day, cells were placed in serum-free medium for 24 h and TPA (100 nM) was added for the times indicated. SPAP activity was determined as described in section 2 and expressed in relation to total cellular protein content. □, control cells; ■, TPA-treated cells. Data represent mean ± S.D. from three experiments performed in quadruplicate.

A.



B.

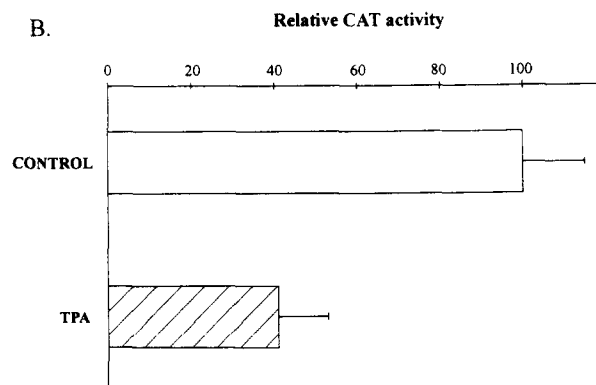


Fig. 5. Effect of TPA on CAT activity in Hep G2 cells transfected with apo A-I promoter (–255/–5)-CAT reporter gene construct. Hep G2 cells transiently transfected with the apo A-I (–253/–4)-CAT construct were grown in serum-free medium for 24 h and treated with or without 100 nM TPA for 24 h. (A) Autoradiogram representing chloramphenicol acetyl transferase (CAT) assays performed in transfected Hep G2 cells. (B) CAT activity was measured as described in section 2 and normalized to the β -galactosidase activity to correct for differences in transfection efficiency. Data represent mean ± S.D. from three separate experiments performed in triplicate. 2AC: 2-acetyl chloramphenicol. 3AC: 3-acetyl chloramphenicol.

nase (GAPDH) gene transcription showed that the apo A-I transcription rate was reduced by one half at 24 h (Fig. 3B). These results therefore indicate that the maximum 2-fold decrease in the rate of apo A-I gene transcription could fully account for the changes in the steady-state level of apo A-I mRNA.

We then investigated the region of the apo A-I gene which responds to TPA by first transfecting Hep G2 cells with the plasmid pA-I-SPAP which contains the –1378 to +11 fragment of the apo A-I promoter fused to the secreted placental alkaline phosphatase (SPAP) reporter gene. Results in Fig. 4 show the absence of any detectable effect of TPA before 9 h of treatment, at which time a slight decrease of SPAP activity of about 15% was observed compared to control cells. At 24 h of TPA treatment, SPAP activity was decreased by 50% of control values, indicating that complete TPA responsiveness was mediated by the –1378 to +11 fragment of the apo A-I promoter. To more precisely delineate the apo A-I promoter region involved in TPA-responsiveness, we transfected Hep G2 cells with the plasmid pEMBL8-CAT which contains the CAT reporter gene under the control of the –253 to –4 region of the apo A-I gene promoter. Results in Fig. 5 show that CAT activity was de-

creased by one half after 24 h of TPA treatment, indicating that TPA-responsive elements were located in the proximal apo A-I gene promoter.

It can be concluded from these data that the nucleotides in the apo A-I promoter responsible for partial inhibition of transcription by TPA are located within the 249 bp segment of DNA between positions –253/–4. The negative effect appears to involve an interaction between a cis-regulatory element and a trans-acting factor(s) whose activity is regulated by TPA-triggered phosphorylation. It is now well established, for a number of regulatory systems, that gene transcriptional responses to TPA can be transduced by AP-1, AP-2 or NF κ B binding sites [17–20]. Inspection of the sequence of the human apo A-I gene promoter [28] between position –253/–4, 5' to the transcription start site revealed a single potential NF κ B binding site located at –22, and two CCCGCCCC and CCCTGCCC motifs located at –228 and –199, respectively, which closely resemble the AP-2 elements [29]. Involvement of AP-2 in TPA-mediated responsiveness can be excluded, since Hep G2 lacks endogenous AP-2 mRNA and protein [30]. Apo A-I gene expression could therefore be modulated by an NF κ B regulatory element, as previously observed for the apo C-III gene promoter [15]. However, although a perfect AP-1 binding site [29] has not been found in the apo A-I promoter, the AP-1 transcription complex may be involved in the effect of TPA via another *cis*-regulatory element, as demonstrated for other genes [31]. Footprinting and gel-retardation analysis should identify the specific DNA sequence(s) and facilitate characterization of the regulatory protein(s) responsible for the effect of TPA on apo A-I gene transcription.

As shown for apolipoproteins E and CIII [15,16], this study demonstrates that TPA modulates apo A-I gene expression at the transcriptional level in the Hep G2 cell line. TPA treatment results in partial inhibition of apo A-I gene transcription and this effect is mediated by elements located in the 5'-flanking region of the apo A-I gene between nucleotides –253 to –4 upstream of the transcription start site. To our knowledge, our report is the first demonstration of regulatory elements within the apo A-I gene that respond to extracellular signals.

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