Calcitriol enhances transcriptional activity of lysozyme and cathepsin D genes in U937 promonocytes

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Calcitriol (1,25-dihydroxyvitamin D₃) has been reported to enhance the rate of synthesis of lysozyme [Rouis, Thomopoulos, Louache, Testa, Heroy & Titeux, (1985) *Exp. Cell Res.* **157**, 539–543] and of cathepsin D [Stein, Braulke, von Figura & Hasilik (1987) *Biol. Chem. Hoppe-Seyler* **368**, 413–418] in human promonocytes U937. In this study we show that the hormone enhances the steady-state levels of cathepsin D and lysozyme mRNAs. The enhancement of the latter but not of the former is strongly inhibited by cycloheximide. The degradation rates of cathepsin D and lysozyme mRNAs as revealed in the presence of actinomycin D are not significantly affected by the treatment of the cells with calcitriol. In nuclei prepared from calcitriol-treated cells, an enhanced rate of synthesis of cathepsin D and lysozyme mRNA precursors is observed. These results suggest that in human promonocytes the rate of the transcription of cathepsin D and lysozyme genes is enhanced in the presence of calcitriol and that the regulation by the hormone of the transcription of lysozyme gene involves a short-lived or an induced protein.

INTRODUCTION

In recent years, certain bone marrow-derived cells have been added to the list of targets of the hormonal control exerted by calcitriol (1,25-dihydroxyvitamin D₃) [1–5]. In these cells calcitriol enhances the phagocytic and chemotactic activities and the activity or the rate of synthesis of several enzymes, including lysozyme [6,7] and cathepsin D [8]. Cathepsin D (EC 3.4.23.5) is a typical lysosomal enzyme. In U937 cells it is synthesized as a precursor of larger molecular mass [9], which is efficiently packaged into lysosomes. In contrast, lysozyme (EC 3.2.1.17) is a predominantly secreted protein. In U937 cells about one-third of lysozyme remains inside the cells [10]. In sucrose-density gradient centrifugation the intracellular lysozyme co-fractionates with β -hexosaminidase indicating lysosomal localization [11].

The mechanism of the regulated expression of cathepsin D and lysozyme in human myeloid cells has not been studied so far. Based on the previous work from other laboratories describing cDNA clones of cathepsin D [12] and lysozyme [13], we used hybridization probes to study the levels, stability and rate of synthesis of mRNAs of lysozyme and cathepsin D in U937 cells. Our results indicate that the treatment of the cells with calcitriol results in an enhanced rate of transcription from both cathepsin D and lysozyme genes.

EXPERIMENTAL

Cell culture

U937 monocytes [14] were cultured in RPMI 1640 medium (Gibco-BRL, Eggenstein, Germany) containing 10% heat-inactivated fetal calf serum.

RNA isolation

Total RNA was isolated from control and treated cells by precipitation with ethanol from guanidinium-HCl extracts [15] and phenol extraction [16]. Poly(A)⁺ RNA was isolated using oligo(dT)-cellulose [16].

Northern blot

Before electrophoresis, RNA was denatured by heating at 65 °C for 10 min in 50% formamide/2.2 M-formaldehyde and electrophoresed in a 1% agarose gel containing 2.2 M-formaldehyde/10 mM-sodium acetate/1 mM-EDTA/40 mM-sodium morpholinopropane sulphonate, pH 7.0, [16] and transferred to nylon membranes by capillary blotting with 20× SSC overnight (1× SSC = 150 mM-NaCl/15 mM-sodium citrate, pH 7.0).

Hybridization of RNA

Total RNA (8 μ g in 100 μ l of water) was added to 60 μ l of 20× SSC and 40 μ l of 37% formaldehyde and incubated for 15 min at 60 °C [17]. Serial dilutions were made in 10× SSC and aliquots were applied to a nylon membrane.

cDNA probes

The following nucleic acids were used as the hybridization probes. (i) An oligodeoxyribonucleotide corresponding to amino acids 325–336 of human cathepsin D [12] for Northern blot analysis and a full-length human cathepsin D cDNA which was isolated from a commercial human placenta cDNA λ gt11 library (Clontech, Palo Alto, CA, U.S.A.). This library has been screened with the above oligonucleotide and positive clones were re-screened with an oligonucleotide corresponding to amino

Abbreviations used: SMH, 0.32 M-sucrose/3 mM-MgCl₂/1 mM-Hepes, pH 7.5; SSC, 150 mM-NaCl/15 mM-sodium citrate, pH 7.0; SSPE, 150 mM-NaCl/10 mM-NaH₂PO₄/1 mM-EDTA, pH 7.4

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acids -64 to -59 of human cathepsin D [12]. The oligonucleotides were synthesized in a Gene Assembler (Pharmacia LKB, Freiburg, Germany). The clone selected for further work was 1.99 kb long. Its 5'- and 3'-ends were sequenced [18,19] and found to correspond to nucleotides 46 and 2036 of the cathepsin D clone pHKCD45 [12]. (ii) *Hind*III-*Bam*HI insert of the plasmid pHL14-23 [13] coding for human lysozyme. (iii) A 3.6 kb *Hind*III fragment of the human β -actin-related pseudogene H β Ac-42 [20].

Radioactive labelling and hybridization

The oligonucleotide was labelled with [γ - 32 P]ATP using polynucleotide kinase [16]. The hybridization was performed in $6 \times$ SSC/0.1% SDS/0.05% sodium phosphate/1 $\times$ Denhardt's solution [16]/250 μ g of sonicated salmon sperm DNA per ml/50% formamide at 42 °C for 16–20 h and washed with $6 \times$ SSC/0.1% SDS twice at room temperature for 15 min and four times at 65 °C for 30 min each.

The cDNA probes for actin, cathepsin D and lysozyme mRNAs were labelled by 'random-primed' polymerase reaction [21] and hybridization was performed in $6 \times$ SSC/0.1% SDS/5 $\times$ Denhardt's solution/100 μ g of sonicated salmon sperm DNA per ml/50% formamide at 42 °C for 16–20 h and washed twice in $2 \times$ SSC/0.1% SDS at room temperature for 15 min, once in $2 \times$ SSC/0.1% SDS at 65 °C for 30 min and once in $0.2 \times$ SSC/0.1% SDS at 65 °C for 30 min. The probes were used at a radioactivity concentration of 1×10^5 c.p.m./ml. Prior to rehybridization the filters were placed in 1 litre of a solution containing $0.1 \times$ SSPE (1 $\times$ SSPE = 150 mM-NaCl/10 mM-NaH₂PO₄/1 mM-EDTA)/0.1% SDS at 100 °C for 2–4 min. The solution was allowed to cool slowly to room temperature and the filter was removed for further hybridization [22].

Preparation of nuclei

Nuclei were purified by a slightly modified method of Tata [23]. U937 cells were washed twice with ice-cold phosphate-buffered saline and resuspended ($\sim 3 \times 10^7$ cells/ml) in ice-cold SMH-buffer (0.32 M-sucrose/3 mM-MgCl₂/1 mM-Hepes). Then, 0.75 vol. of SMH containing 0.1% Triton X-100 was added and the mixture was stirred on a vortex and diluted with cold water to 0.28 M-sucrose. The sample was layered over 5 ml of SMH and centrifuged at 2500 *g* for 10 min at 4 °C. The pellet was resuspended in SMH and recentrifuged at 2500 *g* for 10 min at 4 °C. The nuclear pellet was suspended in 40% glycerol/5 mM-MgCl₂/50 mM-Tris/HCl, pH 8.0/0.1 mM-EDTA at 2×10^7 nuclei/50 μ l and stored at -70 °C.

'Run-on' transcription and RNA hybridization

The experiments were performed by modifications of the procedure of Groudine *et al.* [24]. In a total volume of 100 μ l, 2×10^7 nuclei were incubated with 60 μ Ci of [α - 32 P]UTP (800 Ci/mmol; NEN, DuPont de Nemours, Dreieich, Germany) in the presence of 20% glycerol/5 mM-MgCl₂/25 mM-Tris/HCl, pH 8.0/0.1 mM-EDTA/70 mM-KCl/2.5 mM-dithiothreitol/10 mM-aurointricarboxylic acid/0.4 mM each of ATP, GTP and CTP for 15 min at 26 °C. The radioactively labelled RNA was purified by a treatment with DNAase I, proteinase K digestion, phenol/CHCl₃ extraction, trichloroacetic acid precipitation, a second DNAase I and proteinase

K digestion, phenol extraction and ethanol precipitation. The pellet was dissolved in 10 mM-Tris/HCl, pH 8.0/1 mM-EDTA/0.2 M-NaCl.

The double-stranded probes were denatured by boiling for 10 min in $0.1 \times$ SSC. After denaturation the DNA was adjusted to $6 \times$ SSC and aliquots of 0.1 μ g were applied to a prewashed nitrocellulose filter in a slot apparatus. The filters were washed with $6 \times$ SSC for 5 min, dried and incubated at 80 °C for 2–4 h [25]. The filters were prehybridized in $5 \times$ SSPE/0.1% SDS/1 $\times$ Denhardt's solution/250 μ g of salmon sperm DNA per ml/50% formamide for 16–20 h at 42 °C. Then the filters were hybridized with 1×10^6 c.p.m. of radiolabelled RNA (denatured for 5 min at 65 °C) in the prehybridization buffer at 42 °C for 5 days. The filters were washed twice in $2 \times$ SSPE/0.1% SDS for 30 min at 42 °C and twice in $0.1 \times$ SSPE/0.1% SDS for 30 min at 65 °C.

RESULTS

Effect of calcitriol on the level of lysozyme and cathepsin D mRNA in U937 cells

We have determined the size of mRNAs hybridizing with our probes in a Northern blot (Fig. 1). In poly(A)⁺ RNA from control U937 cells, lysozyme cDNA recognized an approx. 1.6 kb species. The oligonucleotide probe for cathepsin D and β -actin pseudogene DNA hybridized mainly to 2.1 kb and 2.0 kb RNAs respectively. These sizes were in a good agreement with those estimated previously by other workers [12,26–28]. The three probes recognized hardly any RNA in the break-through fraction from oligo(dT)-cellulose chromatography of the same RNA. Poly(A)⁺ RNA that was prepared from calcitriol-treated cells hybridized several-fold stronger with probes for lysozyme and cathepsin D mRNAs than that from control cells. These were selec-

Fig. 1. Northern blot of poly(A)⁺ RNA from control and calcitriol-treated U937 cells

The cells were incubated with 10^{-7} M-calcitriol for 3 days. Poly(A)⁺ RNA (3 μ g) from control and treated cells were hybridized sequentially to lysozyme, cathepsin D and actin probes. As a control, 10 μ g of a poly(A)⁻ RNA from untreated cells was used. The position of ribosomal RNA standards, which were separated in the same gel and stained with ethidium bromide, are indicated.

Table 1. Concentrations of cathepsin D and lysozyme mRNAs in U937 cells treated with 10^{-7} M-calcitriol

Total RNA was isolated from cells treated for 0, 2, 6 and 10 h (first experiment) and for 0, 10, 14, 18, 22, 36 and 46 h (second experiment). Aliquots of 0.5 and 1 μ g of RNA were applied to nylon filters and hybridized sequentially with lysozyme and cathepsin D cDNAs and β -actin pseudogene fragment. The intensity of hybridization was determined by densitometry. Ratios were calculated based on the concentration of actin mRNA. The ratios were normalized to 1 for RNA from untreated cells and means and S.D. of the population were calculated.

Preincubation with calcitriol (h)	Relative hybridization intensity	
	Lysozyme	Cathepsin D
0	1.0	1.0
2	1.4 ± 0.2	1.4 ± 0.2
6	1.6 ± 0.1	2.0 ± 0.1
10	3.3 ± 1.8	2.3 ± 0.2
14	8.3 ± 4.1	3.5 ± 0.8
18	10.8 ± 5.1	3.8 ± 0.6
22	11.7 ± 6.3	4.7 ± 0.9
36	10.1 ± 6.2	5.3 ± 1.2
46	10.2 ± 6.5	6.1 ± 1.0

Calcitriol	—	—	—	+	+
Actinomycin D (d)	0	1	2	0	1
Cathepsin D					
	1.0	0.57	0.34	5.8	4.6
Lysozyme					
	1.0	0.81	0.34	3.1	2.7
Actin					
	1.0	0.41	0.12	1.3	0.35

Fig. 2. Stability of cathepsin D, lysozyme and actin mRNAs

Control and calcitriol-treated (10^{-7} M, 72 h) U937 cells were incubated for up to 2 days (d) in the presence of 5 μ g of actinomycin D/ml. Total RNA was isolated and 1 μ g aliquots were hybridized sequentially to cathepsin D, lysozyme and actin probes. Results of three hybridizations were quantified by densitometric scanning of films and the average relative changes in the hybridization intensities are given under each slot.

tively affected by calcitriol, because the hormone did not change the concentration of actin mRNA. It has been observed earlier that calcitriol does not change the level of actin mRNA in HL-60 cells [29,30].

Calcitriol (h)	0	14	36	72
Lysozyme				
Cathepsin D				
Actin				
Lysozyme	1	2.5	3.1	2.4
Cathepsin D	1	7.7	8.7	5.0
Actin	1	1	1	1

Fig. 3. 'Run-on' synthesis of lysozyme, cathepsin D and actin mRNA precursors and its dependence on treatment of cells with calcitriol

U937 cells were treated for the indicated periods of time with 10^{-7} M-calcitriol. Nuclei were isolated and RNA was labelled in the presence of [α - 32 P]UTP. The labelled RNA was hybridized to DNA probes on nitrocellulose filters. In this experiment the hybridization signal with the actin probe was relatively weak. Presumably, the concentration of the actin DNA probe was less than the nominal 100 ng. No significant background signals were observed with pUC18 DNA as a probe (results not shown). The hybridization signals with lysozyme, cathepsin D and actin probes were quantified by densitometric scanning. For lysozyme and cathepsin D mRNA the hybridization signals referring to untreated cells were set to 1 and the relative increases were corrected for the changes in the hybridization signals obtained with the actin probe.

We isolated RNA from U937 cells that were cultured for up to 46 h in the presence of 10^{-7} M-calcitriol. The RNA was subjected to hybridization sequentially with lysozyme, cathepsin D and actin cDNA probes, and the concentrations of the RNAs were determined by densitometry. The concentration of actin mRNA did not change systematically and was taken as the reference. The relative concentration of lysozyme mRNA increased up to 11-fold and that of cathepsin D up to 6-fold (Table 1). After a slow increase in the course of 10 h, the half-maximum increase in the levels of both lysozyme and cathepsin D mRNAs was observed between 10 and 14 h. There was a considerable variation in the increase in the levels of the two mRNAs in different experiments. Also, the response to calcitriol shown in Fig. 1 seemed to be much greater than in Table 1. One of the reasons for this difference could be the purity of RNA, because in the first experiment poly(A)⁺ RNA was used, while in other experiments total RNA was hybridized and might have caused a higher background signal.

Stability of cathepsin D and lysozyme mRNAs in control and calcitriol-treated cells

RNA was isolated from cells which were harvested before or after 24 or 48 h of culturing in the presence of actinomycin D. Two such experiments were performed and in both the yield of RNA from cells that were pretreated with calcitriol and cultured in the presence of actinomycin D for 48 h was an order of magnitude less

Fig. 4. Effects of cycloheximide on the increase in the level of cathepsin D and lysozyme mRNAs in cells treated with calcitriol

U937 cells were cultured in the presence or absence of 10^{-7} M-calcitriol and $10 \mu\text{g}$ of cycloheximide/ml for 1 day (d). In experiment (a), $2 \mu\text{g}$ aliquots of total RNA were applied to two filters. One of them was hybridized sequentially with probes for lysozyme and actin mRNA and the other one with probes for cathepsin D and actin mRNA. The hybridization signals with the actin probe on the two filters were indistinguishable from each other. In experiment (b), $0.5 \mu\text{g}$ aliquots of mRNA on one filter were hybridized sequentially with all three probes. The numbers in the lower part of the figure represent the relative changes in the intensity of the hybridization signals with lysozyme and cathepsin D probes that were corrected for changes observed with the actin probe.

than in the other samples. Therefore the hybridization of this RNA was not evaluated. After 24 h of incubation of the cells with actinomycin D, the level of cathepsin D and lysozyme mRNAs decreased by less than 35%. In calcitriol-treated cells, the concentration of these mRNAs was higher than in control, but the decrements that were seen after 24 h of treatment with actinomycin D were similar to those in controls. An unexpected finding was the higher degradation rate of actin mRNA as compared with cathepsin D and lysozyme mRNAs (Fig. 2).

Rate of transcription from lysozyme and cathepsin D genes in nuclei isolated from control and calcitriol-treated cells

The transcription rate was examined in 'run-on' experiments. The run-on assay was performed with nuclei that were isolated from cells cultured up to 72 h in the presence of 10^{-7} M-calcitriol. Calcitriol caused a several-fold increase in the transcriptional activity of lysozyme and cathepsin D genes without a major change in the transcription from the actin genes (Fig. 3). Altogether three experiments with the same design were performed. The pattern of the transcriptional activity from cathepsin D and actin genes was very similar in all of them. However, as compared with the data of Fig. 3, the stimulation of the transcription from the lysozyme gene was either less pronounced or delayed (results not shown).

Effect of cycloheximide on the augmentation of lysozyme and cathepsin D mRNAs in calcitriol-treated cells

In experiments with calcitriol-treated cells, we observed that in the absence of protein synthesis the increase in the level of lysozyme cDNA was strongly inhibited. In contrast, the increase in the level of cathepsin D mRNA was rather insensitive to cycloheximide (Fig. 4). This experiment was repeated twice. In none of the experiments did the inhibition of the induction of cathepsin D mRNA exceed 20%.

DISCUSSION

Calcitriol has been shown to enhance the activity of lysozyme in U937 cells [6,7]. Oestrogens have been reported to control the expression of the lysozyme gene in hen oviduct [31] and of the cathepsin D gene in human breast cancer cells [26,27,32]. After having observed that calcitriol enhances the synthesis of cathepsin D in U937 cells [8], we performed a preliminary experiment to see if a similar effect is exerted by steroids. We have treated U937 cells with 10^{-7} M-calcitriol, 17β -oestradiol, dexamethasone or testosterone. The synthesis of cathepsin D and lysozyme was accelerated only in the presence of calcitriol (results not shown). Calcitriol inhibited the multiplication of U937 cells by approx. 10%, but the steroids did not. We concluded that the observed changes in the enzyme expression were not due to a cross-reaction of the hormone with a receptor for any of the steroids tested.

We decided to study the mechanism of this increase in the rate of synthesis of lysozyme and of cathepsin D. Our results show that, firstly, this increase is correlated with a several-fold enhancement in the levels of mRNAs of lysozyme and cathepsin D. Secondly, the increase is rather slow, reaching half-maximum between 10 and 14 h. Thirdly, the treatment of the cells has little effect of the stability of these mRNAs. Fourthly, the treatment results in an enhancement in the rate of the synthesis of the precursors of both lysozyme and cathepsin D mRNAs. Our data indicate that calcitriol controls the rate of the transcription from cathepsin D and lysozyme genes in U937 cells. We have not examined whether calcitriol affects other levels of expression such as the processing of the precursor RNAs or the translational yield.

The enhancements in the expression of lysozyme and cathepsin D due to calcitriol do not seem to be tightly coupled to each other and may follow different regulatory pathways. We have observed a considerable variation in the relative levels of the two mRNAs (results not shown). Further, the responses of the concentrations of these RNAs to calcitriol were distinguished in their sensitivity to cycloheximide (Fig. 4). The expression of the lysozyme gene in macrophages is controlled by several positive and negative elements [33]. In U937 cells the increase in the level of lysozyme mRNA was strongly dependent on continued protein synthesis, and this may indicate that calcitriol regulates the expression of lysozyme indirectly. In contrast with lysozyme mRNA, the strong enhancement in the level of cathepsin D mRNA was manifested even in the presence of cycloheximide. This situation is similar to the regulation of the cathepsin D gene expression by oestrogens in human breast cancer cells; like calcitriol in U937 cells, in MCF₇ cells the oestrogens

enhance the rate of transcription and this effect is insensitive to cycloheximide [26]. The present results leave open the possibility that cathepsin D gene is directly regulated by a calcitriol receptor. It will be of interest to study the regulatory sequences of the human cathepsin D gene and to examine them for the presence of a calcitriol receptor responsive element by isolating and characterizing cathepsin D genomic clones.

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