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KINETICS OF NUCLEAR TRANSLOCATION AND TURNOVER OF THE VITAMIN D RECEPTOR IN HUMAN HL60 LEUKEMIA CELLS AND PERIPHERAL BLOOD LYMPHOCYTES—COINCIDENT RISE OF DNA-RELAXING ACTIVITY IN NUCLEAR EXTRACTS*

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Summary—High affinity receptors (VDR) for 1,25-dihydroxycholecalciferol (calcitriol) are expressed in HL60 human leukemia cells and in low numbers in peripheral blood lymphocytes (PBL). HL60 cells, expressing some characteristics of promyelocytes, can be induced to monocytoid differentiation by calcitriol. Specific nuclear translocation of [^{3}H]calcitriol/VDR was examined after exposure of whole cells to 10^{-9} M/l calcitriol in the presence and absence of a 500-fold excess of unlabeled ligand and subsequent isolation of nuclei. Specific nuclear translocation of [^{3}H]calcitriol/VDR was found to be time dependent reaching a maximum of ~2100 binding sites/nucleus after 3 h of incubation in HL60 cells, whereas a maximum of ~310 binding sites/nucleus was found after 3 h in PBL. Pulse exposure of HL60 to radiolabeled hormone for 3 h followed by culture in medium without serum and calcitriol lead to nuclear retention of ~1600 radiolabeled VDR by 8 h and ~1000 VDR by 24 h. Radiolabeled VDR disappeared from the nuclear compartment with a halflife of ~30 min if cells were cultured with identical concentrations of unlabeled hormone after the pulse (pulse/chase-experiments). No difference of VDR retention in pulse and pulse/chase-experiments was seen in PBL, where VDR halflife was ~30 min. No specific translocation into the nuclear compartment was seen when isolated nuclei were incubated in [^{3}H]calcitriol. Radiolabeled hormone/receptor complexes of nuclei isolated from cells exposed for 3 h to radiolabeled hormone—in contrast to identical experiments with intact cells—did not disappear from the nuclear compartment upon incubation of nuclei with identical concentrations of the unlabeled compound. The activity of DNA relaxing enzymes (e.g. topoisomerases I and II) in nuclear extracts was measured using a PBR 322-relaxation-assay. Enhanced overall enzyme activity was found in nuclear extracts by 1 h after incubation with calcitriol (final ethanol concentration 0.0001% v/v) in HL60 and PBL. The enhanced activity disappeared after 2 h in PBL, whereas it was still enhanced by 4 h in HL60. No effect was seen in ethanol treated controls. We conclude that a specific nuclear translocation mechanism exists for calcitriol in both cell types examined, most likely due to translocation of receptor proteins after hormone binding. Translocated hormone/receptor complexes compete for a limited number of specific nuclear binding sites. Enhanced activity of topoisomerases in nuclear extracts upon translocation of VDR might reflect interaction of both within the nuclear compartment, thus initiating DNA-unwinding, a prerequisite of transcription initiation.

INTRODUCTION

During the last decade all receptor proteins for classical steroid hormones have been cloned and sequenzed. Besides the receptors for sex steroids and gluco- and mineralocorticoid steroids the receptors for vitamin A and D and thyroid hormone as well as the oncogen V *erb* B proved to belong to the family. In addition several "orphan receptors" without known ligand and function have been cloned [1–4]. Most recently a new member of the family was described to be activated by peroxisome proliferators [5]. Functional dissection of receptor proteins revealed considerable similarity of the organization of functional domains and high homology of conserved amino acid sequences like the DNA-binding domains. Immunochemical

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analysis showed preferential nuclear localization of receptor proteins in the absence and presence of hormones [6–8]. Domains for nuclear translocation have been characterized in several molecules allowing to block hormone-mediated nuclear translocation by site-directed mutagenesis or deletion experiments. It is generally accepted that the glucocorticoid receptor is localized in the cytoplasm and is translocated to the nucleus upon hormone binding. Similar events have been shown for the estrogen receptor protein as well as for the progesterone receptor, requiring further experiments [9–12].

Binding of dimerized and translocated hormone/receptor complexes to highly specific hormone responsive elements is a well characterized event *in vitro* in cell free systems [9, 13]. Cooperation between DNA-binding proteins, structural nuclear proteins and DNA/RNA-modifying enzymes is being investigated [14, 18]. Transcription initiation after binding of a transcription factor among other nuclear events requires DNA-relaxation to facilitate accessibility for other DNA-modifying enzymes and other DNA-binding proteins. The unwinding of a supercoiled defined DNA, pBR 322, can be monitored in a functional assay to characterize DNA-relaxing activity in nuclear extracts [19]. The interactions between such modifying enzymes and ligand-induced transcription factors may be important to characterize the mechanisms of cooperation during the process of transcription initiation.

Calcitriol induces monocytoid differentiation in human HL60 leukemia cells [20, 21]. No dramatic effects are reported for calcitriol in resting peripheral blood lymphocytes (PBL). The induction of a differentiation program requires considerable modification of nucleic acids. HL60 cells can therefore be looked upon as target cells for calcitriol. Recent work has described the necessity of high numbers of glucocorticoid receptors and specific responsive elements to induce transcription/translation and lympholysis in lymphoblasts [22]. Nuclear retention and turnover of hormone/receptor complexes, possibly representing the presence of high affinity binding sites for the hormone/receptor complexes, has not been extensively studied. Previous work has demonstrated a time course of nuclear translocation of calcitriol in monocytes and a breast cancer cell line [23, 24]. The mechanism of translocation of steroid hormones to the nucleus as well as the turnover and further processing of steroid hormone receptors after termination of nuclear binding remain to be investigated, while protein import into the cell nucleus in general has been studied extensively [25].

We now report the kinetics of nuclear translocation and turnover of the vitamin D receptor in HL60 and PBL. The amount of DNA-relaxing activity in nuclear extracts following hormone treatment and nuclear binding of receptors is shown by relaxation of pBR 322 in a DNA-relaxation-assay.

EXPERIMENTAL

Reagents

[^{3}H]1,25-Dihydroxycholecalciferol ([^{3}H]calcitriol) (160 Ci/mmol) was from Amersham (Braunschweig), the unlabeled compound was a gift from Dr Calcanis, Hoffmann-La Roche, (Grenzach-Whylen).

HL60 cells were from ATTC. PBLs were from buffy coats from healthy donors purified by Ficoll gradient preparation. pBR 322 was from Boehringer (Mannheim).

Preparation of nuclei

Cells were washed and resuspended in ice cold STM-buffer (0.05 M Tris, pH 7.4, 0.25 M sucrose, 0.005 M $MgSO_4$) for 15 min. This suspension was made 1% v/v Triton X 100 and incubation continued for 15 min at 4°C under overhead tumbling. Nuclei were pelleted for scintillation counting (1800 rpm, 10 min, 4°C). For one single point of analysis a minimum of 1×10^6 cells was used, usual amounts were 3×10^6 cells/sample. Nuclei were prepared in triplicates where one was used for counting in a Rosenthal chamber.

Nuclear translocation and pulse/chase-experiments

Calcitriol was dissolved in ethanol and stored at −70°C. Secosteroids were transferred into glass tubes, which were vacuum dried shortly before use. Harvested cells were washed twice with phosphate buffered saline (PBS) pH 7.4 and consecutively incubated in RPMI medium without fetal calf serum (FCS) at 37°C in glass tubes with gentle shaking. Final concentration of [^{3}H]calcitriol was 1×10^{-9} M in the absence or presence of a 500-fold excess of unlabeled hormone for various times.

For pulse experiments cells were incubated in the presence of [^{3}H]calcitriol for 3 h in RPMI

without FCS, washed twice in PBS, resuspended in RPMI and cultured at 37°C until analyzed. In pulse/chase experiments cells were treated as above and subsequently cultured in the presence of identical concentrations of unlabeled hormone until analyzed.

Radioactivity was counted in a liquid scintillation counter. Binding sites were calculated after correction for unspecific binding.

For DNA-relaxation experiments HL60 in their logarithmic proliferation phase or freshly isolated PBL were incubated in unlabeled calcitriol 10^{-8} M. Identical amounts of ethanol (final concentration 0.0001% v/v) with and without calcitriol were directly pipetted into the culture medium (RPMI, 5% FCS).

Control experiments with isolated nuclei

Nuclei were isolated from HL 60 cells, resuspended in STM-buffer 0.1% Triton X 100 in the presence of 1×10^{-9} M [^{3}H]calcitriol with and without a 500-fold excess of the unlabeled compound. Samples were drawn from the suspension at different times, washed twice in PBS and subjected to scintillation counting.

Nuclei were prepared from batches of cells, which had been exposed to [^{3}H]calcitriol 1×10^{-9} M for 3 h. Nuclei were resuspended in STM-buffer 0.1% Triton X 100 with and without 1×10^{-9} M calcitriol and incubated at 37°C under gentle shaking. Samples were drawn every 1 h up to 3 h, nuclei were pelleted and subjected to scintillation counting.

DNA-relaxation-assay

Relaxation of pBR 322 was measured as described [19]. Briefly, nuclei were prepared as above, counted and equal numbers resuspended in extraction buffer [0.005 M potassium phosphate, pH 7.5, 0.1 M NaCl, 0.01 M 2-mercaptoethanol, 0.0005 M phenylmethylsulfonyl fluoride (PMSF)]. The suspension was made 0.35 M NaCl by the addition of 5 M NaCl, gently mixed, incubated on ice for 60 min and centrifuged at 1000 *g* for 10 min, at 4°C. 5 μl aliquots of the supernatant in different dilutions were mixed with 5 μl reaction buffer, (0.1 M Tris–HCl, pH 7.5, 0.2 M KCl, 0.001 M EDTA, 0.3 mg/ml bovine serum albumin) and 200 ng pBR 322 plasmid DNA. The incubation solution was made 0.01 M $MgCl_2$ and 0.01 M ATP.

Incubation at 37°C for 60 min was followed by electrophoresis in 1% agarose (25 V, 16 h, room temperature) and subsequent visualization by ethidium bromide staining.

RESULTS

In HL60 cells nuclear transfer of [^{3}H]calcitriol/VDR was seen reaching a maximum of ~2100 binding sites/nucleus at 3 h, slightly declining thereafter. In PBL transfer of a lower number of molecules took place reaching a maximum of ~310 binding sites/nucleus after 3 h (Fig. 1). All numbers are corrected for unspecific binding. Unspecific radioactivity did not exceed 10% of the total radioactivity.

Pulse experiments showed a low turnover of VDR in the nucleus when cells were cultured in the absence of hormone after a 3 h pulse. When cultured under restrained conditions (in 200 μl/sample RPMI medium without FCS under standard cell culture conditions) for 8 and 24 h the still viable cells were found to contain approx. 1600 and 1000 binding sites/nucleus, respectively. Upon addition of unlabeled hormone after the 3 h pulse a rapid decline of radiolabeled nuclear VDR was seen with a halflife of approx. 30 min only, exponentially declining to 0 by 3 h (Fig. 2).

PBL subjected to the same procedure showed a rapid turnover of nuclear VDR no matter whether cultured in the presence or absence of unlabeled hormone (Fig. 3).

Viability of cells after exposure to hormone and after culture in serum-free RPMI medium was >95% as judged by trypan blue exclusion. Purity of the nuclear preparation, judged by counting nuclei, cell detritus and intact cells in a Rosenthal chamber, was >90% in all experiments.

When freshly isolated nuclei of HL60 or PBL were incubated with [^{3}H]calcitriol (+unlabeled hormone) no significant specific binding was

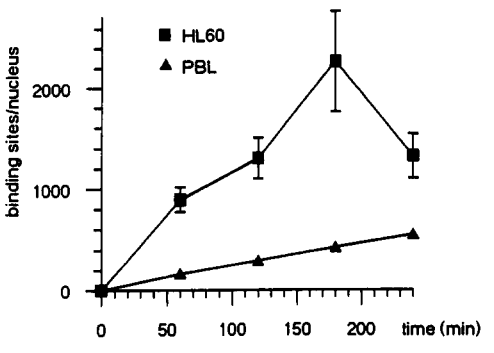

Fig. 1. Translocation kinetics of [^{3}H]calcitriol/VDR to nuclei of HL60 and PBL. Numbers represent binding sites/nucleus corrected for unspecific binding. Numbers are given as the mean of 3 (PBL) and 6 (HL60) independent experiments and standard deviation of the mean.

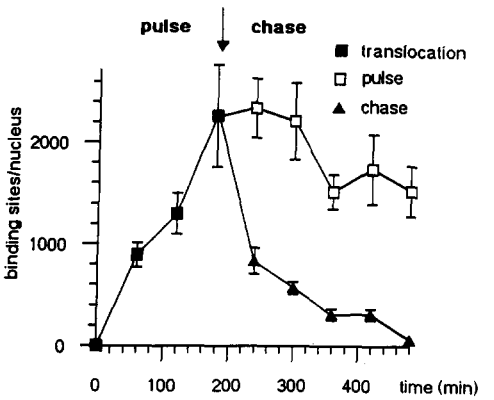

Fig. 2. Nuclear turnover of [^{3}H]calcitriol/VDR in HL60 in pulse- and pulse/chase-experiments. Binding sites are corrected for unspecific binding. Numbers represent the mean of three independent experiments. Cells were incubated in the presence of [^{3}H]calcitriol 1×10^{-9} M (pulse) for 3 h, washed twice and incubated in the presence (chase) and absence of 1×10^{-9} M unlabeled calcitriol. Radiolabeled hormone/VDR complexes very slowly disappear from the nuclear compartment in contrast to chase-experiments, where VDR halflife is ~30 min.

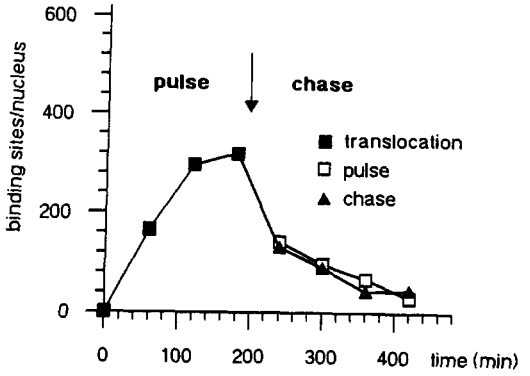

Fig. 3. Nuclear turnover of [^{3}H]calcitriol/VDR in PBL in pulse- and pulse/chase-experiments. Numbers represent the mean of three independent experiments and are corrected for unspecific binding. There is no difference between pulse and pulse/chase conditions in contrast to HL60 cells.

obtained. Nuclei of HL60 cells prepared after 3 h exposure of whole cells to radiolabeled hormone and resuspended in STM-buffer did not release significant amounts of radioactivity over a time period of 3 h in the absence and in the presence of identical concentrations of unlabeled hormone. Further observations were limited by the viability of isolated nuclei.

Overall extractable activity of DNA-relaxing enzymes measured by the decrease of the supercoiled form of pBR showed enhanced activity by 1 h after exposure of cells to the hormone in HL60 and PBL (Figs 4 and 5). No change of random activity was seen in control dishes, where ethanol was applied to cell cultures. After 60 min of culture in the presence of 1×10^{-8} M calcitriol a rise of DNA-relaxing activity in nuclear extracts could be observed, which lasted for more than 4 h in HL60 and vanished after 2 h in PBL. For controls pBR 322 alone (−control) and an undiluted extract of nuclei of untreated cells (+control) were monitored.

DISCUSSION

Cytoplasmic localization of hormone receptors and their nuclear translocation upon ligand

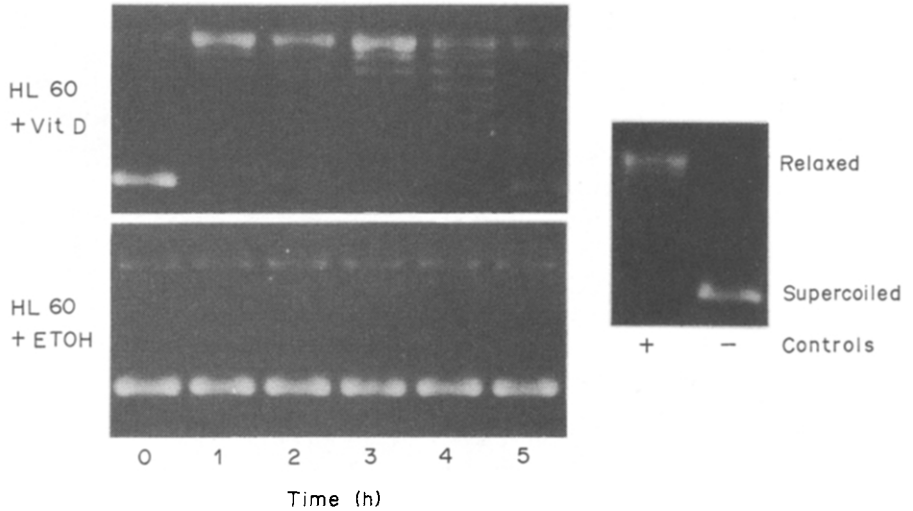

Fig. 4. DNA-relaxing activity in nuclei of HL60 after exposure to calcitriol and ethanol as controls. Extracts from 0.9×10^5 cells are applied to one lane. Controls with pBR 322 alone (negative control) and with the undiluted nuclear extract (representing extract from 3.5×10^5 cells) of untreated cells (positive control) are shown as examples for the localization of relaxed and supercoiled DNA in the gel. DNA relaxing activity dramatically rises in nuclear extracts by 1 h after the addition of calcitriol. There is no effect if cells are treated with the solvent of secosteroids, e.g. 0.0001% v/v final concentration of ethanol. DNA-relaxing activity gradually decreases from 3 to 5 h but does not reach baseline.

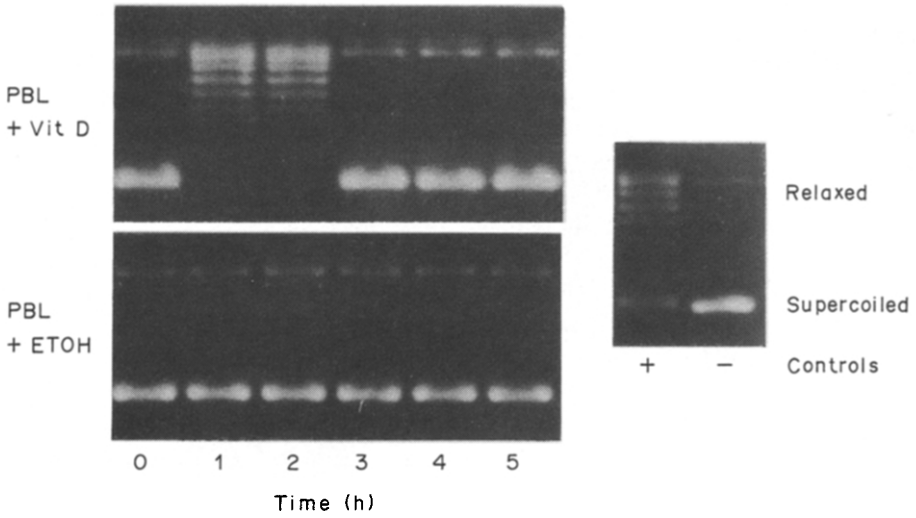

Fig. 5. DNA-relaxing activity in PBL after exposure to calcitriol and ethanol as controls. Extracts from 0.9×10^5 nuclei are applied to one lane, extract from 3.5×10^5 nuclei for +control. DNA-relaxing activity increases by 1 h after the addition of calcitriol. pBR 322 is not completely relaxed as shown in HL60 cells in Fig. 4, baseline level is reached by 3 h.

binding and dimerization is a matter of discussion and research. The present state of discussion favors cytoplasmic and nuclear localization of steroid hormone receptors in the absence of hormone. Functional domains apparently mediate nuclear translocation, which can be blocked in site-directed mutagenesis or deletion experiments. Clearly the translocation is enhanced in the presence of hormone [1, 6–8, 10–12]. In our experiments the translocation of radiolabeled hormone to the nucleus can be competed by an excess of the unlabeled compound. Moreover, isolated nuclei do not accumulate radioactive hormone. From these data we conclude that a pool of vitamin D receptor molecules resides in the cytoplasm and is translocated to the nucleus after hormone binding. The turnover of nuclear radioactivity most likely represents the turnover of hormone/receptor complexes. The difference of VDR halflife in nuclei of HL60 cells and PBL after a 3 h hormone pulse may reflect the difference of affinity and/or accessibility of nuclear binding sites for the hormone/receptor complex. The number of (accessible?) high affinity nuclear binding sites for the hormone/receptor complex is limited in a single nucleus. Molecular events however, which cause the complexes to leave the nucleus after having competed for the responsive element, remain to be elucidated. Constant turnover of receptors might cause repetitive signals necessary for induction of major nuclear events, which is supported by the finding that sufficient amounts of receptors are required for induction of transcription/translation [22].

Relaxation of DNA is one prerequisite for DNA modification. Transcription initiation requires DNA unwinding. Interactions and cooperation between ligand-induced transcription factors, the nuclear matrix and proteins involved in DNA-modification and gene regulation are being investigated [14–18, 26]. In proliferating cells there is always a basal activity of topoisomerases, which should be unchanged under constant conditions of the environment, although dependent on the cell cycle of the individual cell [18, 26]. In non-synchronized cell cultures there is a mean random activity if cells are in their logarithmic phase of proliferation. Changing a single relevant parameter should cause an increase of the mean activity. It has been shown that the addition of calcitriol induces monocytoid differentiation in HL60 cells [21]. We show that the amount of DNA-relaxing activity extracted/nucleus is enhanced after exposure to calcitriol and is time-coincident with the nuclear translocation of hormone/receptor complexes. This rise may be due to changes in extractability of the enzymes, enzyme liberation by binding of receptors or direct enzyme activation. The possibility of enzyme activation by the secosteroid itself cannot be excluded. Although relatively unspecific, the relaxation assay applied mainly represents ATP-dependent topoisomerase II-activity. Further characterization of the enzymes involved is in progress.

Our experiments present a whole cell model to demonstrate cooperation between ligand-induced transcription factors and the machinery of enzymes active in DNA-modification. Other investigators performed experiments on steric interactions with constructs in cell free systems [16, 17]. Test systems specific for single enzymes shall help to further characterize the interactions. The influence of inhibitors of topoisomerase activity on nuclear retention and turnover and on differentiation events remains to be investigated.

In summary we conclude, that in HL60 cells and PBL different amounts of VDR are translocated to the nucleus upon hormone binding. VDR do bind with high affinity to the nuclear matrix in HL60 cells but not in PBL. Hormone/receptor complexes do not accumulate in the nucleus but do show a high turnover in pulse- and pulse/chase-experiments. This indicates the presence or accessibility of a limited number of high affinity nuclear VDR binding sites, possibly hormone responsive elements. The number of the latter is higher in HL60 cells compared to PBL. Coincidentally the activity of DNA-relaxing enzymes in nuclear extracts rises upon VDR translocation, lasting longer in HL60 cells. Further experiments to characterize the interactions of steroid hormone receptors with the nuclear matrix and with DNA-modifying enzymes are in progress.

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