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Interactions of $1,25(OH)_2D_3$ and retinoic acid in the regulation of IEC-6 cell alkaline phosphatase activity

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The goal of the present work was to use IEC-6 cells to investigate the possible mechanisms underlying the regulation of alkaline phosphatase (ALP) activity (ALPA) by 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) and retinoids. Here we demonstrate that the vitamin D analogs, $25(OH)_2$-16-ene-23-yne-D_3 and $1\alpha,24S\text{-}(OH)_2$-22-en-26,27- dehydrovitamin D_3, which have been shown by others to bind to the intracellular vitamin D receptor (VDR), have similar effects to $1,25(OH)_2D_3$ in increasing ALPA of IEC-6 cells. A third vitamin D analog, 25-(OH)-16-ene-23-yne-D_3 (AT), which activates membrane $1,25(OH)_2D_3$ effects, but binds poorly to the intracellular VDR, did not stimulate ALPA of IEC-6 cells. These data suggest that the effects of $1,25(OH)_2D_3$ to increase ALPA are mediated by intracellular VDR rather than by membrane actions of the hormone. The all-trans and 9-*cis* retinoic acids alone each caused increased ALPA of IEC-6 cells without altering steady-state levels of ALP mRNA, suggesting that retinoic acids may regulate ALPA of IEC-6 cells at a post-transcriptional level. Vitamin D analogs which bind intracellular receptors showed synergistic effects with either retinoid to increase ALPA, but there was no interaction with AT. Although the retinoids alone did not alter ALP mRNA levels, addition of $1,25(OH)_2D_3$ in combination with either retinoid increased ALP mRNA more than did $1,25(OH)_2D_3$ alone. These data suggest that the synergistic effects of $1,25(OH)_2D_3$ and retinoids on IEC-6 cell ALPA are mediated by intracellular VDR. The results of these experiments indicate that $1,25(OH)_2D_3$ alters IEC-6 cell ALPA via increased mRNA levels, while retinoids appear to both have post-transcriptional effects and the capacity to interact with $1,25(OH)_2D_3$ in altering ALP mRNA levels.

Keywords: retinoic acid; alkaline phosphatase; vitamin D; post-transcriptional regulation

Introduction

The active metabolite of vitamin D, $1,25(OH)_2D_3$, mediates its biological effects via both genomic and non-genomic pathways (Farach-Carson *et al.*, 1991; Ozono *et al.* 1991; Boland & Nemere, 1992; Norman *et al.*, 1992). The non-genomic pathway for $1,25(OH)_2D_3$ is via the activation of $1,25(OH)_2D_3$-dependent calcium channels (Norman *et al.*, 1992). This pathway has been shown to be responsible for transmembrane calcium influx in ROS cells (Farach-Carson *et al.*, 1991) and for the activation of transcaltachia in chicken intestine (Nemere & Norman, 1987; de Boland *et al.*, 1990; Zhou *et al.* 1992). The genomic pathway for $1,25(OH)_2D_3$ involves binding to intracellular receptors which belong to the steroid/thyroid nuclear receptor superfamily and cause either gene activation or suppression (Ozono *et al.*, 1991).

Several vitamin D analogs have been synthesized which exhibit the ability to discriminate between the nuclear receptor and the membrane receptor activating calcium transport (Norman *et al.*, 1990; Farach-Carson *et al.*, 1991; Zhou *et al.* 1992). Analogs of $1,25(OH)_2D_3$ such as $1,25(OH)_2$-16-ene-23-yne-D_3 (V) and $1\alpha,24S\text{-}(OH)_2$-22-en-26,27- dehydrovitamin D_3 (BT) have been demonstrated to bind to intracellular $1,25(OH)_2D_3$ receptors (VDR) and induce differentiation of myeloid leukemic cells (HL-60) while having little effect on either intestinal calcium absorption or bone mobilization (Norman *et al.*, 1990). Other vitamin D analogs such as 25-(OH)-16-ene-23-yne-D_3 (AT) have been demonstrated to stimulate transmembrane calcium influx, but bind poorly to VDR (Farach-Carson *et al.*, 1991; Zhou *et al.*, 1992). These vitamin D analogs were used in our study to distinguish between the genomic and non-genomic responses of IEC-6 cells to $1,25(OH)_2D_3$.

At least two different nuclear signalling pathways appear to play a role in the genomic actions of $1,25(OH)_2D_3$. Dependent upon the sequence of response elements located on $1,25(OH)_2D_3$-responsive genes, the VDR can bind as either a homodimer or as a heterodimer with retinoid X receptors (RXR) (Kliewer *et al.*, 1992; Carlberg *et al.*, 1993). The presence of an RXR ligand and the nature of the response element determine whether the $1,25(OH)_2D_3$ response is co-regulated by RXR.

The retinoids are known to exert multiple physiological effects in the regulation of vision, embryogenesis, growth, and differentiation of normal or malignant tissues (DeLuca, 1991). They achieve their biological effects by binding to specific intracellular receptors also belonging to the steroid/thyroid nuclear receptor superfamily. A sub-family of retinoic acid receptors has been reported. The effects of all-trans retinoic acid (*t*-RA) are mediated by binding to a family of RA receptors (RARα, RARβ and RARγ) while 9-*cis* retinoic acid (9-*cis* RA) is a ligand for either the RAR or RXR receptors (RXRα, RXRβ and RXRγ) (Hashimoto & Shudo, 1991; Heyman *et al.*, 1992; Mangelsdorf *et al.*, 1993). The retinoic acids play an important role in modulating a variety of cell functions, with tissue specificity and diversity achieved via the multiple receptor subtypes and the ability to form homo- or hetero-dimers with other nuclear receptors. Both RAR and RXR exist in many different types of tissues (DeLuca, 1991; Hashimoto & Shudo, 1991; Leid *et al.* 1993) and transcriptional activity of RAR and RXR can be reciprocally modulated by direct

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interactions between RARs and RXRs (Schräder *et al.*, 1993).

We have recently reported that 1,25(OH)$_2$D$_3$ increased alkaline phosphatase (ALP) activity of IEC-6 cells (Thomas *et al.*, 1993), a non-transformed cell line which shows morphological and immunological characteristics of small intestinal crypt cells (Quaroni *et al.*, 1979). We also characterized the ALP present in the IEC-6 cells as the liver/bone/kidney isoenzyme (Jeng *et al.*, 1994). In the present paper, we demonstrate that co-treatment of IEC-6 cells with *t*-RA or 9-*cis* RA and 1,25(OH)$_2$D$_3$ showed additive to synergistic effects in increasing ALP activity. Most of the work to date concerning the interaction of 1,25(OH)$_2$D$_3$ and the retinoids has been done in transfection systems. Therefore, the objective of the present work was to investigate the mechanism underlying the interaction of retinoic acids and 1,25(OH)$_2$D$_3$ to increase ALP activity of IEC-6 cells using the endogenously expressed receptors in these cells.

Results

To investigate whether the genomic or non-genomic pathway was being utilized by 1,25(OH)$_2$D$_3$ in increasing IEC-6 cell ALP activity, different analogs of 1,25(OH)$_2$D$_3$ exhibiting different abilities to bind to VDR were used (Norman *et al.*, 1990; Farach-Carson *et al.*, 1991; Zhou *et al.*, 1992). As shown in Figure 1, 1,25(OH)$_2$D$_3$ stimulated ALP activity of IEC-6 cells at concentrations from 10^{-8} M to 10^{-7} M. Compounds V and BT, two vitamin D analogs which have been demonstrated to bind to the VDR and induce differentiation of HL-60 cells, also increased ALP activity of IEC-6 cells at concentrations between 10^{-8} M to 10^{-7} M with equal or greater potency than 1,25(OH)$_2$D$_3$. Compound AT, which exhibits high ability to induce transmembrane calcium influx but low nuclear receptor binding, did not significantly increase ALP activity of IEC-6 cells at concentrations between 10^{-10} to 10^{-7} M (Figure 1). These results suggest that the regulation of ALP activity by 1,25(OH)$_2$D$_3$ in IEC-6 cells is probably via binding to the intracellular VDR. Concentrations greater than 10^{-7} M of the vitamin D analogs decreased the viability of IEC-6 cells (data not shown), therefore it was not possible to determine the maximal effects of these analogs.

The data in Figure 2 show that both *t*-RA and 9-*cis* RA were able to stimulate IEC-6 cell ALP activity in a dose-dependent manner. The maximum response was not achieved even when 10^{-6} M *t*-RA or 9-*cis* RA were tested. There was no significant difference in ALP activity between cells treated with *t*-RA or 9-*cis* RA at any concentration used. This result suggests that the effects of both 9-*cis* RA and *t*-RA in the stimulation ALP activity of IEC-6 cells may be via activation of the same retinoic acid receptors. Northern analysis showed that neither 10^{-6} M 9-*cis* RA nor *t*-RA alone caused an increase in mRNA levels of ALP in IEC-6 cells (Figure 3), although the same concentration of 9-*cis* RA and *t*-RA resulted in a four-fold increase in ALP activity (Figure 2). This result suggests that the effects of retinoic acid to increase ALP activity of IEC-6 cells may be due to activation of existing enzyme rather than an increase in synthesis of mRNA.

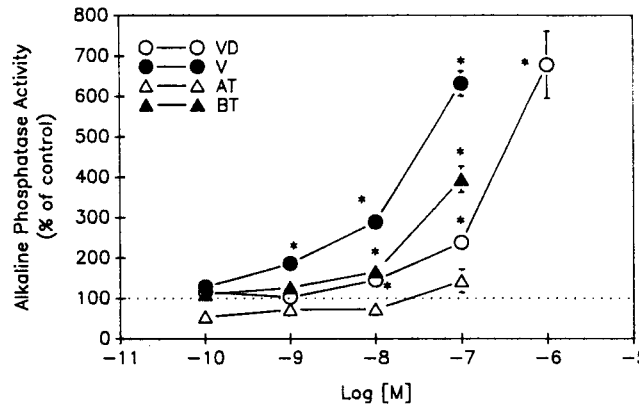

Figure 1 Alkaline phosphatase activity of IEC-6 cells treated with different vitamin D analogs (10^{-10} M to 10^{-7} M) (V,AT and BT) and 1,25(OH)$_2$D$_3$ (10^{-10} M to 10^{-6} M) (VD) for 3 days. Control values for each point were determined in parallel wells treated for the same amount of time in the presence of vehicle (the alkaline phosphatase activity in vehicle treated cells was designated as 100%). Each point represents the mean ± SEM for four wells. *$P<0.05$ compared to control

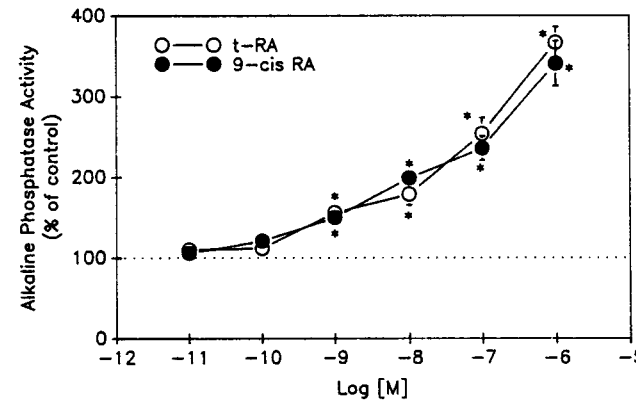

Figure 2 Alkaline phosphatase activity of IEC-6 cells treated with *t*-RA or 9-*cis* RA (10^{-11} M to 10^{-6} M) for 3 days. Control values for each point were determined in parallel wells treated for the same amount of time in the presence of vehicle (the alkaline phosphatase activity in vehicle treated cells was designated as 100%). Each point represents the mean ± SEM for four wells. *$P<0.05$ compared to control

When IEC-6 cells were co-treated with 10^{-8} M 9-*cis* RA or *t*-RA and different vitamin D analogs, we found that both 9-*cis* RA and *t*-RA potentiated the effect of vitamin D analogs dependent on the ability of the analog to bind to the nuclear VDR (Figure 4); when V or BT were added with either 9-*cis* RA or *t*-RA, the effect on ALP activity was even greater than the additive effects of 1,25(OH)$_2$D$_3$ plus the respective retinoic acid analog. Addition of AT with either retinoic acid was not significantly different from AT alone.

Alkaline phosphatase mRNA of IEC-6 cells was measured by quantitative Northern analysis after 48 h treatment with 1,25(OH)$_2$D$_3$ in the absence or presence of 9-*cis* RA or *t*-RA. While 1,25(OH)$_2$D$_3$ alone increased steady-state transcript levels, the presence of either retinoic acid analog resulted in an even greater increase in mRNA levels (Figure 5). These data suggest that retinoic acids may regulate the synthesis of some

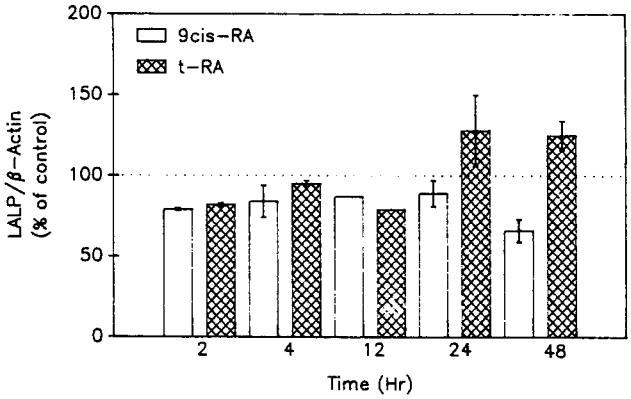

Figure 3 LALP/β-actin mRNA levels determined by Northern analysis in IEC-6 cells treated with 10^{-6} M *t*-RA or 9-*cis* RA for different times. The ratio of LALP/β-actin mRNA in vehicle-treated IEC-6 cells was designated as 100%

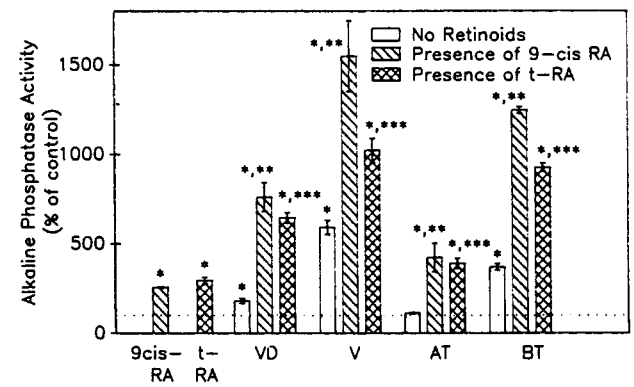

Figure 4 Alkaline phosphatase activity of IEC-6 cells treated with *t*-RA or 9-*cis* RA (10^{-8} M) in the absence or presence of 10^{-7} M different vitamin D analogs for 3 days. The open bars are cells treated with the indicated vitamin D analogs only. The hatched bars are cells treated with 9-*cis* RA alone or in the presence of indicated vitamin D analogs. The cross hatched bars are cells treated with *t*-RA alone or in the presence of indicated vitamin D analogs. Control values for each point were determined in parallel wells treated for the same amount of time in the presence of vehicle (the alkaline phosphatase activity in vehicle treated cells was designated as 100%). Each point represents the mean ± SEM for four wells. $*P<0.05$ compared to control. $**P<0.05$ compared to cells treated with 9-*cis* RA alone. $***P<0.05$ compared to cells treated with *t*-RA alone

nuclear protein(s) which then potentiate the effect of 1,25(OH)$_2$D$_3$ to increase ALP activity of IEC-6 cells.

Discussion

Alkaline phosphatase (ALP) is known to catalyze the hydrolysis of various phosphate ester compounds. At least two ALP genes have been demonstrated in the rat. One is expressed in tissues such as bone, liver, kidney and placenta (LALP) (Misumi et al., 1988), and the other in the intestine (IALP) (Seetharam *et al.*, 1987; Eliakim *et al.*, 1990; Engle & Alpers *et al.*, 1992). Although the physiological functions of ALP remain unclear, IALP has been demonstrated to be related to phosphate uptake (Kempson *et al.*, 1979; Hirano *et al.*, 1985) and calcium transport in the small intestine (Dupuis *et al.*, 1990). The LALP is more generally distributed among different tissues, and has been demonstrated to be able to bind extracellular matrix proteins such as collagen (Vittur *et al.*, 1984). We have previously demonstrated that it is the LALP and not the IALP isoenzyme which is present in IEC-6 cells (Jeng *et al.*, 1994), and that the ALP activity in IEC-6 cells is regulated by 1,25(OH)$_2$D$_3$ (Thomas *et al.*, 1993). The present studies were carried out to determine whether the effect of 1,25(OH)$_2$D$_3$ on IEC-6 cell ALP is regulated via the genomic pathway by binding to the intracellular VDR, and how retinoic acids interact with 1,25(OH)$_2$D$_3$ to regulate the ALP activity.

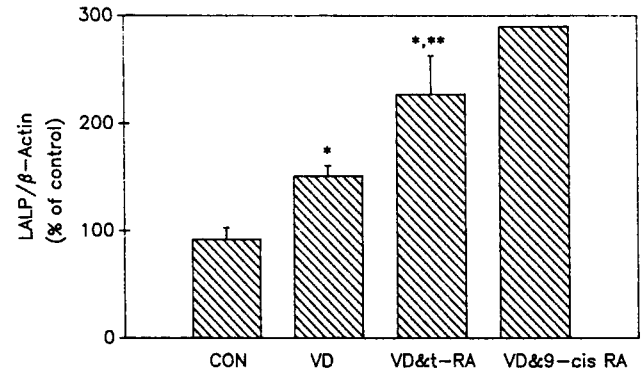

Figure 5 LALP/β-actin mRNA levels determined by Northern analysis in IEC-6 cells treated with 10^{-7} M 1,25(OH)$_2$D$_3$ (VD) ($n = 3$) in the absence or presence of 10^{-6} M *t*-RA ($n = 3$) or 9-*cis* RA ($n = 2$) for 48 h. The ratio of LALP/β-actin mRNA in vehicle-treated IEC-6 cells was designated as 100%. $*P<0.05$ compared to control. $**P<0.05$ compared to VD. For the VD&9-*cis* group, statistical comparison to other groups could not be carried out, because the bar represents the results from only two different experiments

The use of vitamin D analogs enabled us to discriminate between the genomic actions of 1,25(OH)$_2$D$_3$ and membrane calcium channel activation and suggested that the effects of 1,25(OH)$_2$D$_3$ on ALP activity were exclusively due to the genomic actions of 1,25(OH)$_2$D$_3$. The ability of vitamin D analogs to increase ALP activity was correlated with the ability of the analogs to bind to VDR. Since addition of 1,25(OH)$_2$D$_3$ alone resulted in significantly increased ALP activity in IEC-6 cells, the regulation of ALP activity by 1,25(OH)$_2$D$_3$ alone may be via a VDR-VDR homodimer.

The alkaline phosphatase activity of IEC-6 cells was significantly increased by *t*-RA and 9-*cis* RA in a dose-dependent manner. Work from other laboratories has shown that both *t*-RA and 9-*cis* RA are equally efficient ligands for RAR receptors, while 9-*cis* RA, but not *t*-RA, is a ligand for RXR receptors (Heyman *et al.*, 1992; Allenby *et al.*, 1993). Binding assays have shown that 9-*cis* RA binds to RAR receptors with the same affinity as *t*-RA (kd = 0.4 nM); the affinity for RXR is lower (kd = 15 nM). Since in the present experiments there was no significant difference in the ALP activity between IEC-6 cells treated with 9-*cis* RA or *t*-RA, we propose that the effect of retinoic acids in increasing ALP activity of IEC-6 cells may be regulated via RAR receptors. It has been reported that *t*-RA can be metabolized into 9-*cis* RA *in vivo* (Heyman *et al.*, 1992). Therefore, it is also possible that the effects of 9-*cis* RA and *t*-RA are due to the formation of RAR-RXR heterodimers in IEC-6 cells. In this case it is hypothesized that when *t*-RA was

added to the cells, enough of it was metabolized to 9-*cis* RA to provide ligand for the RXR. When only 9-*cis* RA was added, it was able to bind both RAR and RXR.

We have previously shown that 1,25(OH)$_2$D$_3$ increases the level of ALP mRNA in IEC-6 cells (Jeng *et al.* 1994). In the present experiments, 9-*cis* RA and *t*-RA had no effect on ALP mRNA levels in IEC-6 cells. These data suggest that different mechanisms may be utilized by 1,25(OH)$_2$D$_3$ and retinoic acids to regulate ALP activity of IEC-6 cells. While the 1,25(OH)$_2$D$_3$ effects may occur via increased mRNA levels of ALP, retinoic acids presumably produce gene products which cause post-transcriptional and/or post-translational regulation of ALP activity (Heath *et al.*, 1992). For example, ALP is known to be activated by phosphorylation (Sarrouilhe *et al.*, 1993). Therefore, the retinoids may increase a kinase activity which subsequently alters ALP activity.

Co-administration of 1,25(OH)$_2$D$_3$ or its genomically-active analogs with *t*-RA or 9-*cis* RA showed additive to synergistic effects to increase ALP activity of IEC-6 cells. Northern analysis of IEC-6 cells treated with 1,25(OH)$_2$D$_3$ only, or co-treated with *t*-RA or 9-*cis* RA showed higher mRNA levels of ALP. These data suggest that the interaction between retinoic acids and 1,25(OH)$_2$D$_3$ is very complex. Presumably the mechanism for 1,25(OH)$_2$D$_3$ alone to regulate ALP is via formation of VDR-VDR homodimers, but other mechanisms may be involved when IEC-6 cells are co-treated with retinoic acids. Two possible mechanisms may explain the combined effects of 1,25(OH)$_2$D$_3$ and retinoic acid. As described above, it is possible that *t*-RA may be partially metabolized into 9-*cis* RA, therefore addition of either *t*-RA or 9-*cis* RA may provide sufficient 9-*cis* RA to bind to intracellular RXR receptors and form RXR-VDR heterodimers, resulting in potentiated effects of 1,25(OH)$_2$D$_3$. Another possible mechanism is that *t*-RA or 9-*cis* RA produce some nuclear factor (e.g. VDR) which then potentiates the effects of 1,25(OH)$_2$D$_3$ to increase ALP mRNA.

The distribution of retinoic acid receptor is well studied in the mouse embryo. RAR receptors are more generally distributed among different tissues, while RXR-β receptors are more specifically present in the mouse intestine. One obvious solution to address the exact mechanism involved in the interactions between 1,25(OH)$_2$D$_3$ and retinoic acid will be to determine whether IEC-6 cells posses RAR, RXR, or both receptors. The IEC-6 cell line was derived from neonatal rat small intestinal crypts and maintains characteristics of non-transformed crypt epithelial cells (Quaroni *et al.*, 1979). Although the exact physiological function of ALP in IEC-6 cells remains to be determined, due to the low circulating plasma level of 1,25(OH)$_2$D$_3$ in neonatal rats, the synergy between 1,25(OH)$_2$D$_3$ and retinoic acids may cause significant biological responses.

Materials and methods

IEC-6 cell culture

IEC-6 cells were obtained from the American Type Culture Collection (Rockville, MD). Cells, passages 20 to 25, were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma, St. Louis) with 10% NuSerum (Collaborative Research, Bedford, MA).

For measuring ALP activity, cells were plated at low density (3000 cells/cm^2) in multiwell plates. After two days, culture media were changed to test media containing the appropriate steroid hormones, or ethanol as a vehicle control. Cells were harvested on day 3 by washing twice with PBS and then freeze-thawing three times at − 70°C to produce lysates.

Alkaline phosphatase activity

Alkaline phosphatase activity was measured using *p*-nitrophenyl phosphate as substrate at pH 9.8 (Thomas & Ramp, 1979). The cell lysates were incubated with substrate at 37°C for different periods of time, and the product was measured spectrophotometrically at 410 nm. ALP activity is expressed as per cent of control to facilitate comparison between experiments.

DNA determinations

DNA was assayed by fluorescence spectrophotometry using the dye, H33258 (Sigma). Fluorescence was measured using an excitation wavelength of 356 nm and a 458 nm emission wavelength (Labarca & Paigen, 1980). DNA and ALP activity were measured on the same samples.

Northern analysis of LALP mRNA

For preparation of RNA, cells were grown in T-75 flasks and treated with retinoic acids and vitamin D analogs as described. Total cellular RNA was prepared using guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski & Sacchi, 1987). The RNA was electrophoresed on 1% agarose slab gels in the presence of 0.66 M formaldehyde. The RNA was then blotted onto a nylon membrane (MSI, Magna-Graph) by capillary transfer, dried, and baked for 2 h at 80°C (Thomas, 1980). Standard prehybridization and hybridization conditions were employed (Sambrook *et al.*, 1989). Briefly, hybridization was carried out using a 960 bp cDNA probe which was an amplimer prepared by the polymerase chain reaction and labeled with [^{32}P]dCTP (Jeng *et al.*, 1994). Hybridization was carried out for 18 h at 65°C. The blots were washed with 2 × SSC, 0.1% SDS three times at room temperature for 10 min, and 0.1 × SSC, 0.1% SDS two times at 65°C for 15 to 20 min. The blots were then exposed to X-ray film (X-OMAT, Kodak), using intensifying screens at − 70°C. Blots were stripped and reprobed to detect β-actin mRNA (Cleveland *et al.*, 1980). Densitonmetric quantitation of LALP and β-actin mRNA was carried out (Applied Image, CA).

Statistics

Statistical significance of differences between groups was determined using one-way analysis of variance with *post-hoc* Bonferroni *t*-tests.

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