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# Potential Involvement of Calcineurin in Regulating the State of Differentiation and Apoptosis of HL-60 Cells During Methylprednisolone-Treatment

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## ABSTRACT

To evaluate the role of calcineurin (protein phosphatase type 2B, PP2B) in methylprednisolone-induced differentiation and apoptosis of leukaemic cells, we have investigated the induction of apoptosis, calcineurin specific protein phosphatase activity and expression of regulatory and catalytic subunits of calcineurin and calmodulin after induction of HL-60 leukaemic cells with methylprednisolone.

The cells underwent differentiation and apoptosis within 72 hours time period after methylprednisolone added to cell culture media. Before apoptosis occurred, the specific calcineurin enzyme activity revealed gradual increase during the differentiation process.

However, immunoblots of catalytic and regulatory subunits of calcineurin showed no amplification in the amount of these cellular signaling mediators during methylprednisolone-induced differentiation and apoptosis but calmodulin expression gradually increased during the process. Significant increase in the specific calcineurin enzyme activity during differentiation and apoptosis might be crucial to the posttranslational modifications of calcineurin during methylprednisolone-induced differentiation.

**Key Words:** Calcineurin, Methylprednisolone, Differentiation, Apoptosis, HL-60 cells.

## ÖZET

### HL-60 Hücrelerin Metilprednizolon Altında Apoptoz ve Farklılaşmasının Calcineurin ile Düzenlenmesi

Calcineurin'in (protein fosfataz tip 2B, PP2B) metilprednizolona bağlı lösemi hücre farklılaşması ve apoptozundaki rolünü anlamak amacı ile HL-60 lösemik hücrelerin metilprednizolon altında apoptozisi, calcineurin spesifik protein fosfataz aktivitesi ve calcineurin'in regülatör ve katalitik alt birimlerinin ve calmodulin'in ekspresyonunu inceledik.

Hücre kültür ortamına metilprednizolon eklendikten sonraki 72 saatlik dönemde hücreler apoptoz ve farklılaşma gösterdiler. Apoptoz oluşmadan, farklılaşma döneminde spesifik calcineurin enzim aktivitesinde kademe bir artış izlendi.

Ancak bu sırada katalitik ve regülatör calcineurin alt birimlerinin immünblotlarında artış olmazken, calmodulin ekspresyonu giderek arttı. Metilprednizolona bağlı apoptoz ve farklılaşmada spesifik calcineurin enzim aktivitesinde belirgin artış olması calcineurin'in posttranslasyonel modifikasyonlarında belirgin öneme sahip olabilir.

**Anahtar Kelimeler:** Calcineurin, Metilprednizolon, Farklılaşma, Apoptoz, HL-60 hücreler.

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## INTRODUCTION

Glucocorticoids are essential hormones that influence a wide range of cellular events<sup>[1]</sup>. In many types of cells, glucocorticoids produce an antiproliferative response that is associated with the regulation of cellular progression through the G1/S-phase transition. The glucocorticoid response is initiated in the cytoplasm by the interaction of hormone with a associated intracellular receptor complex. Upon glucocorticoid binding, the activated GR enters the nucleus, where it acts as a ligand-activated transcriptional stimulator or repressor of primary response genes by binding to glucocorticoid hormone-responsive elements (GRE) in the promoter regions of steroid responsive elements<sup>[2]</sup>.

Methylprednisolone is a member of the family of steroid hormones including retinoic acid and vitamin D<sub>3</sub>, and act by binding to nuclear hormone receptors (NHR)<sup>[3]</sup>. These receptor proteins bind directly to specific DNA recognition sequences in the promoter region of target genes, resulting in the alteration of the transcription initiation rate. High-dose methylprednisolone (HDMP) treatment has been shown to induce in vivo differentiation of myeloid leukaemia cells to mature granulocytes in patients with acute promyelocytic leukaemia and other subtypes of acute myeloblastic leukaemia (AML)<sup>[4,5]</sup>.

The ability of methylprednisolone to induce growth suppression appears to arise from complex signalling networks in which

NHR-mediated transcription of primary response genes regulates the subsequent expression or activity of a diverse set of downstream proteins, some of which regulate cell cycle progression and apoptosis. Molecular mechanism(s) by which NHR activation leads to growth arrest is (are) unclear. Phosphorylation events are major regulatory mechanisms of signal transduction pathways that control cell growth and differentiation. Achievement of such control requires the interconverting enzymes, the protein kinases and protein phosphatases<sup>[6]</sup>. One apparent target of methylprednisolone-induced signalling network is a family of serine/threonine protein phosphatases. In mammals, at least 13 closely related serine/threonine protein phosphatases (Ppases) have been identified<sup>[7]</sup>. The major serine/threonine protein phosphatase catalytic subunits in mammalian cells consist of four forms which have been designated as phosphatases type 1 (PP1), phosphatases type 2A (PP2A), phosphatases type 2B (PP2B, calcineurin), and phosphatases type 2C (PP2C), based on a classification system proposed by Cohen<sup>[8]</sup>. The activity of several of these enzymes has been linked to the mechanisms that control the cell cycle, and therefore, potentially some of these members might be involved in aberrant cell growth and tumorigenesis.

Calcineurin (also called PP2B) is a serine/threonine phosphatase that is activated by Ca<sup>+2</sup>-calmodulin. Calcineurin dephosphorylate nuclear factor of activated T-cells

(NFAT) which then translocates to nucleus where it acts combinatorially with other transcriptional factors to activate downstream targets. The role of calcineurin in these processes has been reviewed in detail elsewhere<sup>[9]</sup>.

Human myeloid leukaemia cell line, HL-60 cells differentiate along the granulocytic or the monocytic pathway when treated with the certain inducer molecules. These characteristic features of HL-60 cells have attracted researchers' interest and have become frequently described model system for leukaemic differentiation *in vitro*<sup>[10,11]</sup>.

In our previous study, we reported the augmentation effect of PP1 and PP2A inhibitors and the role of up-regulation of PP2A regulatory subunit expression on methylprednisolone-induced HL-60 cell differentiation<sup>[12,13]</sup>. PP2A and calcineurin are highly homologous with respect to the sequence of the catalytic domain but they differ in substrate specificity and type of regulation. We also demonstrated increased calcineurin activity during vitamin D<sub>3</sub>-induced monocytic differentiation of HL-60 cells<sup>[14]</sup>. In this study, we elucidated the role of calcineurin on methylprednisolone-induced HL-60 cell differentiation and apoptosis.

## **MATERIALS and METHODS**

### **Reagents**

All of the chemical reagents and cell culture mediums were purchased from Sigma (Deisenhofen, Germany) unless otherwise specified. Rabbit antiserine/threonine PP2B antibody to CNA, CNB and calmodulin were obtained from Biogenesis Ltd (Poole, UK). Bradford reagent and Immunoblot assay kit were purchased from Bio-Rad (Hercules, CA, USA).

### **Tumor Cells**

The human myelogenous leukaemia cell line, HL-60, was generously supplied by Erdem Göker, Ege University, İzmir, Turkey. The cell line was maintained in RPMI-1640

medium supplemented with 1% nonessential amino acids, 1% L-glutamine, 10.000 units/mL penicillin, 10 mg/mL streptomycin, 10% heat inactivated fetal calf serum. HL-60 was grown in a humidified atmosphere at 37°C in 5% CO<sub>2</sub>.

### **Experimental Protocols**

1 mM stock solution of methylprednisolone was prepared with RPMI-1640. HL-60 cells (2 x 10<sup>6</sup> cells/mL) were cultured with 0.1 mM methylprednisolone between 24-96 hours. Control group was only cultured in RPMI-1640.

At the end of each experiment period HL-60 cells (2 x 10<sup>6</sup>) were washed three times with ice-cold TBS (pH 7.4) and then disrupted in 1 mL of homogenization buffer containing 20 mM Tris-HCl, pH 7.4, 2 mM dithiothreitol, 2 mM EDTA, 2 mM EGTA, 0.25 mM sucrose, protease inhibitors 0.1 mM leupeptin and 0.02 mM TLCK (N  $\alpha$ -p Tosyl-L lysine chloro methyl ketone) by a glass to glass Potter-Elvehjem homogeniser. The homogenates were immediately centrifuged at 1000 x g for 10 min and supernatant was centrifuged at 100.000 x g for 1 h. The resulting supernatant was used as the cytosolic fraction for protein assay and western blotting.

### **Protein Assay**

Total protein concentrations in the cell culture extracts were measured by using the Bradford reagent (Bio-Rad, Hercules, CA, USA). The assay was standardised with bovine serum albumin.

### **Morphological Evaluation**

Effects of methylprednisolone on apoptosis and differentiation of cultured HL-60 cells were determined morphologically and documented photographically after staining with May-Giemsa by light microscopy (Nikon, Japan).

### **Detection of Apoptosis by Acridine Orange/Ethidium Bromide Staining**

Apoptosis was determined morphologically after staining with acridine orange and

ethidium bromide by fluorescence microscopy. Cells were washed in cold PBS and adjusted to the cell density to  $1 \times 10^6$  cells/mL in PBS. Acridine orange and ethidium bromide (1:1) (v:v) were added to the cell suspension in final concentrations of 100  $\mu\text{g}/\text{mL}$  and then cells were incubated for 30 minutes. The cellular morphology was evaluated by fluorescent microscopy (Olympus, Japan) using an excitation wavelength of  $380 \pm 10$  nm and a bandpass filter of  $\geq 520$  nm for emission. Apoptotic cells were essentially characterised by nuclear condensation of chromatin and/or nuclear fragmentation. Three hundred cells were evaluated for apoptosis and/or necrosis for each sample. When more than 50% of the preapoptotic plus apoptotic to total cell ratio were positive, the result was accepted positive for apoptosis.

#### **Measurements of Calcineurin Phosphatase Activity**

The activity of PP2B was assayed by the serine/threonine phosphatases system (Promega Corporation, Madison WI, USA) according to the manufacturer's instructions. Cell supernatants were extracted from the columns in PP2B specific medium. The enzyme activity was measured by a nonradioactive method based on the determination of free phosphate with color development. Dye (ammonium molybdate-malachite green) absorbance was measured at 630 nm with a plate reader (Bio-Rad-Coda, Richmond, CA). The control values were the phosphatase activity in the samples without the addition of methylprednisolone.

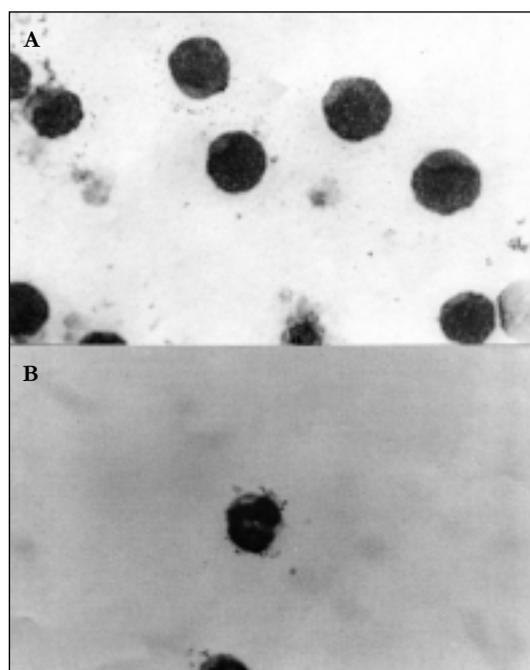
#### **Immunoblot Analysis of Calcineurin Subunits and Calmodulin**

Homogenate supernatants were analysed for quantitation of total proteins by using Bradford reagent and adjusted to mg of protein per mL. On the day of electrophoresis, stored samples were heated in heating block ( $95^\circ\text{C}$ , 1 min). The samples were allowed to cool at room temperature for at least 10 minutes then centrifuged at  $15,000$  g for 2 min. Homogenate supernatants were analyzed by

SDS-PAGE (10% gels) followed by Western blotting onto PVDF membranes using samples of 20 mg protein/well. After blocking with blotto (5% milk, 0.1% Tween 20, 10 mM Tris-HCl, pH 7.5, 150 mM NaCl) for 1 h, the membranes were probed to the primary antibodies according to the manufacturer's instructions. The secondary antibodies were coupled to biotinylated alkaline phosphatase, and were detected by using the enhanced calorimetric revelation system (Bio-Rad Immunoblot<sup>®</sup> Assay Kit, Hercules, CA, USA).

#### **Statistical Analysis**

The enzyme activity data were presented as mean from at least three independent experiments and analysed using one-way ANOVA with Scheffe's test. A p value less than 0.05 was considered as statistically significant. Statistical analysis was performed by using SPSS 10.0 (SPSS Inc., Chiago, USA) software program.



**Figure 1.** Morphological analysis of differentiated HL-60 cells after induction with methylprednisolone (Giemsa, x 100). (A) Before induction with methylprednisolone, and (B) After induction with methylprednisolone at 72<sup>nd</sup> hour.

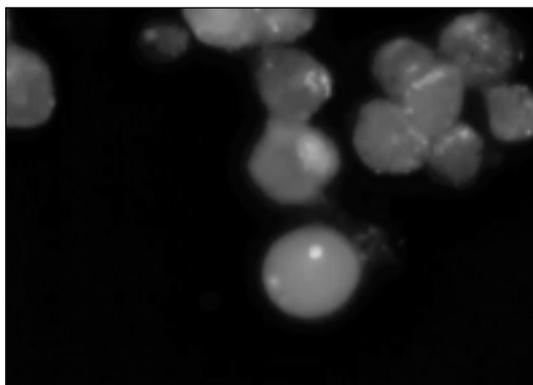
## RESULTS

### Morphological Assessment of Methylprednisolone-induced Differentiation

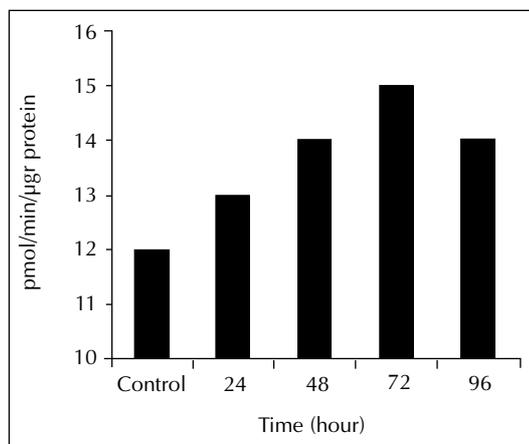
The optimum nontoxic differentiation-inducing concentration of methylprednisolone for HL-60 was 0.1 mM<sup>[8]</sup>. At these concentrations, methylprednisolone suppressed the proliferation of HL-60 cells and induced these cells to differentiate into granulocytic cells. Morphological examinations of May-Giemsa stained cytospin preparations showed that methylprednisolone-treated cells gradually differentiated to mature myeloid cells (Figure 1).

### Identification of Apoptotic Cells After Nuclei Staining with Acridine Orange and Ethidium Bromide

Fluorescence microscopy revealed an array of nuclear changes, including chromatin condensation and margination and increased stainability with acridine orange. Double staining with acridine orange and ethidium bromide clearly demonstrated that nuclear alterations shown occurred before the cells took up ethidium bromide (Figure 2).



**Figure 2.** Cells were double stained with the membrane-permeable fluorochrome acridine orange and DNA-binding fluorochrome ethidium bromide, which are impermeable to the normal plasma membrane but stains the nuclei of necrotic and late apoptotic cells. Acridine orange is a vital dye and will stain both live and dead cells. Live cells stain uniformly green. Note that chromatin condensation and margination as well as ruffling of nuclear membranes had already occurred in cells that did not take up ethidium bromide (x 400 magnification).



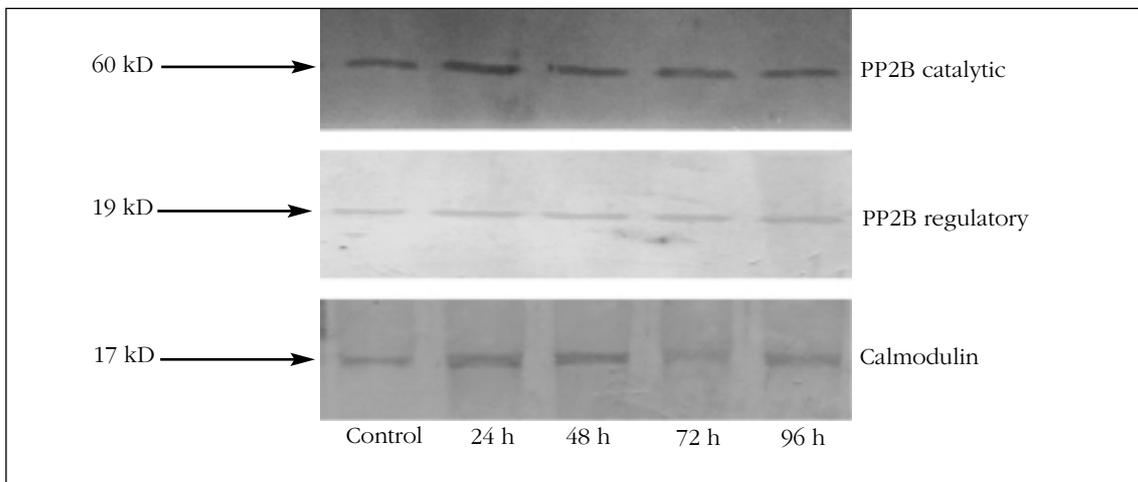
**Figure 3.** Serine/threonine protein phosphatase 2B specific phosphatases activities in cytosolic fractions of HL-60 cells. PP2B activity was measured after the addition of methylprednisolone and compared with the control cells. Each point represents the mean from three separate experiments (SD were < 10%).

The cells were evaluated at 72<sup>nd</sup> hour after incubation with methylprednisolone and in all of the samples 50 to 70% of the cells were found to be apoptotic.

### Phosphatase Activity and Protein Expression of Calcineurin Subunits and Calmodulin During Methylprednisolone Induced Differentiation of HL-60 Leukaemic Cells

Based on the measurements of phosphate content in the extracts, calcineurin activity was studied (Figure 3). PP2B activity was elevated after induction with methylprednisolone at 24<sup>th</sup> hour. The most significant alteration was detected at 72<sup>nd</sup> hours ( $p < 0.05$ ). The total increase in the activity at 72<sup>nd</sup> hours was 25% more than the control activity (Figure 3).

Immunoblots demonstrated no significant change during the time course of changes in PP2B subunits in HL-60 cells over a three days period following incubation with methylprednisolone. However, there occurred a gradual increase in calmodulin expression during differentiation process (Figure 4).



**Figure 4.** Homogenates of HL-60 cells were fractionated by SDS-PAGE (10% gel, 20  $\mu$ g protein/well), blotted onto nitrocellulose membranes and immunoblotted with PP2B catalytic, PP2B regulatory subunit, and calmodulin. 0, 1 mM methylprednisolone added to culture medium at the beginning of experiments. Control cells had no treatment.

## DISCUSSION

The differentiation and apoptosis of cells is controlled by complex regulatory events that must be coordinated to meet the demands of cells. The fundamental decision of a cell to either DNA fragmentation or withdrawal from the cell cycle appears to take place in mid-to-late G1 phase of the cell cycle.

Calcineurin, the  $\text{Ca}^{2+}$ /calmodulin-regulated protein phosphatase has been identified in various tissues, and was first detected in skeletal muscle and brain, has been found in all cells from yeast to mammals<sup>[15]</sup>. The calcineurin holoenzyme purified from mammalian cells is a heterotrimeric complex consisting of a large ~ 60 kD catalytic subunit, calcineurin A (CNA), an ~ 19 kD  $\text{Ca}^{2+}$ -binding regulatory subunit, calcineurin B (CNB), and calmodulin, an ~ 17 kD  $\text{Ca}^{2+}$  receptive protein. Multiple catalytic subunits of calcineurin are derived from at least 2 structural genes, type 1 (calcineurin A-alpha) and type 2 (calcineurin A-beta; CALNA2), each of which can produce alternatively spliced transcripts<sup>[16]</sup>. The A subunit binds calmodulin whereas the B subunit attaches to four molecules of  $\text{Ca}^{2+}$ . Calcineurin is incapable of functioning as a

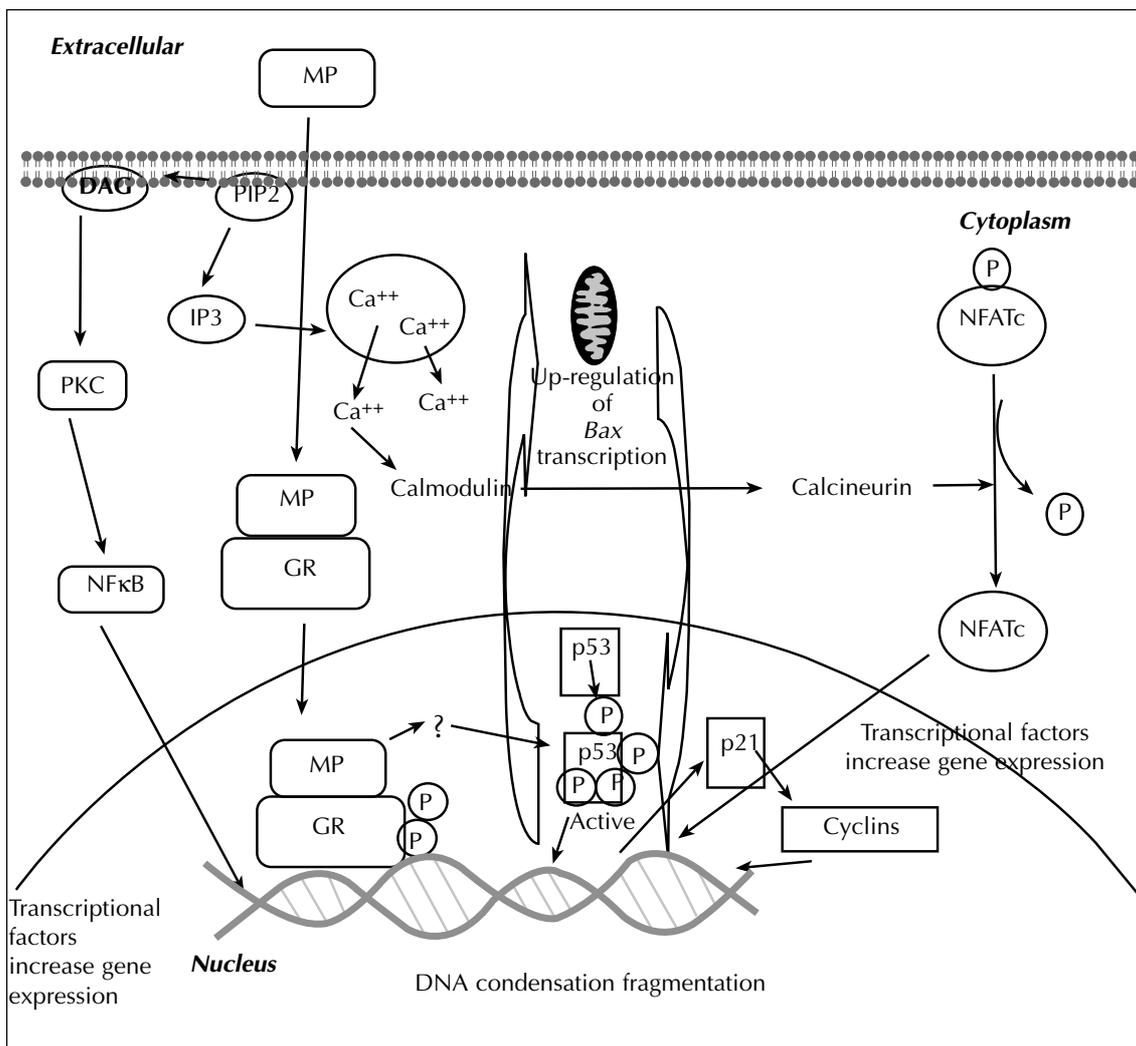
phosphatase *in vitro* when calmodulin or CNB is absent. Calcineurin plays a pivotal role in the T-cell receptor-mediated signal transduction pathway and serves as a common target for the immunosuppressants FK-506 and cyclosporin A<sup>[17-19]</sup>. Calcineurin activity is regulated by calmodulin, which is the archetype of the family of calcium-modulated proteins of which nearly 20 members have been found. Calmodulin contains 149 amino acids and has 4 calcium-binding domains. They are identified by their occurrence in the cytosol or on membranes facing the cytosol and by a high affinity for calcium<sup>[20]</sup>.

Calcineurin-mediated second messenger functions are regulated at multiple molecular levels through intra- and intermolecular protein-protein interactions as well as by post-translational modification through phosphorylation. Posttranslational modification has long been recognized, as a way in which the properties of proteins may be subtly altered after synthesis of the polypeptide chain is complete. Amongst the moieties most commonly encountered covalently attached to proteins are oligosaccharides, phosphate, acetyl, formyl and nucleosides. Protein phosphorylation and dephosphorylation is

one of the most prevalent and best understood modifications employed in cellular regulation[21].

We have demonstrated the up-regulation of calcineurin during vitamin D<sub>3</sub>-induced differentiation of HL-60 cells. Cyclosporin A and FK-506 augmented the leukaemic cell proliferation of HL-60 cells[14]. To examine the role for calcium/calmodulin-regulated

calcineurin in the differentiation process, we examined the specific calcineurin activity for the effects of methylprednisolone on differentiation and apoptosis. The present results suggest (i) increase in the activation of calcineurin specific phosphatase activity by methylprednisolone treatment and (ii) induction of apoptosis and differentiation in the leukaemia cell line, HL-60 consequently. Furt-



**Figure 5.** Proposed role of calcineurin in the regulation of apoptosis and differentiation initiated by methylprednisolone. The data presented here suggest that calcineurin is a key regulator of cellular differentiation and apoptosis by steroid hormone signalling network to impede or inhibit progression through the increased gene expression of transcriptional factors and up regulation of *Bax* transcription through p53 activation. DAG: Diacylglycerol, PKC: Protein kinase C, PIP2: Phosphatidylinositol diphosphate, MP: Methylprednisolone, GR: Glucocorticoid receptor.

hermore, increased calcineurin activity in HL-60 cells was associated with up-regulation of calmodulin.

As the biological effect of methylprednisolone is through up-regulation of the calcium/calmodulin regulated phosphatase calcineurin activity, this suggests a role for glucocorticoids in agonizing calcineurin activity. The experiments suggest that calmodulin is important in one or more of the processes that lead to activation (trimerization, nuclear localization, DNA binding) and which becomes dephosphorylated due to the activation of a calcium/calmodulin/calcineurin complex. Numerous intermediate signaling proteins might also be activated including the Ras and Rap1, which are small molecular weight GTPases and these ultimately lead to the activation of MAP kinases including Erk, JNK and p38 and p53 which activate proapoptotic *Bax* gene transcription<sup>[22-25]</sup>. Abundant opportunities for cross talk between the glucocorticoid receptors and other signaling pathways increases the complexity of glucocorticoid induced apoptosis and its regulation. This involves counterbalancing influences of prosurvival and proapoptotic factors, and the execution stages which involves caspases and endonuclease activation<sup>[26]</sup>. Reddick et al reported that the effect of the glucocorticoids on Ca<sup>++</sup>-ionophore-stimulated 5-lipoxygenase product in human monocytes<sup>[27]</sup>. Most probably also in our study, methylprednisolone induces Ca<sup>++</sup>- ionophore effect from intracellular Ca stores. The demonstrated increase of calmodulin expression is also related with the increase in cytoplasmic calcium concentration. Semserian et al have also shown that treatment with IGF-1 or insulin and dexamethasone mobilizes intracellular calcium, activates the Ca<sup>2+</sup>/calmodulin-dependent phosphatase calcineurin, and induces the nuclear translocation of the transcription factor NF-ATc1<sup>[28]</sup>.

From the data presented here and those available in the literature, we propose that calcineurin is a key regulator of cellular dif-

ferentiation and apoptosis by up regulation of *Bax* transcription through p53 activation and steroid hormone signalling network to impede or inhibit progression through the increased gene expression of transcriptional factors (Figure 5). We also propose that methylprednisolone-induced differentiation and apoptosis are also mediated by calcium mobilization and are critically regulated by calcineurin/NF-ATc1 signalling pathway in HL-60 cells. Further experiments from the windows opened by our data related to the proposed pathway steps, will shed light to differentiation of leukaemic cells.

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