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Anti-inflammatory and Corticosteroid Enhancing Actions of Vitamin D in the Monocytes of Steroid Resistant and Steroid Sensitive Asthmatics

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Abstract

Background—Vitamin D is known for its anti-inflammatory effects.

Objective—Vitamin D regulation of responses in steroid resistant (SR) versus steroid sensitive (SS) asthmatics has not been studied.

Methods—Peripheral blood cells from eleven SR and eight SS asthmatics were preincubated with 1,25-dihydroxyvitamin D (VitD) followed by dexamethasone (DEX) treatment and LPS stimulation. LPS-induced phospho-p38 MAPK (p-p38) in monocytes was examined by flow cytometry. Mitogen-activated protein kinase phosphatase-1 (MKP-1) mRNA expression that inhibits p-p38 was analyzed by real-time PCR. Glucocorticoid receptor (GR) binding and histone H4 acetylation in the glucocorticoid response element (GRE) of monocytes' MKP-1 promoter were analyzed by chromatin immunoprecipitation

Results—DEX significantly inhibited LPS-induced p-p38 in monocytes from SS, but not from SR asthmatics ($p < 0.01$). VitD inhibited LPS-induced p-p38 in monocytes from both patient groups ($p < 0.01$), but enhanced DEX suppression of LPS-induced p-p38 only in monocytes from SS asthmatics ($p < 0.01$). VitD induced MKP-1 expression and enhanced DEX induction of MKP-1 in both SS and SR asthmatics. VitD/DEX-induced MKP-1 mRNA levels remained significantly lower in SR asthmatic monocytes ($p < 0.05$). DEX-stimulated recruitment of GR and histone H4 acetylation at GRE 4.6kbp upstream of MKP-1 gene were significantly lower in SR, as compared to SS, asthmatic monocytes. VitD pretreatment enhanced DEX-induced GR binding and histone acetylation in monocytes from both patient groups. GR binding and histone H4 acetylation, however, remained significantly lower in SR asthmatic monocytes.

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Conclusion—VitD demonstrated anti-inflammatory and corticosteroid enhancing effects in monocytes of SR and SS asthmatics. However, the responses to corticosteroids in SR asthmatics remained significantly lower than SS asthmatics.

Keywords

asthma; steroid resistance; corticosteroids; vitamin D

Introduction

Current scientific literature supports the importance of anti-inflammatory function of vitamin D in health and disease beyond its known role in calcium metabolism and bone health^{1, 2}. It has been reported that vitamin D deficiency defined as serum concentration of vitamin D pro-hormone, 25-hydroxyvitamin D, 25(OH)D, less than 20ng/ml is very common in the general population^{1, 3}. Epidemiologic studies have suggested that adequate levels of 25(OH)D are critical, as studies have linked vitamin D insufficiency to a variety of conditions, including cancers, autoimmune diseases, infections, diabetes. Vitamin D deficiency has been reported to be associated with higher rate of asthma risk^{4, 5}. Recent publications by our research group and others have found that asthmatics with low serum vitamin D have increased corticosteroid requirements^{6–10}. We recently reported^{6, 7} that in adult and pediatric patients with mild-to-moderate asthma, reduced vitamin D levels are associated with impaired lung function, increased airway hyperreactivity and reduced corticosteroid response.

Corticosteroids are currently the gold standard for controlling inflammation in asthma¹¹. The anti-inflammatory effects of corticosteroids are mediated through cognate binding to glucocorticoid receptor (GR), which translocates to the cell nucleus from cytoplasm and binds to glucocorticoid response elements (GRE) to regulate the transcription of specific genes, including mitogen-activated kinase phosphatase (MKP-1)^{12, 13}. MKP-1 is a phosphatase that selectively inactivates p38 MAPK¹⁴, thus inhibiting the production of pro-inflammatory cytokines regulated by p38 MAPK, which is critical for the anti-inflammatory functions of corticosteroids¹⁵. In a recent study of monocytes from healthy subjects we demonstrated that 1,25-dihydroxyvitamin D (1,25(OH)₂D, VitD) mediated inhibition of LPS-induced p38 MAPK activation and proinflammatory cytokine production. This was mediated by VitD induced MKP-1¹⁶. We also found *in vitro* evidence for steroid sparing effects of VitD⁷ and reported that VitD enhancement of cellular responses to DEX in human monocytes was mediated by MKP-1¹⁷.

It is estimated that up to 25% of asthmatic patients do not respond to corticosteroids^{18–21}. GR-mediated signaling has been a focus of research to understand the mechanisms that modulate steroid sensitivity and resistance²². Whether vitamin D can exert anti-inflammatory and steroid enhancing effects in steroid resistant (SR) and steroid sensitive (SS) asthmatics is of great interest, as oral supplementation with vitamin D may represent an inexpensive approach to enhance corticosteroid responsiveness, improve asthma control and reduce asthma burden. In this study the anti-inflammatory effects vitamin D and the effects

of vitamin D on cellular responses to corticosteroids *in vitro* were examined in the monocytes of SR and SS asthmatics.

Methods

Materials

LPS, DEX, 1,25(OH)₂D₃ (VitD), an active form of vitamin D, and monoclonal anti-β-actin antibody were purchased from Sigma (St. Louis, MO). HyQTase was purchased from Hyclone Laboratories (Logan, UT). Rabbit polyclonal antibody to GR and Rabbit IgG were purchased from Abcam, Inc. (Cambridge, MA). Rabbit polyclonal antibody to GR phosphorylated at S211 was purchased from Cell Signaling (Danvers, MA). Rabbit polyclonal antibody to histone H4 and acetylated histone H4 were purchased from Millipore (Temecula, CA). Anti-mouse and anti-rabbit horseradish peroxidase (HRP)-labeled IgG were purchased from Amersham Biosciences (Piscataway, NJ). Chemiluminescent reagent was purchased from Perkin Elmer Life Sciences (Waltham, MA). CHIP-IT Express kit was purchased from Active Motif (Carlsbad, CA). Mouse monoclonal antibody to GR and all the antibodies and reagents used for flow cytometry were purchased from BD Biosciences (San Diego, CA).

Study subjects

Nineteen adult asthmatics with a baseline FEV₁ % predicted less than 85% were recruited. None of the subjects had received systemic corticosteroids for at least 6 months before the study. Smokers were excluded from participation in the study. All study subjects were symptomatic as determined by Juniper Asthma Control Questionnaire²³. The mean ACQ-7 scores are provided in Table I, which details characteristics of the study subjects. Some of the study participants were not on ICS at the time of enrollment, but had used ICS in the past. Patients' clinical response to corticosteroids was determined based on change in prebronchodilator morning FEV₁ % predicted after one week oral prednisone burst (40 mg/day). Asthmatics were defined as SR if they had less than 10% improvement in FEV₁ and as SS if they had more than 12% improvement in FEV₁. Eight patients were classified as SS, 11 were SR asthmatics (Table I). Blood samples were collected from all patients. The study was approved by the Institutional Review Board at National Jewish Health, Denver, CO.

Cell Culture and Treatment

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized, venous blood of asthmatic donors by Ficoll-Hypaque density gradient centrifugation. Cells were cultured in hormone free medium (phenol-red free RPMI containing 5% charcoal stripped FCS, 50μg/ml streptomycin, and 50 units/ml penicillin) during hormone and LPS treatments. PBMC from SS and SR asthmatics were pre-incubated with 10nM 1,25(OH)₂D₃ (VitD) for 21h, followed by 10nM dexamethasone (DEX) treatment for 3h. Adherent PBMC fraction was collected in the RNA preservation solution (Qiagen) or lysed for total protein extraction. To examine cellular responses to LPS following VitD±DEX treatment the cells were stimulated with 10ng/ml LPS for 10min for MAPK activation. Phospho-p38 MAPK (p-p38) expression in CD14+ cells was examined by flow cytometry as described by us earlier²⁴. To examine the effects of VitD pretreatment on the LPS-induced cytokine

production PBMC were cultured in the presence of 10nM VitD for 24h followed by cell stimulation by 10ng/ml of LPS with and without 10nM DEX for 24h. Cell culture supernatants were collected and stored at -80°C for cytokine analysis by ELISA.

Real-Time PCR

Total RNA from cells was prepared using RNeasy Mini kit (Qiagen, Valencia, CA). After reverse transcription, cDNA from each sample was analyzed by real-time PCR using the dual-labeled fluorogenic probe method on an ABI Prism 7300 real Time PCR system (Applied Biosystems) as described²⁵. Specific gene expressions were determined using primers for MKP-1, IL-6, β -actin purchased from Applied Biosystems (Foster City, CA).

Western Blot

Protein lysates were prepared from adherent PBMC fraction cultured with 10nM VitD for 21h, followed by media or 10nM DEX culture for 3h. Cells were rinsed with ice-cold PBMC and lysed in complete RIPA buffer supplemented with protease inhibitor cocktail (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 10 min on a plate, transferred to the Eppendorf tubes and lysed for additional 30 min on ice. Cell lysates were spun at 10,000rpm for 10min at 4°C and supernatants were stored at -80°C. Protein samples were resolved on Invitrogen 4–12% Bis-tris gel and transferred to PVDF membranes. The membranes were incubated in PBS containing specific antibodies, 5% dry milk, and 0.1% Tween 20 at 4°C overnight. Subsequently, membranes were washed in PBS/0.1% Tween 20, incubated for 1h at room temperature with horseradish peroxidase labeled secondary antibodies, washed, incubated with chemiluminescent reagent and processed for autoradiography.

Developed X-ray films were scanned and densitometry of the bands was quantified with the ImageJ (version 1.47) software (this software is available on the Internet at <http://rsbweb.nih.gov/ij>). The densitometry of the bands was adjusted by subtracting the background densitometry readings. In this study blots detected by antibody against Ser211 phosphorylated GR and total GR were developed together for cell lysates from each study subject.

ELISA analysis

IL-6 levels in cell culture supernatants were tested using Human IL-6 ELISA ready-set-go! Kit from eBioscience, Inc. (San Diego, CA).

Chromatin Immunoprecipitation Assay (ChIP)

GR binding to the glucocorticoid response element (GRE) in the MKP-1 promoter in monocytes of SR and SS asthmatics was assessed by ChIP assay using ChIP-IT Express kit following the manufacturer's instructions. PBMC were pre-incubated with 10nM VitD for 21h, followed by DEX treatment at 10nM for 3h. GR binding to the GRE in the human MKP-1 gene promoter and histone H4 acetylation in this region were examined. Rabbit polyclonal antibodies were used in the chromatin precipitations. Precipitated DNA was quantified by quantitative real time PCR using SYBR green (Applied Biosystems). Primers used to detect MKP-1 promoter GRE were synthesized according to reference²⁶. The quantity of anti-GR antibody precipitated DNA was normalized to Input DNA; anti-

acetylated histone H4 (Anti-acH4) antibody precipitated DNA was normalized to anti-histone H4 (Anti-H4) antibody precipitated DNA.

Statistical analyses

Results were expressed as the Mean $\pm$ SEM or Mean $\pm$ SD. Statistical analysis was conducted using GraphPad Prism, version 5 (GraphPad Software, La Jolla, CA). Data were analyzed by the paired Student's *t* test (pairing by experimental conditions within the same patient group) and unpaired Student's *t* test (comparing the same treatment condition between two patient groups, i.e. SR asthma and SS asthma) if the data followed normal distribution; non-parametric tests were applied for the data that was not normally distributed. Differences were considered significant at $p < 0.05$. A minimum of three independent experiments was conducted to allow for statistical comparisons. In all figures values represent Mean $\pm$ SEM.

Results

Study subjects

Clinical characteristics of the study participants are summarized in Table I. In this study patients were defined as SR or SS based on changes in lung function after one week of oral prednisone treatment (40mg/day). Asthmatics were defined as SR if they had less than 10% improvement in prebronchodilator FEV₁ % predicted after 7-day prednisone burst and as SS if they had more than 12% improvement in FEV₁. This definition of SR and SS asthmatics has been used in all of our research studies; our previous publications indicate that most SR asthmatics have less than 5% improvement in FEV₁ after one week oral prednisone burst^{27–32}. As documented by prebronchodilator FEV₁ % predicted all study participants had bronchoconstriction with a FEV₁ % predicted (Mean $\pm$ SD) of 66.9 $\pm$ 16.2% and 65.0 $\pm$ 11.3 % for SR and SS asthma groups, respectively. After 1 week prednisone burst patients defined as SS asthmatics had significant improvement in prebronchodilator FEV₁ % predicted, with a FEV₁ % predicted (Mean $\pm$ SD) of 85.4 $\pm$ 11.7% ($p = 0.0002$), while no significant change in lung function was observed in the SR asthma group: FEV₁ % predicted (Mean $\pm$ SD) of 66.1 $\pm$ 16.4% ($p = 0.96$). Suppl. Fig. 1 summarizes changes in individual prebronchodilator FEV₁ % predicted in SR and SS asthma patients before and after one-week oral prednisone burst. After 7 days of oral prednisone treatment SS asthmatics had a substantial improvement in lung function with FEV₁ % predicted (Mean $\pm$ SD) of 33.1 $\pm$ 17.5%. No significant change in FEV₁ % predicted was observed in the SR asthma group with FEV₁ % predicted (Mean $\pm$ SD) 0.1 $\pm$ 5.3% ($p < 0.0001$ as compared to SS asthmatics). Both SR and SS asthma patients demonstrated greater than 12% improvement in FEV₁ % predicted in response to bronchodilator administration (albuterol), consistent with the ATS definition of asthma³³ (see Table I).

VitD inhibits LPS-induced p38 phosphorylation and enhances corticosteroid responses in SS asthmatics

In this study, we examined the effects of VitD and DEX on suppression of responses to LPS in the cells from SS versus SR asthmatics. PBMC from SS and SR asthmatics were pre-incubated with 1,25(OH)₂D₃ for 21h, followed by DEX treatment for 3h. Then, the cells were stimulated with 10ng/ml LPS for 10min. As shown by flow cytometry (Fig. 1),

4.6±1.1% and 2.4±0.6% CD14⁺ cells expressed phosphorylated p-p38 prior to LPS treatment in SS and SR asthmatics, respectively. LPS treatment significantly increased the percentage of CD14⁺ cells that express p-p38 to 46.1±6.3% (n=8, p<0.01) and 50.3±6.7% (n=9, p<0.01) in SS and SR asthmatics, respectively. DEX significantly inhibited LPS induced p-p38 by 29% (n=8, p<0.01) in SS asthmatics, but no significant inhibition of LPS induced p-p38 by DEX was observed in SR asthmatics. At the same time 1,25(OH)₂D₃ pretreatment significantly inhibited LPS induced p-p38 by 56% (n=8, p<0.01) and 50% (n=9, p<0.01) in SS and SR asthmatics, respectively. Combination of 1,25(OH)₂D₃ and DEX inhibited LPS induced p-p38 by 74% (n=8, p<0.01) in SS asthmatics, this was significantly higher than the inhibition by 1,25(OH)₂D₃ or DEX alone (n=8, p<0.01). However, the combination of 1,25(OH)₂D₃ and DEX did not further enhance the inhibitory effect caused by 1,25(OH)₂D₃ alone (n=9) in the cells from SR asthmatics.

VitD enhances DEX inhibition of LPS-induced IL-6 production in monocytes from SS and SR asthmatics

To examine whether the differential suppression of LPS-induced p38 MAPK activation by VitD and DEX influenced cytokine production, PBMC from SS and SR asthmatics were pre-incubated with 1,25(OH)₂D₃ for 24h, followed by LPS stimulation with or without DEX for additional 24h. IL-6 levels in cell culture supernatants were examined by ELISA. Upon stimulation with LPS, the amount of IL-6 in culture supernatants increased from a basal level of 31.1±8.5 pg/ml and 66.6±10.0 pg/ml to 749.5±31.8 pg/ml (p<0.01) and 1443.2±44.2 pg/ml (p<0.01) in SS and SR asthmatics, respectively. Treatments of 1,25(OH)₂D₃ or DEX significantly inhibited LPS-induced IL-6 production in the cells from SS asthmatics. This was further inhibited by the combination of 1,25(OH)₂D₃ and DEX in the cells from these patients (Fig. 2). Similarly, 1,25(OH)₂D₃ or DEX significantly inhibited LPS-induced IL-6 production in the cells from SR asthmatics. The combination of 1,25(OH)₂D₃ and DEX further lowered IL-6 levels (Fig. 2). However, significantly greater inhibition of LPS-induced IL-6 production was observed in the cells of SS asthmatics in the presence of DEX or VitD/DEX combination as compared to the cells of SR asthmatics (p<0.05 and p<0.01 for DEX and VitD/DEX treatment conditions, respectively) (Fig. 2).

VitD enhances DEX induction of MKP-1 in monocytes from SS and SR asthmatics

We previously reported that VitD suppression of LPS-induced p38 MAPK activation in human monocytes from healthy subjects is mediated by MKP-1¹⁶, and that VitD/DEX enhanced the expression of MKP-1 in human monocytes¹⁷. In this study, we examined the expression of MKP-1 mRNA in response to VitD, DEX or VitD/DEX in monocytes of SR and SS asthmatics. PBMC from SS and SR asthmatics were pre-incubated with 1,25(OH)₂D₃ or vehicle control for 21h, followed by DEX treatment for 3h. The adherent fraction of PBMC, which contains mainly monocytes¹⁶, was collected to examine MKP-1 mRNA expression. 1,25(OH)₂D₃ or DEX individually significantly induced MKP-1 expression in both SS and SR asthmatics (p<0.05 and p<0.01 for VitD or DEX treatment conditions individually as compared to untreated cells, respectfully). The pre-incubation of cells with 1,25(OH)₂D₃ significantly enhanced DEX induction of MKP-1 in both SS and SR asthmatics (p<0.05) (Fig. 3A, B). The level of MKP-1 induced by DEX or by VitD/DEX

combination was significantly higher in SS asthmatics than in SR asthmatics ($p<0.05$ and $p<0.05$ for the corresponding treatment conditions in SS asthmatics) (Fig. 3C).

VitD enhanced GR binding and histone H4 acetylation to the GRE of MKP-1 promoter in monocytes from SS and SR asthmatics

The above data indicated that DEX differentially upregulated MKP-1 in SS and SR asthmatics. To further understand whether this effect was influenced by differential GR binding to the GRE in the MKP-1 promoter, we performed ChIP assays to assess GR binding to the GRE in the MKP-1 promoter. GRE, 4.6kb upstream of the MKP-1 gene transcriptional start site, is a predominant GR binding site in the human MKP-1 gene promoter²⁶. A schematic drawing of the human MKP-1 gene promoter, location of GRE and potential histone acetylation sites are shown in Fig. 4A.

The results of ChIP assays showed that DEX treatment significantly induced GR binding to the GRE by 3.5 ± 0.1 fold ($p<0.05$) and 2.9 ± 0.6 fold ($p<0.05$) respectively comparing media control in SS and SR asthmatics (Fig. 4B). In response to DEX treatment, GR binding to this GRE in the MKP-1 promoter was significantly higher in SS asthmatics as compared to SR asthmatics ($p<0.05$) (Fig. 4B). Pre-incubation with $1,25(\text{OH})_2\text{D}_3$ significantly enhanced DEX induced GR binding to 6.0 ± 0.4 fold ($p<0.05$) and 7.6 ± 1.8 fold ($p<0.05$) respectively in SS and SR asthmatics (Fig. 4B). The amount of GR bound to the MKP-1 promoter in response to VitD/DEX treatment was significantly greater in the cells of SS asthmatics as compared to SR asthmatics ($p<0.05$).

Because histone acetylation is an indication of active gene transcription^{16, 34}, we tested histone H4 acetylation levels around the region of GRE in the MKP-1 promoter by ChIP assay. The results showed that DEX treatment significantly increased histone H4 acetylation by 17.5 ± 4.1 fold ($p<0.05$) and 9.1 ± 1.1 fold ($p<0.05$) respectively comparing with media control in SS and SR asthmatics (Fig. 4C). Significantly higher histone H4 acetylation was observed in SS asthmatics than in SR asthmatics following DEX treatment ($p<0.05$) (Fig. 4C). Pre-incubation with $1,25(\text{OH})_2\text{D}_3$ significantly enhanced DEX-mediated histone H4 acetylation to 36.3 ± 2.8 fold ($p<0.05$) and 18.3 ± 0.9 fold ($p<0.05$) respectively in SS and SR asthmatics (Fig. 4C). In response to VitD/DEX treatment, significantly greater histone H4 acetylation was observed at the MKP-1 promoter of SS asthmatics as compared to SR asthmatics ($p<0.01$).

DEX differentially up-regulated GR phosphorylation at S211 in monocytes from SS and SR asthmatics

To determine whether the differential induction of MKP-1 by DEX in the monocytes of SS and SR asthmatics are related to differences in GR phosphorylation status following treatment with DEX, we tested GR phosphorylation at S211 site. This site is associated with transcriptionally active GR^{24, 35}. PBMC from SS and SR asthmatics were pre-incubated with 10nM VitD for 21h or vehicle control, followed by DEX treatment at 10nM for 3h. An adherent PBMC fraction was collected. Western blot results demonstrated that DEX induced GR phosphorylation at S211 by 2.6 ± 0.1 fold ($p<0.01$), and 2.3 ± 0.1 fold ($p<0.01$) respectively comparing with media control in SS and SR asthmatics (Fig. 5B, D). However,

the S211 phosphorylation induced by DEX was significantly higher in SS asthmatics than in SR asthmatics (Fig. 4D). Pretreatment with 1,25(OH)₂D₃ had no effect on GR phosphorylation at S211. VitD pretreatment did not enhance GR phosphorylation at S211 in response to DEX, and GR S211 phosphorylation levels remained significantly greater in the cells of SS asthmatics as compared to SR asthmatics ($p < 0.05$) (Fig. 5D).

Discussion

In the current study, we examined the anti-inflammatory effects of VitD, DEX and VitD/DEX combination in monocytes from SS and SR asthmatics. We demonstrated that DEX had a significantly reduced ability to suppress LPS-induced cell activation in the cells of SR asthmatics as compared to SS asthmatics. At the same time, we demonstrated significant suppression of LPS-induced p38 MAPK activation and cytokine production in these cells by VitD, irrespectively whether the cells were from SR or SS asthmatics. The data demonstrates that VitD, as compared to DEX, has superior anti-inflammatory function in the cells of SR asthmatics. As well, we showed an enhancement of cellular responses to corticosteroids in the cells from SR and SS asthmatics if the cells were pretreated with VitD. However, regardless of enhancement of DEX responses by VitD, the cells of SR asthmatics remained less responsive to DEX treatment as compared to the cells from SS asthmatics.

Despite the excellent efficacy of corticosteroids in controlling asthmatic symptoms, a proportion of asthma patients (SR asthmatics) do not respond to corticosteroids^{18, 36}. Patients with SR asthma have persistent airway inflammation despite treatment with corticosteroids and therefore could be predisposed to increased airway remodeling and irreversible lung disease³⁰. This has become a challenging health problem that contributes to the high costs of asthma care^{37, 38}. Therefore, it is desirable to know the mechanisms of differential actions of corticosteroids in SS and SR asthmatics, and also develop approaches to enhance responses to corticosteroids in SR asthmatics.

Recent studies demonstrated that administration of vitamin D to healthy individuals and SR asthmatic patients enhanced subsequent responsiveness to dexamethasone for induction of IL-10 *in vitro*³⁹. Also, vitamin D had been shown to inhibit TH17 cytokine production *in vitro* in severe asthmatics, irrespective of their clinical responsiveness to corticosteroids⁴⁰. In our previous study using monocytes from healthy controls donors we found that VitD inhibited LPS-induced cell activation by reducing p38 MAPK phosphorylation due to increased MKP-1 production¹⁶. In the current study, VitD exerted similar effects in the cells from asthmatic patients. However, DEX inhibited LPS-induced p38 phosphorylation only in SS asthmatics. This observation prompted us to test MKP-1 expression in response to DEX and VitD treatment in monocytes of SR and SS asthmatics. The results showed a significantly higher induction of MKP-1 by DEX in SS asthmatics than in SR asthmatics, consistent with our p38 MAPK phosphorylation data. We then tested GR binding to a GRE located 4.6kbp upstream of the MKP-1 promoter; this GRE is reported to be the predominant GR binding site in human MKP-1 promoter²⁶. Increased GR binding to GRE and a concomitant higher acetylation of histone H4 around the GRE in MKP-1 promoter were observed in the cells of SS asthmatics as compared to SR asthmatics, indicating a higher GR activity in SS asthmatics for MKP-1 gene transcriptional activation.

In this study we had limited number of cells available and we were not able to evaluate MKP-1 protein and mRNA expression in response to LPS stimulation after treatment with DEX and VitD both individually and in combination. However, we have previously demonstrated changes in MKP-1 mRNA production after VitD treatment and LPS stimulation in the monocytes from healthy control subjects. LPS treatment resulted in the upregulation of MKP-1 mRNA expression ($p<0.05$). Cellular MKP-1 mRNA levels were further induced (Mean $\pm$ SD) 4.5 ± 1.4 fold in response to LPS/DEX ($p<0.01$ as compared to LPS treated cells). MKP-1 mRNA expression in response to LPS was significantly higher in VitD pretreated cells ($p<0.01$ as compared to the cells cultured in media alone for 24h, followed by 6h LPS treatment). MKP-1 mRNA expression was the highest in the cells pretreated with VitD and stimulated with DEX and LPS as compared to all other treatment conditions¹⁷. As well, we previously reported changes in MKP-1 protein expression in response to VitD treatment in the monocytes from healthy control subjects¹⁶.

We noted that in the monocytes from SR asthmatics the effect of VitD on DEX-induction of MKP-1 does not translate into an effect on p-p38, as some degree of MKP-1 mRNA enhancement by VitD/DEX combination was observed in the monocytes of SR asthmatics, while this enhancement did not result in increased suppression of LPS-induced p38 MAPK activation by VitD/DEX combination as compared to VitD alone. These differences are likely related to the different read-outs used in these assays, i.e. p38 MAPK phosphorylation vs MKP-1 mRNA induction, with MKP-1 mRNA changes being more sensitive. As well, as assessed by VitD and DEX effects on IL-6 production some measure of cooperativity between VitD and DEX was observed in SR asthmatics. However, in the presence of VitD/DEX about 40% of LPS-induced IL-6 was still in culture from SR asthmatics, while only 20% of the LPS-induced levels were present in cultures from SS patients, suggesting that VitD/DEX-enhancing effects were less efficient in the SR asthma cell cultures.

Using the cells from healthy control donors, we recently reported that VitD enhancement of DEX-induced MKP-1 transcription occurred due to increased GR binding to the GRE 4.6kbp upstream of the MKP-1 gene which was mediated by adjacent vitamin D receptor (VDR) binding to the MKP-1 promoter and shared MED14 co-activator between GR and VDR¹⁷. GR transcriptional activity is closely related to the phosphorylation state at three serine residues (S203, S211 and S226) in the N-terminal transcriptional activation region of GR⁴¹. It has been demonstrated that S203 phosphorylated GR localizes to the cytoplasm, S226 phosphorylated GR inhibits gene transcription, and S211 phosphorylated GR is induced by ligand binding to GR and directly correlates with the degree of GR nuclear translocation and transactivation^{42, 43}. We, therefore, tested DEX-induced GR phosphorylation at S211 in asthmatic monocytes. Significantly higher GR phosphorylation levels at S211 in response to DEX was observed in the cells from SS asthmatics as compared to SR asthmatics. VitD treatment did not affect GR phosphorylation at S211 and did not enhance GR phosphorylation at S211 in response to DEX.

It has been reported that S211 GR phosphorylation enhances GR interaction with mediator complex subunit 14 (MED14) co-activator^{35, 43}. Since we previously demonstrated that the enhancement of GR-induced MKP-1 transcription by VitD is mediated by shared with GR MED14 co-activator, we propose that reduced S211 GR phosphorylation in the cells from

SR asthmatics results in reduced efficacy of VitD-mediated enhancement of MKP-1 mRNA transcription in SR asthma monocytes.

Vitamin D levels are normally measured by serum 25(OH)D₃ levels; as this form of vitamin D is more stable, while the active form of vitamin D, 1,25(OH)₂D₃ has a short half-life⁴⁴. Human monocytes have the ability to convert 25(OH)D₃ to an active form^{45, 46}. Studies have concluded that serum concentrations of 25(OH)D₃ are important as the available circulating 25(OH)D₃ influences local tissue concentrations of the active vitamin D^{45, 46}. Our previous data¹⁶ support the idea that in order to achieve optimal anti-inflammatory effects by vitamin D it is important to maintain serum vitamin D levels above 30 ng/ml in the physiologic range^{1, 47}. We previously assessed the anti-inflammatory effects of 25(OH)D₃ doses that are related to vitamin D deficiency (15 ng/ml) and vitamin D sufficiency (30 ng/ml – lower normal range, 50 ng/ml and 70 ng/ml – upper normal range for the serum vitamin D levels⁴⁸) and compared these effects to several doses of active form of vitamin D, 1,25(OH)₂D₃, using the cells from healthy normal controls¹⁶. We demonstrated that 15 ng/ml of the 25(OH)D₃ (a concentration considered as vitamin D deficiency⁴⁸) did not suppress LPS-induced IL-6 and TNF-α production in human monocytes. We found that 25(OH)D₃ at 30 ng/ml and higher, i.e. levels considered vitamin D sufficient in humans, significantly inhibited IL-6 and TNF-α production induced by LPS. Furthermore, the degree of suppression of IL-6 and TNF-α production by 30 ng/ml of 25(OH)D₃ was comparable to the effects achieved by 0.1 nM of the active form of the vitamin D¹⁶. The effects achieved by 50–70ng/ml of 25(OH)D₃ were comparable to the effects of 10nM 1,25(OH)₂D₃¹⁶. In this study we used 10nM 1,25(OH)₂D₃. Based on the comparisons to effects of this dose of active form of vitamin D to the effects of 25(OH)D₃, the concentrations used in these experiments are realistically attainable and safe.

In summary, our study has several novel findings, which may have important clinical implications. First, despite reduced responses to DEX in the cells of SR asthmatics, VitD alone can inhibit LPS-induced activation in the cells from these patients, as shown by inhibition of LPS-induced p38 MAPK activation and IL-6 production. Second, VitD demonstrates corticosteroid-enhancing effects in the monocytes from SR and SS asthmatics as shown by increased MKP-1 production in response to DEX after VitD pretreatment. Third, despite the enhancement by VitD, the responses to corticosteroids, in SR asthmatics' monocytes, remained significantly lower as compared to SS asthmatics. Lower MKP-1 mRNA induction in response to DEX or VitD/DEX treatment was observed in monocytes of SR asthmatics due to reduced GR binding and histone H4 acetylation at MKP-1 promoter in these cells. Reduced GR binding to the MKP-1 promoter may be related to the reduced levels of DEX-induced GR phosphorylation at S211 in the monocytes of SR asthmatics. The conclusions of this work are limited by the small sample size studied and the findings should be replicated. Nevertheless, this work sets the stage to address potential benefits and biomarkers for monitoring clinical VitD supplementation in asthmatic patients treated with corticosteroids.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ChIP	chromatin immunoprecipitation
DEX	dexamethasone
GR	glucocorticoid receptor
GRE	glucocorticoid response element
ICS	inhaled corticosteroids
LPS	lipopolysaccharide
MKP-1	mitogen activated kinase phosphatase 1
PBMC	peripheral blood mononuclear cells
p-p38	phosphorylated p38 MAPK
VDR	vitamin D receptor
VitD	1,25-dihydroxyvitamin D (1,25(OH) ₂ D)

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Key Messages

- Monocytes of SR asthmatics are less responsive to DEX *in vitro* as compared to SS asthmatics as shown by DEX suppression of both LPS-induced p38 MAPK activation and IL-6 production and DEX induction of MKP-1.
- VitD alone demonstrates anti-inflammatory effects in the cells of SR and SS asthmatics, as it inhibits LPS-induced p38 MAPK activation and IL-6 production and stimulates monocyte MKP-1 expression.
- VitD pretreatment enhances DEX responses *in vitro* in the monocytes of SR and SS asthmatics via increased MKP-1 production as a result of increased GR binding to the MKP-1 promoter mediated by VitD receptor.
- Despite the enhancement of corticosteroid responses by VitD, VitD pretreatment does not overcome reduced responses to corticosteroids in the cells of SR asthmatics. The levels of DEX-induced GR phosphorylation at S211 are significantly lower in SR asthmatic monocytes as compared to SS asthmatics monocytes. VitD pretreatment does not affect GR phosphorylation at this site.

Fig. 1.

VitD enhances DEX inhibition of LPS-induced p38 phosphorylation in monocytes from SS asthmatics (n=8), but not from SR asthmatics (n=9) (A) as shown by flow cytometry. (B) Representative flow cytometry data on the effects VitD, DEX and VitD+DEX on LPS-induced p-p38 in CD14+ cells from SS and SR asthmatic is shown.

Fig. 2.

VitD enhances DEX inhibition of LPS-induced IL-6 production in monocytes from SS and SR asthmatics as detected by ELISA. Percentages of LPS-induced IL-6 levels for each treatment condition as compared to the amount of IL-6 produced in response to LPS alone (100%) are shown (n=5 SR asthmatics and n=5 SS asthmatics).

Fig. 3.

VitD enhances DEX induction of MKP-1 in monocytes from SS and SR asthmatics. PBMC from SS and SR asthmatics were pre-incubated with 10nM VitD for 21h, followed by DEX treatment at 10nM for 3h. An adherent PBMC fraction was collected. MKP-1 mRNA levels were detected by real-time PCR and normalized to β -actin mRNA (n=8 SR asthmatics and n=8 SS asthmatics).

A.

B.

C.

Fig. 4.

Differential recruitment of GR to the GRE in the MKP-1 gene promoter in monocytes from SS and SR asthmatics.

(A) Schematic representation of the GR binding to GRE 4.6 and histone acetylation in human MKP-1 promoter. The number represents kbp upstream of the transcriptional start site (TSS). Recruitment of GR (B) and histone H4 acetylation (C) at GRE 4.6 site (n=3).

A. **B.** **Fig. 5.**

S211 GR phosphorylation in response to DEX with and without VitD pretreatment in the cells of SS and SR asthmatics.

(A) Results from a representative Western blot comparing monocytes from SR and SS asthmatics are shown out 5 SR and 5 SS asthmatics studied. (B) Blotting densitometry readings summary of GR S211 phosphorylation normalized to total GR levels.

Table I

Patient characteristics

	SR asthma n=11	SS asthma n=8
Age, yrs, (Mean±SD)	42.5±12.3	41.4±13.8
Gender (Male/Female)	5/6	4/4
Race (C/AA/Other)	7/1/3	7/0/1
BMI, kg/m ² , (Mean±SD)	27.9±5.9	31.3±9.8
IgE, U/ml, (Mean±SD)	178±181	127±87
ACQ-7	2.5±1.2	2.7±0.9
Baseline FEV ₁ % predicted, (Mean±SD)	66.2±16.2	65.0±11.3
FEV ₁ % reversal with Albuterol, (Mean±SD)	27.8±22.4	38.0±24.6
FEV ₁ % change after Prednisone burst, (Mean±SD)	0.1±5.3*	33.1±17.5
Corticosteroid medications***		
ICS/LABA	4	3
ICS	3	0
none	4	5

* p<0.0001 as compared to SS asthmatics

** For the SR and SS asthmatics that received ICS/LABA or ICS the Mean±SE of the ICS dose in budesonide equivalents was 1000±344 µg and 940±681 µg, respectively.