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Vitamin D regulates contractile Profile in Human uterine myometrial Cells via NFκB pathway

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Abstract

OBJECTIVE—Infection triggers inflammation which in turn enhances the expression of contractile associated factors in myometrium and increases the risk of preterm delivery. In this study we assessed vitamin D regulation of inflammatory markers, contractile-associated factors, steroid hormone receptors and NFκB pathway proteins in human uterine myometrial smooth muscle (UtSM) cells cultured in an inflammatory environment.

STUDY DESIGN—Inflammatory environment was simulated for UtSM cells by co-culturing them with monocyte lineage (THP1) cells. We measured the expression of inflammatory markers, contractile-associated factors, steroid hormone receptors and NFκB pathway proteins in UtSM cells cultured with THP1 cells in the presence and absence of vitamin D by RT-PCR and western analysis.

RESULTS—Monocytes secreted MIP-1α, MIP-1 β, IL-1β, IL6, and TNFα into the conditioned medium. In UtSM cells co-cultured with THP1 cells there was a significant (p<0.05) increase in the expression of inflammatory markers IL-1β, IL6, IL13 and TNFα; the contractile associated factors connexin-43, cox-2 and prostaglandin F₂α receptor; the estrogen receptor α and progesterone receptors A and B. Vitamin D treatment of co-cultures decreased (p<0.05) the expression of inflammatory markers and contractile associated factors in UtSM cells. Similarly vitamin D decreased estrogen receptor α and progesterone receptors A to B ratio in UtSM cells co-cultured with THP1 cells. In addition, vitamin D treatment significantly (p<0.05) decreased monocyte induced p-IκBα in cytosol, NFκB-p65 in the nucleus and increased IκBα in cytosol in UtSM cells.

CONCLUSION—Our results suggest that vitamin D treatment decreases inflammation induced cytokines and contractile associated factors in the uterine myometrial smooth muscle cells via NFκB pathway.

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Keywords

Monocytes; Myometrial cells; inflammatory markers; contractile associated factors; steroid hormone receptors; NFκB

INTRODUCTION

Infection induces inflammatory reactions mainly through the production of cytokines.¹ Inflammation is one of the responses of the body, to infection, to remove the injurious stimuli and to initiate the healing process.²⁻⁴ In addition, controlled aseptic inflammations were reported to play a role in normal parturition at term.⁵⁻⁷ On the other hand untimely activation of inflammation has been implicated in preterm labor. Infection not only initiates inflammatory responses but was reported to be responsible for 40% of the premature births.⁸ Cell derived and plasma derived mediators play a role in infection induced inflammatory reactions.⁹ Lipopolysaccharide (LPS), a bacterial endotoxin has been reported to increase the cytokine secretion in human myometrial cells,¹⁰ enhance prostaglandin production in rodent and human myometrium^{6,11,12} and cause preterm delivery in rodents.¹³ In turn cytokine treatments have been reported to increase expression of prostaglandins,^{5,7,14,15} connexin-43,^{16,17} oxytocin and oxytocin receptor^{18,19} suggesting that infection increases cytokines which in turn increase contractile associated proteins and initiate preterm delivery.

The expression levels of progesterone receptor A and estrogen receptor α were reported to be high not only at term but also in preterm deliveries.²⁰⁻²² Even though infection was reported to increase inflammation and cause preterm deliveries it is not clear if the expression of the progesterone and the estrogen receptors was altered in myometrium upon infection-induced inflammation. Furthermore, infection was reported to induce phosphorylation of IκB α , which leads to the translocation of NFκB into nucleus,²³ increase production of the proinflammatory cytokines²⁴ and increase expression of contractile associated factors.²⁵ Vitamin D was reported to play a role in antagonizing the inflammatory effect of infections in addition to its classical effect on calcium and phosphate homeostasis.²⁶ Studies showed that vitamin D treatment has been used successfully to treat tuberculosis.^{27, 28} In addition, recent findings suggest that vitamin D improves cytokine profile in patients with congestive heart failure²⁹ and inhibits both cytokines and contractile associated proteins in myometrial cells treated with LPS.¹⁰ Vitamin D has also been reported to regulate NFκB pathway proteins in decidual cells.³⁰ However, the effect of vitamin D action on myometrial cells in a biological system that mimics in vivo conditions and examines cross talk between immune and non immune cells within the uterus is not yet clearly defined. In this study we used monocyte lineage THP1 cells to simulate an in vivo environment of inflammation for uterine smooth muscle cells and assess if vitamin D regulates proinflammatory cytokines, contractile associated proteins, steroid hormones receptor expression, and NFκB pathway proteins in human uterine myometrial smooth muscle (UtSM) cells.

MATERIALS AND METHODS

Cell lines and Reagents

The UtSM cell line was a generous gift from Dr. Darlene Dixon (National Institute of Environmental Health Sciences; NIEHS). Monocyte lineage cells (THP1) were obtained from ATCC (Manassas, VA). Smooth muscle basal medium (SmBM), growth factors and antibiotics, Gentamicin sulphate and Amphotericin B were obtained from Lonza (Walkersville, MD). RPMI medium and connexin-43 antibody were obtained from Life Technologies (Grand Island, NY). Vitamin D3, absolute alcohol, and β -actin antibody were

obtained from Sigma (St. Louis, MO). SYBR green was obtained from Bio-Rad (Hercules, CA). Progesterone receptor and estrogen receptor α antibodies were purchased from Santa Cruz biotechnology (Santa Cruz, CA), cox-2 antibody from Cayman chemicals (Ann Arbor, MI), and NF κ B-p65, I κ B α and p-I κ B α antibodies from Abcam (Cambridge, MA).

Cell culture

Immortalized adherent UtSM cells were cultured in SmBM medium supplemented with 5% FBS, human epidermal growth factor (0.1%), recombinant human fibroblast growth factor (0.2%), 0.1% insulin and 0.1% antibiotics Gentamicin sulphate and Amphotericin B (Lonza). These cells were maintained at 37°C in a humidified atmosphere of air and 5% CO₂.

Monocyte culture

Non adherent THP1 cells were cultured in RPMI medium supplemented with 10% FBS, 1mM sodium pyruvate, 2mM glutamine, 1mM HEPES, and 0.05mM β -Mercaptoethanol (BME;Sigma). They were maintained at 37°C in a humidified atmosphere of air and 5% CO₂.

Co-culture

UtSM cells grown to 90% confluence in 60 mm dishes were starved for 24h in RPMI medium containing 0.1% BSA (Sigma), sodium pyruvate, glutamine, HEPES, and BME. Inflammatory environment was created for UtSM cells by co-culturing them with THP1 (200 000;200K) cells. Co-cultures of UtSM and THP1 cells were treated in triplicates with 0 (control), 5, 10, 50 150 and 300nM of 1, 25 (OH)₂ vitamin D (Sigma) dissolved in alcohol (Sigma). Myometrial cells cultured alone served as normal control. Co-cultures were maintained at 37°C in a humidified atmosphere of air and 5% CO₂ on a platform shaker at slow speed. After the treatment, adherent UtSM cells were washed once with diluted Trypsin-EDTA (Life Technologies) and twice with ice cold PBS to remove monocytes that might have been attached to the adherent myometrial cells.

Chemokine and cytokine assay

Conditioned media was collected from monocyte (200k) and UtSM cells cultured separately. Monocytes in conditioned medium were removed by centrifugation at 10000 rpm at 4°C and the supernatant was stored at -70°C until further analysis.

We measured the levels of MIP-1 α , MIP-1 β , IL-1 β , IL6, and TNF α in the conditioned media obtained from monocytes and UtSM cell cultures by multiplex assay system (Luminex 100 system; Hormone Assay Core, Vanderbilt University, Nashville TN). Levels of chemokines and cytokines measured were represented as pg/ml.

Isolation of total RNA and Reverse Transcription

Total RNA was extracted using RNeasy kit (Qiagen, Valencia, CA) from the UtSM cells cultured alone and from UtSM cells co-cultured with THP1 cells in the presence and absence of vitamin D. RNA obtained was treated with DNase I (Qiagen) enzyme to remove genomic DNA contamination. As described earlier¹⁰ 1.0 μ g of total RNA was briefly mixed with 3.0 n mol of oligo dT, 200 μ M dNTP, 5U RNase inhibitor and 10 U of avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI) in a total volume of 20 μ l. Complimentary DNA (cDNA) was generated by reverse transcription in a thermal cycler set at 70°C for 5 min, 25°C for 10 min. and 42°C for 60min and stored at -20°C until analysis.

Real Time Polymerase Chain Reaction (RT-PCR)

Briefly 50ng of cDNA were added to the master mix containing SYBR green (Bio-Rad) and specific primers for IL-1 β , IL6, IL13, and TNF α , connexin-43, prostaglandin receptor, oxytocin receptor and GAPDH. Primer sequences were obtained from peer reviewed publications (Table 1). All primer sets used for RT-PCR generated a single amplicon. RT-PCR was performed in a Bio-Rad MyiQ5. Genes were amplified at 95°C for 15 sec and 60°C for 1 min for 40 cycles. Data obtained was normalized using respective GAPDH value.

Whole cell lysates and cytosol preparation

Whole cell lysates, cytosol and nuclear fractions were prepared from UtSM cells as described by Suzuki et al ³¹ with slight modifications. Briefly, monolayer cells in 60 mm dishes were washed once with diluted Trypsin-EDTA and twice with ice cold PBS to remove any attached monocytes. Lysates were prepared using RIPA buffer containing 1X protease inhibitor cocktail, 1mM sodium vanadate, 0.1% NP40 and 1mM sodium fluoride (Sigma). Part of the whole cell lysate was subjected to brief sonication using microprobe from Misonix Incorporated (Farmingdale, NY) and stored at -80°C until analysis. Remaining lysate was centrifuged at 10,000 RPM, for 10 min at 4°C on a bench top centrifuge. Supernatant (cytosol) was stored at -80°C until further analysis. Pellet was used for nuclear protein preparation.

Nuclear protein preparation

Pelleted nuclei were suspended in 75 μ L of RIPA buffer containing 1X protease inhibitor cocktail and 1 mmol/L dithiothreitol (Sigma), and incubated for 15 minutes on ice. Nuclear pellets were sonicated twice for 5 sec using microprobes from Misonix (Farmingdale, USA) and centrifuged at 16000 x g for 15 minutes at 4°C. Supernatants containing the nuclear proteins were stored at -80°C until analysis.

Western blot analysis

Equal amounts of protein were resolved on a 10% Tris-Bis gels and transferred onto PVDF membranes. Western blot analysis was performed using primary antibodies against cox-2 (1:500), connexin-43 (1:500), estrogen receptor α (1:500), progesterone receptors A (1:500) and B (1:250), I κ B α , p-I κ B α , NF κ B-p65 (2ng/ml) and β -actin (1:5000). The protein signal intensity was quantified using an image documentation system (ProteinSimple, Santa Clara, CA) and normalized with the corresponding β -actin values.

RESULTS

Monocytes secreted chemokines and cytokines into culture media

We measured chemokines and cytokines in conditioned media obtained from both monocytes and UtSM cells. Chemokines MIP-1 α and MIP-1 β and cytokines IL-1 β , IL6, and TNF α were significantly higher in monocyte cultures, while their levels were below the detection limit in UtSM cultures (Table. 2).

Vitamin D decreased mRNA expression of proinflammatory markers in UtSM cells cocultured with monocytes

Real time PCR was performed to assess the effects of vitamin D on inflammatory cytokines in UtSM cells co-cultured with monocytes. UtSM cells when cultured with THP1 cells showed a significant ($p < 0.01$) increase in the mRNA expression of IL-1 β , IL6, IL13 and TNF α . However, treatment of co-cultures with vitamin D significantly ($p < 0.05$) decreased their expression in UtSM cells when compared to that in the vehicle treated co-cultures (Fig. 1).

Vitamin D reduced monocyte induced contractile associated proteins in uterine myometrial smooth muscle cells

We measured the mRNA expression of contractile associated proteins in UtSM cells co-cultured with monocytes in the presence and absence of vitamin D. Real time PCR analysis showed a significant ($p<0.05$) increase in the expression of connexin-43, prostaglandin F2 α receptor and, oxytocin receptor in UtSM cells co-cultured with monocytes (Fig. 2). However, treatment of co-cultures with vitamin D significantly ($p<0.05$) decreased their expression in UtSM cells in comparison to that in the vehicle treated co-cultures (Fig. 2).

Western blot analysis performed showed similar increases ($p<0.05$) in cox-2 and connexin-43 expression in UtSM cells co-cultured with monocytes while vitamin D treatment significantly ($p<0.05$) decreased their expression in comparison to that in the vehicle treated co-cultures (Fig. 3).

Vitamin D decreased estrogen receptor α in UtSM cells co-cultured with monocytes

We measured estrogen receptor α expression in UtSM cells co-cultured with monocytes and treated with various concentrations of vitamin D. Western analysis of nuclear protein obtained from UtSM cells co-cultured with monocytes showed a significant ($p<0.01$) increase in the expression of estrogen receptor α in UtSM cells in co-culture when compared to vehicle treated control (Fig. 4). Vitamin D treatment of co-cultures significantly ($p<0.01$) decreased estrogen receptor α expression in UtSM cells at 150 and 300nM concentrations when compared to that in the vehicle treated co-cultures.

Vitamin D treatment altered progesterone A to B ratio in uterine myometrial smooth muscle cells co-cultured with monocytes

We measured the changes in the individual progesterone receptors expression and assessed if vitamin D regulates their expression in UtSM cells co-cultured with monocytes by western analysis. When cultured along with monocytes, UtSM cells showed a significant ($p<0.01$) increase in the expression of progesterone receptor A and decrease in progesterone receptor B. However treatment of co-cultures with vitamin D significantly ($p<0.05$) reversed the increase and decrease observed in progesterone receptor A and B respectively in UtSM cells (Fig. 5).

Vitamin D reduced phosphorylation of I κ B α and nuclear translocation of NF κ B-p65 in UtSM cells co-cultured with monocytes

Inflammation during parturition was reported to cause phosphorylation of I κ B α and nuclear translocation of NF κ B-p65 leading to the activation of NF κ B pathway. In this study we assessed if vitamin D inhibits phosphorylation of I κ B α and nuclear translocation of NF κ B-p65 in UtSM cells co-cultured with monocytes. Western analysis of cytosolic fraction obtained from UtSM cells co-cultured with monocytes showed a significant ($p<0.05$) increase in the expression of p-I κ B α . Addition of vitamin D (150nM) to co-cultures significantly ($p<0.01$) decreased cytosolic p-I κ B α expression in UtSM cells. On the contrary, vitamin D treatment of co-cultures significantly ($p<0.05$) increased the expression of I κ B α in cytosol in UtSM cells (Fig. 6). Western analysis of nuclear protein obtained from UtSM cells co-cultured with monocytes showed a significant ($p<0.01$) increase in the expression of NF κ B-p65 while vitamin D treatment of co-cultures significantly ($p<0.01$) decreased its expression (Fig. 7). On the other hand expression of NF κ B-p65 showed a significant ($p<0.05$) decrease in the cytosolic fraction obtained from UtSM cells co-cultured with monocytes, while vitamin D (150 and 300nM) treatment of co-cultures significantly ($p<0.01$) increased its expression (Fig. 7).

Comment

In this study we established an inflammatory environment for UtSM cells by co-culturing them with human monocyte lineage (THP1) cells. We assessed vitamin D regulation of the inflammatory markers, contractile-associated factors, estrogen receptor α , progesterone receptors A and B, and members of inflammatory pathway in UtSM cells co-cultured with THP1 cells. Our studies demonstrated that THP1 cells secrete chemokines and cytokines. Furthermore, UtSM cells co-cultured with monocytes showed an increase while vitamin D treatment decreased the mRNA/protein expression of IL-1 β , IL6, IL13, TNF α , connexin-43, prostaglandin (FP) receptor, oxytocin receptor, estrogen receptor α , progesterone receptor A/B ratio, p-I κ B α in cytosol, NF κ B-p65 in the nuclear fraction. These results suggest that vitamin D decreases inflammation induced inflammatory markers and contractile associated factors in UtSM cells through NF κ B pathway.

Our aim in this study was to assess paracrine effects of monocytes and their regulation by vitamin D. As transformed macrophages tend to attach and removal of attached macrophages by physical or chemical methods can compromise the integrity of the myometrial cells we did not transform monocytes to macrophages. We observed significant amounts of MIP-1 α , MIP-1 β and moderate levels of IL-1 β , IL6, and TNF α in the conditioned media obtained from monocyte cultures. Similar increases in chemokines and cytokines irrespective of external stimulus were reported in monocytes.^{32,33} Real time PCR analysis in UtSM cells co-cultured with monocytes in the current study showed an increase in the mRNA expression of inflammatory markers IL-1 β , IL6, IL13 and TNF α compared to the normal (control) cells. It was reported that enhanced expression of proinflammatory markers, chemokines and cytokines contributes to inflammation reported during parturition at term delivery.³⁴ Studies also showed involvement of cytokines in the intrauterine infection-induced preterm labor^{35,36} suggesting a common mechanism in term and preterm labor. In co-cultures treated with vitamin D, we observed a decrease in the expression of IL-1 β , IL6, IL13 and TNF α in UtSM cells which suggests that vitamin D decreases inflammation induced proinflammatory cytokine environment. Vitamin D treatment was also reported to decrease the synthesis of cytokines in decidual NK cells³⁷ suggesting a role for vitamin D in regulation of infection induced inflammation. Since infection is known to increase the risk for preterm birth, vitamin D could play an important role in the maintenance of pregnancy and prevention of infection and inflammation-induced preterm birth. The human myometrial cells co-cultured with monocytes in this study showed higher expression of contractile associated factors connexin-43, prostaglandin receptor and oxytocin receptor compared to that in control. Results from our studies¹⁰ and others¹ assessing effects of bacterial infections during pregnancy also reported similar increases in contractile associated factors. These results suggest that inflammation plays a role in induction of myometrial contractility at labor by enhancing expression of contractile associated proteins in uterine myometrial cells. Furthermore, vitamin D treatment of co-cultures in our current study decreased the expression of contractile associated factors in myometrial cells suggesting that vitamin D may play a role in the maintenance of pregnancy by inhibiting inflammation induced contractile associated proteins.

Because THP1 cells in co-cultures of control and treated cells were removed using the same process any mRNA contribution from the THP1 cells still left behind should be same in both the control and treated UtSM cells. Therefore, we believe that the increases in inflammatory markers and contractile associated factors observed in myometrial cells in our study were because of the paracrine effect of cytokines secreted by monocytes. However, to assess if cell to cell interaction is essential, we plan to co-culture transformed macrophages with myometrial cells and measure the difference in response in our future studies. In this study we wanted to assess if vitamin D inhibits the effects of cytokines secreted by the unstimulated monocytes on UtSM cells. Therefore, we did not treat monocytes with LPS.

However, we will assess the role of vitamin D in the regulation of cytokines and contractile associated factors in myometrial cells co-cultured with THP1 cells in the presence of LPS and with monocyte conditioned media in our future studies.

Estrogen plays a role in the process of labor while progesterone plays a role in the maintenance of gestation.³⁸ UtSM cells co-cultured with monocytes showed an increase in estrogen receptor α suggesting that inflammatory environment increases the expression of estrogen receptors in myometrium. This is in agreement with prior observation that inflammation is involved in the process of labor³⁴ and that estrogen receptor α increase during the process of labor and delivery.³⁸ Vitamin D treatment of co-cultures in the present study decreased the expression of estrogen receptor α in UtSM cells suggesting that vitamin D plays a role in regulating estrogen receptor expression during pregnancy. In UtSM cells co-cultured with monocytes there was an increase in the expression of progesterone receptor A and a decrease in progesterone receptor B. Literature suggests that the ratio of the progesterone receptors determine the affinity of the receptors. A low progesterone receptor A to B ratio indicate high affinity to progesterone and in turn uterine quiescence; while an increase in progesterone receptor A to B ratio indicate low affinity to progesterone leading to functional withdrawal of progesterone and onset of labor.²⁰ However, vitamin D treatment decreased progesterone receptor A to B ratio in UtSM cells in the present study. Decrease observed with vitamin D in both estrogen receptor α and progesterone receptor A to B ratio in UtSM cells co-cultured with THP1 cells suggests a role for vitamin D in uterine quiescence and maintenance of pregnancy.

In the present study we observed an increase in p-I κ B α in cytosol and NF κ B-p65 in the nuclear fraction in UtSM cells co-cultured with monocytes, in the same way as infection was reported to activate inhibitory kinases, increase phosphorylation and degradation of I κ B α , nuclear translocation of NF κ B-p65 and transactivation of proinflammatory markers.³⁹ The results suggest that infection activates NF κ B pathway and increases cytokine secretion. Increase in nuclear localization of NF κ B-p65 was also reported in the decidual and fetal membranes with advancing gestation^{40, 41} suggesting that NF κ B plays a role both in infection induced preterm deliveries and in inflammation induced normal delivery at term pregnancy. In our study, vitamin D treatment decreased the expression of p-I κ B α in cytosol and NF κ B-p65 in the nuclear fraction and increased the expression of I κ B α in the cytosol of myometrial cells co-cultured with THP1 cells. Vitamin D has also been reported to decrease NF κ B activity in the macrophages, suggesting that vitamin D inhibits NF κ B activated inflammation and may help in maintenance of myometrial quiescence during gestation.³⁰ In summary, our findings suggest that vitamin D treatment inhibits monocyte-induced increases in inflammatory markers, contractile associated factors, estrogen receptor α and PRA/PRB ratio in UtSM cells. Our results also suggest that vitamin D prevents inflammation induced increases in contractile associated factors through NF κ B pathway. However additional studies are required to assess if administration of vitamin D can prevent infection induced preterm births in humans. Since vitamin D administered at higher concentrations can lead to hypercalcemia, use of less calcemic vitamin D analogues can be beneficial and can be a novel therapeutic approach for treating subjects with vitamin D deficiency.

Statistical Analysis

One way ANOVA was used to compare results between the control and test reagents. The data was represented as the mean $\pm$ SEM of three separate experiments. Real time PCR Data analysis was performed using the Bio-Rad iQ5 Optical System Software Version 2.

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Figure 1. Effects of vitamin D on mRNA expression of inflammatory markers in UtSM cells co-cultured with monocytes

The effects of vitamin D on the expression of cytokine genes in human uterine myometrial smooth muscle (UtSM) cells co-cultured with monocyte lineage THP1 cells. UtSM cells co-cultured with THP1 cells were treated with different concentrations of vitamin D for 24h, and the effects on IL-1 β , IL6, IL13, TNF α and GAPDH mRNA expression were measured by real time Polymerase chain reaction analysis. The data were normalized with respective GAPDH value. The bars represent the mean $\pm$ SEM from three replicates in each group. Groups with different alphabets at the top of the bar are significantly different ($p < 0.05$).

Figure 2. Effects of vitamin D on mRNA expression of contractile associated proteins in UtSM cells co-cultured with monocytes

Real time -polymerase chain reaction analysis of contractile-associated proteins in human uterine myometrial smooth muscle (UtSM) cells co-cultured with monocyte lineage THP1 cells and treated with vitamin D. UtSM cells co-cultured with THP1 cells were treated with different concentrations of vitamin D for 24h, and the RNA obtained was subjected to reverse transcription and real time PCR analysis using specific primers for connexin-43, prostaglandin F2α receptor, oxytocin receptor and GAPDH. The data were normalized with their respective GAPDH values. The bars represent the mean ± SEM from three replicates in each group. Groups with different alphabets at the top of the bar are significantly different (p<0.05).

Figure 3. Effects of vitamin D on contractile associated protein expression in UtSM cells co-cultured with monocytes

Western analyses of contractile associated factors in human uterine myometrial smooth muscle (UtSM) cells co-cultured with monocyte lineage THP1 cells and treated with vitamin D. UtSM cells co-cultured with THP1 cells were treated with different concentrations of vitamin D for 48h. Cell lysates prepared were subjected to western blot analysis using connexin-43, cox-2 and β-actin antibodies. β-actin was used as a loading control. The bars represent the mean ± SEM from three replicates in each group. Groups with different alphabets at the top of the bar are significantly different (p<0.05) from each other.

Figure 4. Effects of vitamin D on estrogen receptor α in UtSM cells co-cultured with monocytes
 The effects of vitamin D on the expression of nuclear estrogen receptor α in human uterine myometrial smooth muscle (UtSM) cells co-cultured with monocyte lineage THP1 cells. UtSM cells co-cultured with THP1 cells were treated with different concentrations of vitamin D for 48h. Nuclear fractions were subjected to western blot analysis using estrogen receptor α and β -actin antibodies. β -actin was used as a loading control. The bars represent the mean $\pm$ SEM from three replicates in each group. Groups with different alphabets at the top of the bar are significantly different ($p < 0.01$) from each other.

Figure 5. Effects of vitamin D on PRA/PRB ratio in UtSM cells co-cultured with monocytes
 The effects of vitamin D on the expression of progesterone receptors in human uterine myometrial smooth muscle (UtSM) cells co-cultured with monocyte lineage THP1 cells. UtSM cells co-cultured with THP1 cells were treated with different concentrations of vitamin D for 48h. Whole cell lysates prepared were subjected to western blot analysis using a) PRA, PRB and β -actin antibodies. β -actin was used as a loading control. The bars are a ratio between progesterone receptor A and B and represent the mean $\pm$ SEM from three replicates in each group. Groups with different alphabets at the top of the bar are significantly different ($p < 0.05$) from each other.

Figure 6. Effects of vitamin D on IκBα and p-IκBα expression in UtSM cells co-cultured with monocytes

The effects of vitamin D on the expression of IκBα and p-IκBα in immortalized human uterine myometrial smooth muscle (UtSM) cells co-cultured with monocyte lineage THP1 cells. UtSM cells co-cultured with THP1 cells were treated with different concentrations of vitamin D for 48h. Cytosol fractions were subjected to western blot analysis using IκBα, p-IκBα and β-actin antibodies. β-actin was used as a loading control. The bars represent the mean ± SEM from three replicates in each group. Groups with different alphabets at the top of the bar are significantly different (p<0.05) from each other.

Figure 7. Effects of vitamin D on NFκB-p65 nuclear translocation in UtSM cells co-cultured with monocytes

The effects of vitamin D on the expression of NFκB-p65 in immortalized human uterine myometrial smooth muscle (UtSM) cells co-cultured with monocyte lineage THP1 cells. UtSM cells co-cultured with THP1 cells were treated with different concentrations of vitamin D for 48h. Cytosol and nuclear fractions prepared were subjected to western blot analysis using NFκB-p65 and β-actin antibodies. β-actin was used as a loading control. The bars represent the mean ± SEM from three replicates in each group. Groups with different alphabets at the top of the bar are significantly different ($p < 0.05$) from each other.

Table 1

Human Forward and Reverse Primers for Real Time polymerase chain reaction

Gene	Primer sequence	Amplicon length	NCBI Accession#
IL-1 β Forward	5-CAA ATT CGG TAC ATC CTC GAC -3	73	NM_000576
IL-1 β Reverse	5-GTC AGG GGT GGT TAT TGC ATC -3		
IL6 Forward	5-AAA CAG ATG AAG TGC TCC TTC CAG G -3	339	NM_000600
IL6 Reverse	5-TGG AGA ACA CCA CTT GTT GCT CCA-3		
IL13 Forward	5-TGA GGA GCT GGT CAA CAT CA-3	76	NM_002188
IL13 Reverse	5-CAG GTT GAT GCT CCA TAC CAT-3		
TNF α Forward	5-CAG AGG GAA GAG TTC CCC AG -3	84	NM_000594
TNF α Reverse	5-CCT TGG TCT GGT AGG AGA CG-3		
Connexin-43 Forward	5'-CCT ATG TCT CCT CCT GGG TA-3'	176	NM_000615
Connexin-43 Reverse	5'-GGG AAA TCA AAA GGC TGT G-3'		
Prostaglandin F2 α Receptor Forward	5-GCA GCT GCG CTT CTT TCAA-3	81	NM_000959
Prostaglandin F2 α Receptor Reverse	5-CAC TGT CAT GAA GAT TAC TGA AAA AAA TAC-3		
Oxytocin Receptor Forward	5-CTG AAC ATC CCG AGG AAC TG-3	84	NM_000916
Oxytocin Receptor Reverse	5-CTC TGA GCC ACT GCA AAT GA-3		
GAPDH Forward	5-TGA TGA CAT CAA GAA GGT GGT-3	240	NM_002046
GAPDH Reverse	5-TCC TTG GAG GCC ATG TGG GCC-3		

Table 2

Levels of chemokines and cytokines in condition medium obtained from myometrial and monocyte cell cultures (n = 3)

Cells	MIP-1 α (pg/ml)	MIP-1 β (pg/ml)	IL-1 β (pg/ml)	TNF α (pg/ml)	IL-6 (pg/ml)
UtSM cells	<3.20	<3.20	<3.20	<3.20	<3.20
THP1 cells	86.61 $\pm$ 11	245.00 $\pm$ 45	5.20 $\pm$ 1.1	8.48 $\pm$ 1.5	10.06 $\pm$ 2.0