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$1,25(OH)_2D_3$ suppresses COX-2 up-regulation and thromboxane production in placental trophoblast cells in response to hypoxic stimulation

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Abstract

In this study, we determined if vitamin D could inhibit oxidative stress-induced thromboxane production by placental trophoblasts. Trophoblast isolated from normal placentas were stimulated with $CoCl_2$, a hypoxic mimicking agent, with or without pretreatment of $1,25(OH)_2D_3$. Soluble phospholipase-A_2, metabolites of thromboxane-A_2 and prostacyclin, and 8-isoprostane were measured. Expression of cyclooxygenase-1 (COX-1), COX-2, and heme oxygenase-1 (HO-1) were determined. We found that pretreatment of trophoblasts with $1,25(OH)_2D_3$ significantly reduced 8-isoprostane and the ratio of thromboxane-A_2 to prostacyclin production, and blocked COX-2 expression induced by $CoCl_2$. These results provide evidence of the beneficial effects of vitamin D on placental trophoblasts.

Keywords

vitamin D; COX-2; thromboxane; trophoblast

Introduction

Recent studies have shown that vitamin D is beneficial to both maternal and fetal health. Sufficient vitamin D intake during pregnancy reduces risk of pregnancy complications such as gestational diabetes and preterm birth [1,2]. In contrast, vitamin D deficiency during pregnancy has been linked to several adverse pregnancy outcomes including those associated with placental insufficiency such as preeclampsia and low birth weight [1,3–5]. Vitamin D exerts many biological effects on the placenta. It regulates lactogen expression [6], human chorionic gonadotropin secretion [7], and calcium transport [8]. Vitamin D also

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down-regulates inflammatory cytokine TNFα and IL-6 expression in placental trophoblasts [9].

Increased thromboxane and decreased prostacyclin production is a characteristic of placental trophoblast dysfunction in preeclampsia [10–12]. Thromboxane is a potent vasoconstrictor and prostacyclin is a potent vasodilator. However, effects of vitamin D on thromboxane and prostacyclin production have never been studied. Since hypoxia/oxidative stress promotes trophoblast thromboxane production [13], in this study we specifically investigated the role of vitamin D in hypoxia/oxidative stress-induced thromboxane production in placental trophoblasts.

Materials and Methods

Placental trophoblasts were isolated by trypsin digestion as previously described [14]. All placentas were delivered by uncomplicated pregnancies. Placental collection was approved by the IRB at Louisiana State University Health Sciences Center-Shreveport. Freshly isolated trophoblasts were seeded into 6 well/plates (5×10^6 cells/well) and cultured with Dulbecco's Modified Eagle Medium containing 5% fetal bovine serum and antibiotics. On day-2, cells were treated with $1,25(OH)_2D_3$ in the presence or absence of cobalt (II) chloride ($CoCl_2$). $CoCl_2$ is a chelate interfering Fe^{2+} on hemoglobin [15]. It can initiate oxygen-sensing signal transduction pathway and upregulates erythropoietin and HIFα expression [16,17]. Thus, $CoCl_2$ has been widely used as a hypoxic mimicking agent to induce tissue or cell hypoxia/oxidative stress in *in vivo* and *in vitro* studies [18–22]. After 48hrs of culture, medium and total cellular protein were collected and stored at −80C until assay. All chemicals and reagents were from Sigma (St. Louis, MO) unless otherwise noted.

Medium levels of TXB_2 and 6-keto PGF1α (stable metabolites of thromboxane-A_2 and prostacyclin), soluble phospholipase-A_2 ($sPLA_2$), and 8-isoprostane were measured by enzyme immunoassay. Assay kits were purchased from Cayman (Ann Arbor, MI). An aliquot of 100μl of sample was assayed in duplicate. Within- and between-assay variations were < 8% for all assays.

Protein expression for cyclooxygenase-1 (COX-1), COX-2, and HO-1 were determined by Western blot. Antibody for COX-1 (sc-1752) and COX-2 (sc-19999) were from Santa Cruz (San Diego, CA) and for HO-1 (610713) was from BD Biosciences (San Jose, CA). β-actin expression was determined and used as the loading control for each sample. Densities were analyzed by NIH Image 1.16.

Data is presented as mean ± SE and analyzed by analysis of variance (ANOVA). Student-Newman-Keuls test was used as post hoc tests. A probability level less than 0.05 was set as statistically significant.

Results and Discussion

Results of trophoblast production of TXB_2, 6-Keto PGF1α, and $sPLA_2$ are shown in Figure 1. We found that cells treated with $CoCl_2$ produced significantly more TXB_2, $p<0.01$, over 6-Keto PGF1α, $p<0.05$, but had no effect on $sPLA_2$ release. $1,25(OH)_2D_3$ alone had no

affect on TXB_2, 6-Keto PGF1α, and $sPLA_2$ production (Figure 1A and B), but significantly reduced TXB_2 production and the ratio of TXB_2 to 6-Keto PGF1α induced by $CoCl_2$, $p<0.05$. To determine $CoCl_2$-induce oxidative stress, we examined 8-isoprostane production. Isoprostanes are a family of eicosanoids of non-enzymatic origin produced by the random oxidation of tissue phospholipids by oxygen radicals. Increased 8-isoprostane production is a marker of increased oxidative stress and lipid peroxide production [23]. Our results showed that the pattern of 8-isoprostane production (Figure 1C) was similar to TXB_2. Suppression of $CoCl_2$-induced 8-isoprostane production by $1,25(OH)_2D_3$ provided evidence of the anti-oxidative effect of vitamin D on placental trophoblasts. These observations are very important because oxidative stress-induced thromboxane production by trophoblasts is believed to contribute to increased placental vasoconstriction in preeclampsia [11]. Thromboxane facilitates platelet aggregation. Thus, reduce lipid peroxide and thromboxane production or reduced ratio of thromboxane to prostacyclin would result in prostacyclin dominance that promotes circulation and retards thrombosis in the placenta.

Inhibition of $CoCl_2$-induced COX-2 up-regulation by $1,25(OH)_2D_3$ is another important finding in this study (Figure 2). PLA_2 liberates AA from membrane phospholipids and then COX and peroxidase convert AA to thromboxane, prostacyclin, and prostaglandins. Our results showed that $CoCl_2$ had no effect on $sPLA_2$ release and COX-1 expression, but significantly up-regulated COX-2 expression, which could be inhibited by $1,25(OH)_2D_3$. We did not examine thromboxane synthase and prostacyclin synthase expression, because $CoCl_2$-induced thromboxane and prostacyclin production could be blocked by specific COX-2 inhibitor NS-398 (Figure 1 supplement). Therefore, inhibition of $CoCl_2$-induced COX-2 up-regulation could be a mechanism of $1,25(OH)_2D_3$ suppression of hypoxia/oxidative stress-induced thromboxane and 8-isoprostane production.

We also examined HO-1 expression. HO-1 is a fundamental ‘sensor’ of cellular stress and directly contributes to limit or prevent tissue damage [24]. HO-1 is induced not only by the substrate heme but also by a variety of agents causing inflammation and oxidative stress [24]. HO-1 induction participates in cellular adaptation to stress and are involved in the mechanisms of defense [25]. Our results showed that HO-1 expression was increased when cells were exposed to $CoCl_2$ even in the presence of $1,25(OH)_2D_3$ (Figure 2), which suggest that up-regulation of COX-2 and HO-1 induced by $CoCl_2$ are regulated through different mechanisms and also imply the specificity of COX-2 suppression by $1,25(OH)_2D_3$.

Maternal vitamin D levels are lower in preeclampsia than in normotensive pregnant women [5,26]. Vitamin D insufficiency/deficiency has emerged as an independent risk factor not only for preeclampsia [5] but also for cardiovascular diseases [27,28]. Although studies have suggested that vitamin D metabolic/generating system, including vitamin D binding protein, 25-hydroxylase, 1α-hydroxylase, 24-hydroxylase, and vitamin D receptor, is present in placental trophoblasts [3,21,29,30], the role of vitamin D and its down-stream effects on trophoblast function are largely unknown. Nonetheless, results from the present study, i.e. $1,25(OH)_2D_3$ suppresses hypoxia/oxidative stress-induced COX-2 up-regulation and thromboxane production by trophoblasts, provides further evidence of the beneficial effects of vitamin D on the placenta.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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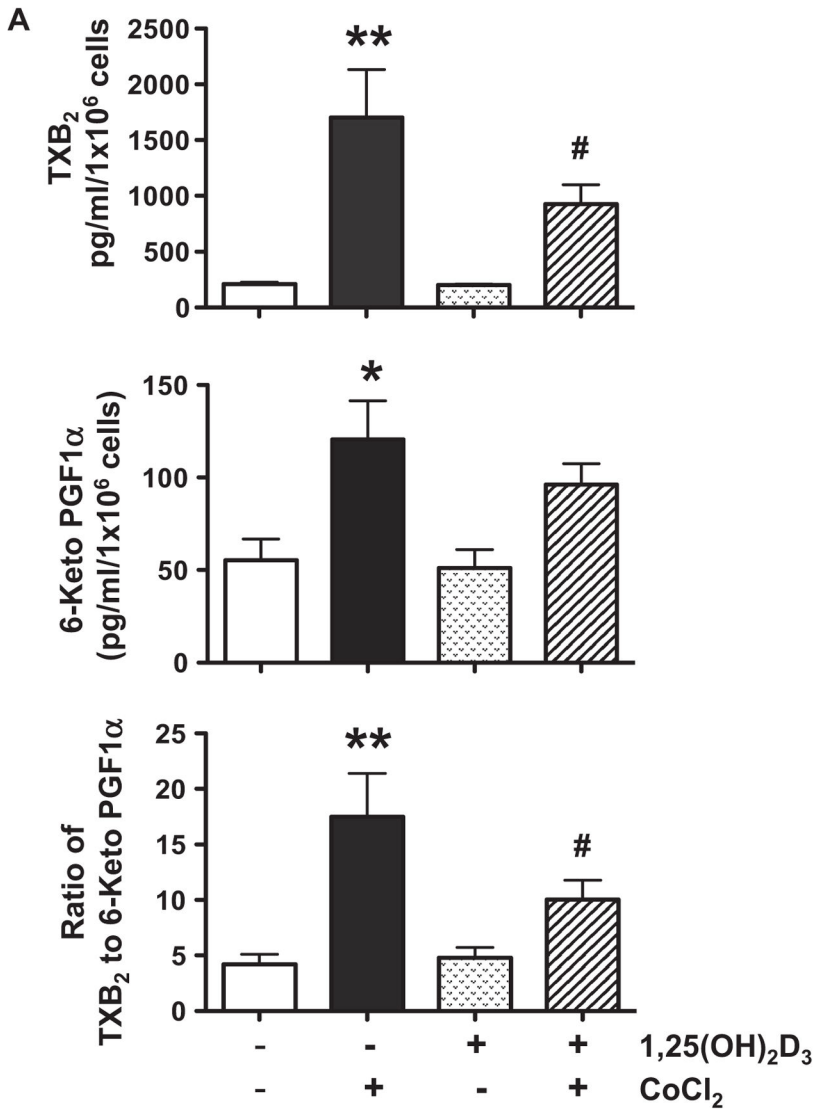

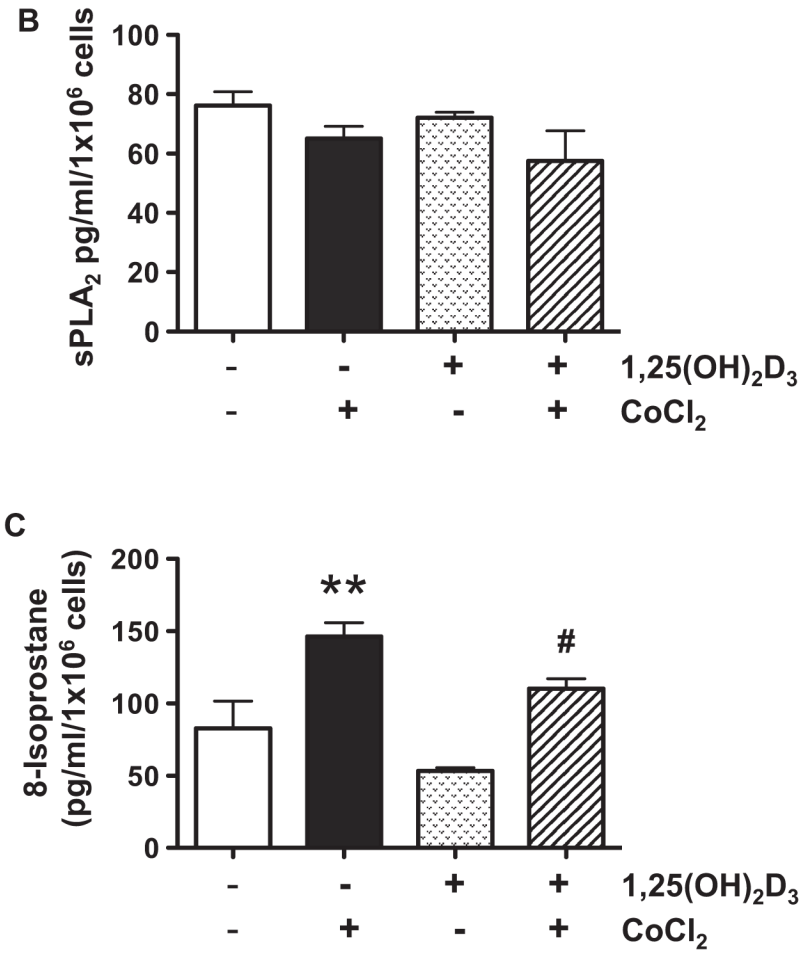

Figure 1.

Trophoblast production of TXB $_2$, 6-Keto PGF1α, $sPLA_2$, and 8-isoprostane. Data are expressed as mean ± SE from 6 independent experiments. **A:** TXB_2 and 6-Keto PGF1α production and the ratio of TXB_2 to 6-Keto PGF1α. **B.** sPLA2 release. **C.** 8-isoprostane production. * $p<0.05$ and ** $p<0.01$: cells treated with $CoCl_2$ vs. control untreated cells; # $p<0.05$: cells pretreated with $1,25(OH)_2D_3$+ $CoCl_2$ vs. cells treated with $CoCl_2$. Cells treated with $CoCl_2$ produced significantly more TXB_2, 6-Keto PGF1α, and 8-isoprostane, but had no effect on $sPLA_2$ release. $1,25(OH)_2D_3$ significantly reduced TXB_2 and 8-isoprostane production and the ratio of TXB_2 to 6-Keto PGF1α induced by $CoCl_2$.

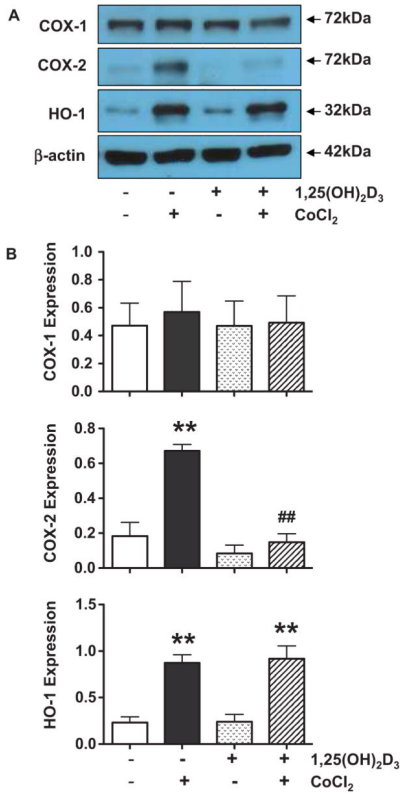

Figure 2.

Trophoblast expression of COX-1, COX-2, and **A:**HO-1. Representative blots for COX-1, COX-2, and HO-1 expression in control cells, cells treated with $CoCl_2$ with or without pretreated with $1,25(OH)_2D_3$. **B:** Relative COX-1, COX-2, and HO-1 expression after normalized by β-actin expression in each sample. ** $p<0.01$: cells treated with $CoCl_2$ vs. control untreated cells; ## $p<0.01$: cells pretreated with $1,25(OH)_2D_3$+ $CoCl_2$ vs. cells treated with $CoCl_2$. $CoCl_2$ had no effect on COX-1 expression, but significantly increased COX-2 and HO-1 expression. Pretreatment of the cells with $1,25(OH)_2D_3$ blocked $CoCl_2$ induced COX-2, but not HO-1, expression.