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Regulation of 25-hydroxyvitamin D-1α-hydroxylase by epidermal growth factor in prostate cells☆

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Abstract

Accumulating data suggest that local production of 1α,25-dihydroxyvitamin D (1α,25(OH)$_2$D) could provide an important cell growth regulatory mechanism in an autocrine fashion in prostate cells. Previously, we demonstrated a differential expression of 1α-OHase enzymatic activity among noncancerous (PZHPV-7) and cancer cells (PC-3, DU145, LNCaP), which appears to correlate with 1α-OHase m-RNA synthesis and its promoter activities. Since it is well-established that EGF regulates the proliferation of prostate cells via autocrine and paracrine loops and 1α,25(OH)$_2$D inhibites prostate cell proliferation, we investigated if EGF also regulated 1α-OHase expression in prostate cells. We found that EGF upregulated 1α-OHase promoter activity and enzyme activity in PZ-HPV-7 and that 1α,25(OH)$_2$D$_3$ inhibited EGF-dependent up-regulation of 1α-OHase enzymatic activity. Moreover, the EGF-stimulated promoter activity was inhibited 70% by the MAPKK inhibitor, PD98059, suggesting that the MAPK pathway may be one pathway involved in the regulation of prostatic 1α-OHase by EGF to increase1α,25(OH)$_2$D synthesis as a feedback regulator of cell growth. Because EGF has no effect on 1α-OHase promoter activity in LNCaP cells, we propose that the ability of EGF to stimulate 1α,25(OH)$_2$D synthesis may be abolished or diminished in cancer cells.

Keywords: Vitamin D; EGF; Prostate; Hydroxylases; MAPK; PKA

1. Introduction

In addition to its role in calcium homeostasis, Vitamin D plays important roles in regulating cellular proliferation and differentiation [1]. To become biologically active, Vitamin D must be hydroxylated first at the C-25 position to form 25(OH)D, catalyzed by Vitamin D-25-hydroxylase (25-OHase), and then at the 1α-position catalyzed by 1α-OHase, to form 1α,25(OH)$_2$D$_3$ [2]. Another cytochrome P$_{450}$ enzyme that plays a critical role in Vitamin D metabolism is 25-hydroxyvitamin D-24R-hydroxylase (24R-OHase) [2,3]. The substrates for 24R-OHase include 25(OH)D and 1α,25(OH)$_2$D. 24R-OHase is likely to be responsible for the clearance of excess 25(OH)D and 1α,25(OH)$_2$D in circulation [2,3]. These three major hydroxylases involved in Vitamin D metabolism have been cloned and belong to the type I cytochrome P$_{450}$ enzymes which use NADPH and molecular oxygen as their electron sources [3].

Epidermal growth factor (EGF) exerts its effects through its transmembrane EGF receptor (EGFR or ErbB-1), which forms either ErbB-1/ErbB-1 homodimers or ErbB-1/ErbB-2 heterodimers upon ligand binding, which in turn activates EGFR tyrosine kinase, an extracellular signal regulated kinase (ERK), and trans-autophosphorylation [4,5]. The consequence of this series of interaction is to recruit transcription factors and to control transcriptional activity. Activation of the EGFR/ERK signaling pathway has many effects including increased proliferation, metastasis and angiogenesis, and decreased apoptosis.

1α-OHase is expressed in prostate cells [6]. Unlike the renal enzyme, 1α-OHase in prostate cells appears to be regu-

Abbreviations: cAMP, cyclic AMP; CREB, cAMP response element binding protein; DPPD, 1,2-dianilinoethane; EGF, epidermal growth factor; EGFR(or ErbB-1), EGF receptor; ERK, extracellular signal regulated kinase; FBS, fetal bovine serum; MAPK, mitogen activated protein kinase; 1α-OHase, 25-hydroxyvitamin D-1α-hydroxylase; 24R-OHase, 25-hydroxyvitamin D-24R-hydroxylase; 25(OH)D$_3$, 25-hydroxyvitamin D$_3$; 1α,25(OH)$_2$D$_3$, 1α,25-dihydroxyvitamin D$_3$; PKA, protein kinase A; VDR, Vitamin D receptor

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lated in an autocrine/paracrine fashion. In the prostate, EGF regulates the proliferation of prostate cells via autocrine and paracrine loops [7]. The growth and survival of prostate tumors depends on EGFR [7]. The aberrant activity of EGFR may play a key role in the development of prostate cancer [7]. In this report, we studied the effects of EGF on the promoter and enzymatic activity of 1α-OHase in prostate cells.

2. Methods and materials

2.1. Prostate cell cultures

PZ-HPV-7 cell line (CRL-2221) was obtained from ATCC (Manassas, VA). The cells were maintained in a serum-free defined prostate epithelial cell growth medium (PEGM) containing MCDB 153 (Sigma, St. Louis, MO), supplemented with EGF, 20 ng/ml; PGE_1, 50 ng/ml; insulin, 5 μg/ml; and bovine pituitary extract, 30 μg/ml. LNCaP prostate cancer cells are grown on RPMI supplemented with 5% FBS.

2.2. Analysis of 1α-OHase gene promoter activity

1α-OHase gene promoter activity was analyzed in cells transfected with a truncated promoter fragments (AN2) inserted upstream of luciferase reporter gene using a pGL2 reporter vector and cotransfection with pRL-SV40 receptor vector as internal control for the normalization of the transfection efficiency (Dual-Luciferase® Reporter Assay System from Promega, Madison, WI). The construct was transiently transfected into cells cultured in 24-well dishes at 60–70% confluence. Each well was transfected with 0.8 μg of the constructed reporter gene and 0.2 μg of control plasmid DNA by using 2 μl of Lipofectamine 2000 (Life Technology). Four hours after transfection, media were replaced with the original culture media without serum or growth factors overnight before cells received EGF treatment for a period of time as specified. Cells were harvested in 100 μl of lysis buffer (Promega). The protein content of the extracts was determined by the Bradford procedure (Bio-Rad, Hercules, CA). Extracted samples (10 μg protein in 20 μl) were subjected to the luciferase assay using a Monolight 2010 luminometer.

2.3. Analysis of 1α-OHase enzyme activity

The transformed PZ–HPV–7 cells were subcultured in the PEGM medium into 35 mm dishes for the enzyme activity studies. When cultures reached about 80% confluence, the media were removed and replaced with basal media plus 50 nM of $25(OH)D_3$ containing 0.1 μCi ^{3}H-$25(OH)D_3$ and 10 μM 1,2-dianilinoethane (DPPD) and incubated for 2 h at 37 °C. At the end of incubation, media were removed, and cells were extracted with methanol twice. The combined extracts were dried down and applied to a high-performance liquid chromatography (HPLC) using methylene chloride/isopropanol (19:1) as the mobile phase to determine the amounts of $1\alpha,25(OH)_2D_3$ produced. This solvent system is used to prevent 10-oxo-19-nor-$25(OH)D_3$ contamination as described [8]. DPPD, an antioxidant, was added during the incubation to prevent the free radical, nonenzymatic autoxidation of $25(OH)D_3$ to $1\alpha,25(OH)_2D_3$.

3. Results

Previously, we reported that the removal of EGF from culture media reduced 1α-OHase enzymatic activity in primary cultures of normal prostate cells by approximately 50% [9]. Similar results were observed in a transformed PZ-HPV-7 prostate cell line. PZ-HPV-7 cells were derived from epithelial cells of the peripheral zone of the normal prostate tissue by transfecting with HPV18 DNA [10]. The cells express high 1α-OHase promoter activity and enzymatic activity. Therefore, these cells are ideal for studying the regulation of this enzyme. Since 20 ng/ml of EGF is routinely included in media for the optimal growth of prostate cells in cultures, we first compared the 1α-OHase enzyme activity in the presence of 0, 20, and 40 ng/ml of EGF to determine the maximal dose for the stimulation of this enzyme. We found that no significant difference in 1α-OHase enzyme activity between 20 and 40 ng/ml, suggesting that 20 ng/ml of EGF is the maximal dose.

Next, we studied the time course of EGF stimulation. As shown in Fig. 1, EGF (20 ng/ml) caused a time-dependent stimulation of 1α-OHase enzyme activity. Fig. 2 demonstrates that $1\alpha,25(OH)_2D_3$ inhibited EGF-dependent and EGF -independent 1α-OHase enzyme activity in PZ-HPV-7 prostate cells in a dose-dependent manner.

Previously, we showed that the promoter activity of 1α-OHase was regulated by mitogen activated protein kinase kinase (MAPKK) inhibitor, PD98059 [11]. Therefore, we studied the influence of EGF and PD98059 on 1α-OHase

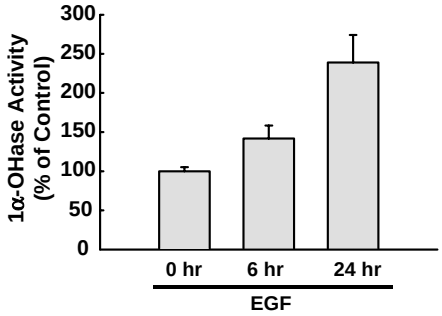

Fig. 1. Time course of EGF stimulation on 1α-OHase activity in PZ-HPV-7 prostate cells. When PZ-HPV-7 cells were grown to 70–80% confluence in PEGM, media were changed to basal media in the absence of growth factors for 18 h, and then cells were incubated with EGF (20 ng/ml) for 0, 6 or 24 h before enzyme activity was determined. Data are mean ± S.D. of three separate determinations.

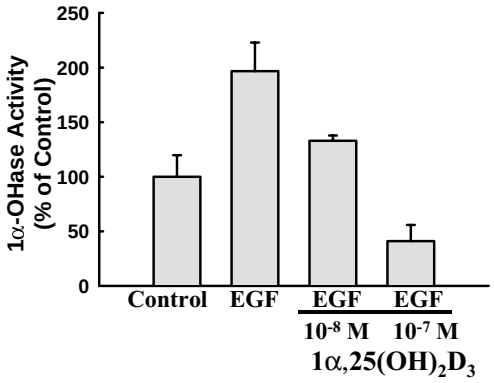

Fig. 2. Effect of EGF and $1\alpha,25(OH)_2D_3$ on 1α-OHase activity in PZ-PHV-7 cells. EGF was removed from media for 18 h after cells were grown to 60% confluence, Cells were then treated with either vehicle, EGF (20 ng/ml) alone or a combination of EGF (20 ng/ml) and $1\alpha,25(OH)_2D_3$ (10^{-8} and 10^{-7} M) for 24 h before the enzyme activity was determined. Data are mean ± S.D. of three separate determinations.

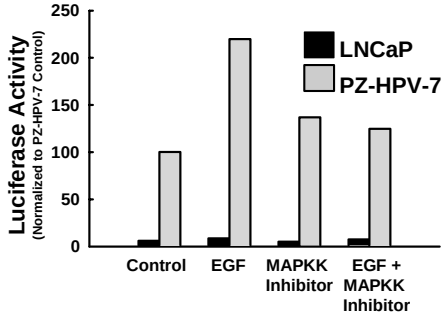

Fig. 3. Regulation of the 1α-OHase promoter activity in PZ-PHV-7 and LNCaP cells by EGF and MAPKK inhibitor (PD98059). Cells were transfected with a 1α-OHase promoter–luciferase reporter construct. Following transfection, cells were treated with either vehicle, EGF, MAPKK inhibitor or a combination for 24 h before the luciferase analysis was performed.

promoter activity in PZ-HPV-7 and LNCaP cells. We found that EGF stimulated the promoter activity of 1α-OHase, whereas it had no effect on LNCaP cells (Fig. 3). In the presence of PD98059, the EGF-stimulated 1α-OHase promoter activity was inhibited 70% in PZ-HPV-7 cells. PD98059 by itself did not inhibit the 1α-OHase promoter activity.

4. Discussion

Previously, we reported that prostatic 1α-OHase promoter activity was up-regulated by forskolin, a protein kinase A (PKA) agonist, and down-regulated by H-89, a PKA antagonist [11]. Moreover, cyclic AMP (cAMP) response element binding protein (CREB), which regulates gene expression [12], was differentially phosphorylated in cancerous and noncancerous prostate cell lines. Both 1α-OHase promoter activity and gene transcription were also differentially expressed. The decreased CREB phosphorylation, promoter activity, and mRNA synthesis in prostate cancer cells appear to correlate with a diminished enzyme activity and with a decreased synthesis of $1\alpha,25(OH)_2D$ [11].

Here, we report that EGF upregulates 1α-OHase promoter activity and enzyme activity in a noncancerous prostate cell line. In addition, we show that $1\alpha,25(OH)_2D_3$ inhibited EGF-dependent and EGF-independent 1α-OHase enzymatic activity (Fig. 2). The inhibition of 1α-OHase expression by $1\alpha,25(OH)_2D_3$ is believed to be at the gene promoter level [3]. However, no sequence related to known inhibitory VDRE has been identified in the 1α-OHase promoter [3].

The EGF-stimulated promoter activity was inhibited 70% by the MAPKK inhibitor, PD98059. Therefore, these results suggest that the MAPK pathway may be one pathway involved in the regulation of prostatic 1α-OHase by EGF to increase $1\alpha,25(OH)_2D$ synthesis as a feedback regulator of cell growth. These observations suggest that EGF has a dual role in growth regulation of normal prostate cells: in addition to stimulating cell proliferation, EGF upregulates 1α-OHase to increase $1\alpha,25(OH)_2D$ syntheses. Because EGF has no effect on 1α-OHase promoter activity in LNCaP cells, it is likely that the ability of EGF to stimulate $1\alpha,25(OH)_2D$ synthesis may be abolished or diminished in cancer cells.

In summary, our data suggest that the prostatic 1α-OHase is regulated by both PKA [11] and MAPK signaling pathways at the gene promoter level. These results also suggest that a defect in the regulation of 1α-OHase activity and/or expression which lead to insufficient synthesis of $1\alpha,25(OH)_2D$ could contribute to growth dysregulation of human prostate cancer cells.

Acknowledgements

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