Calcitriol Down-regulates Human Ether à go-go 1 Potassium Channel Expression in Cervical Cancer Cells

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Abstract. Background/Aim: Human ether à-go-go-1 (EAG1) potassium channels are promising anticancer targets. Calcitriol has antitumoural properties. This study investigated EAG1 regulation by calcitriol in normal and cancer cells. Materials and Methods: Cancer cell lines from cervix, prostate, mammary gland, and normal placenta trophoblasts were cultured. Calcitriol was determined by HPLC. Gene and protein expression were assessed by real-time RT-PCR and western blot analysis, respectively. Calcitriol-synthesising enzyme CYP27B1 or vitamin D receptor (VDR), were transfected in cervical cancer SiHa cells. Cell proliferation was assayed with XTT. Results. Calcitriol decreased EAG1 mRNA in all cell types, and EAG1 protein and proliferation in SiHa cells. VDR antagonist ZK-159222 prevented the calcitriol effect on EAG1 mRNA. CYP27B1-transfected cells produced more calcitriol and less EAG1 mRNA. EAG1 mRNA was more potently inhibited by calcitriol in VDR-transfected cells. Conclusion: EAG1 is a calcitriol target in normal and cancer cells and calcitriol is a potential therapy for cervical cancer.

Human ether à-go-go-1 (EAG1, KCNH1, Kv10.1), voltage-gated potassium channel displays oncogenic properties (1). EAG1 transcripts in normal tissues are expressed mainly in brain, but low amounts can be detected in placenta, testes, adrenal glands and transiently in myoblasts (1-3). EAG1 is overexpressed in many human tumours including cervical, lung, breast, colon, and prostate cancer (3-5). Therefore, EAG1 has been suggested as a tool in cancer diagnosis. Inhibition of either EAG1 gene expression or channel activity with antisense oligonucleotides, siRNA, monoclonal antibodies, or non-specific EAG1 channel inhibitors, reduces tumour cell proliferation in vitro and in vivo, thus, EAG1 has been also proposed as a target for anticancer therapy (1, 5-11). EAG1 channel activity is also reduced by retinoic acid and Ca2+/calmodulin (12, 13). However, despite the promising relevance of EAG1 in oncology, little is known about other, naturally occurring, EAG1 channel inhibitors.

Calcitriol (1,25-dihydroxyvitamin D3) is the active hormonal form of vitamin D3. In the liver, the 25-hydroxylation of vitamin D3 produces 25-hydroxyvitamin D3 (25OHDK), which is considered as a marker of vitamin D nutritional status (14). Synthesis of calcitriol from 25OHDK occurs mainly in the kidney and is catalysed by 25-hydroxyvitamin D3 1α-hydroxylase (CYP27B1). Calcitriol bioavailability is mainly regulated by the enzyme 24-hydroxylase (CYP24A1). Most calcitriol effects are mediated by the vitamin D receptor (VDR), a transcription factor belonging to the superfamily of nuclear steroid hormone receptors. Liganded-VDR regulates the expression of vitamin D-responsive genes by binding to different types of vitamin D response elements (VDREs) (14). Besides its role in mineral homeostasis and normal skeletal architecture, calcitriol regulates cell proliferation, differentiation, apoptosis and angiogenesis (15). Recently, it was shown that calcitriol inhibits EAG1 expression in breast cancer cell primary cultures and in the cell line MDA-MB-231 from pleural effusions of breast adenocarcinoma (11). Such effect was mediated by the VDR and inhibition of EAG1 expression was suggested a potential mechanism of the antiproliferative effects of calcitriol on breast cancer cells (11). Whether calcitriol inhibits EAG1 expression in other cancer cell types or in normal cells remains unknown.
Materials and Methods

Reagents. Calcitriol and the VDR antagonist ZK 159222 were donated from Hoffmann-La Roche Ltd (Basel, Switzerland) and Schering (Berlin, Germany), respectively. 25OHD3 (specific activity 30 Ci/mmol) was purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). Anti-EAG1 antibody was kindly provided by Walter Stühmer and Luis Pardo (Max Planck Institute, Göttingen, Germany). Antibodies against VDR, 1α-hydroxylase and β-tubulin were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), The Binding Site Ltd. (Birmingham, UK) and Sigma (Saint Louis, MO, USA), respectively.

Cell culture and transient transfection. SiHa, HeLa, PC-3 and MCF7 human cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA). Human syncytiotrophoblast cells from normal term placenta were obtained from patients attending the Hospital General ‘Dr. Manuel Gea González’ in Mexico City following local ethical considerations and cultured as previously described (17). Cells were seeded at a density of 1×10⁶ per 100 mm-cell culture dish in supplemented medium (DMEM medium without phenol red with 10% charcoal-stripped-heat-inactivated fetal calf serum and 50 U/ml penicillin and 50 μg/ml streptomycin) and cultured under cell culture standard conditions for 24 h prior to vitamin D treatment. For transfection, SiHa cells were plated at a density of 2.5×10⁴ cells/well/6-well plate in supplemented medium. The next day, cells were transfected using Polyfect reagent according to the manufacturer’s instructions (Qiagen, Germany). VDR expression vector was donated by Dr. M. Nguyen (Hôpital Saint Vincent de Paul, Paris, France). The expression vector for human 1α-hydroxylase was constructed by a PCR-based strategy using human placental cDNA as template.

Activity of 1α-hydroxylase in SiHa cells. The ability of SiHa cells to convert [3H]25OHD₃ into [3H]-calcitriol was studied as previously described (18). Briefly, a day after transfection, cells were incubated for 2 h in serum-free medium (DMEM-F12) in the presence of 10 nM [3H]25OHD₃, and analysis of synthesised [3H]-calcitriol was performed by double standard phase HPLC.

Cell proliferation. Five hundred SiHa cells were grown in 96-well microplates in supplemented DMEM-F12 medium and incubated in the presence of 1×10⁻⁷ M calcitriol or its vehicle (ethanol) for variable durations with daily refresh of each treatment. Every other day proliferation was determined by using the cell proliferation Kit II from Roche following manufacturer’s instructions.

Western blot analysis. Cells were lysed in RIPA buffer (9.1 mM dibasic sodium phosphate, 1.7 mM monobasic sodium phosphate, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, pH 7.4) in the presence of protease inhibitors and resulting homogenates (100 μg) were separated by SDS-PAGE on 10% polyacrylamide gels. After electroblotting, membranes were incubated with primary antibodies at appropriate dilutions (EAG1 1:50; VDR 1:100 and 1α-hydroxylase 1:100) for 1.5 h at room temperature. Blots were incubated with HRP-conjugated secondary antibodies for 1 h. Detection was performed by using the ECL detection system (GE Healthcare, Buckinghamshire, UK). After detection of the primary antigen, blots were stripped and re-probed with β-tubulin antibody (1:50000 dilution) as loading control.

Gene expression studies. Cellular RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and five micrograms of total RNA was used for cDNA synthesis (Roche, Germany). Real time polymerase chain reactions (qPCR) were performed using the LightCycler Taqman Master System and the LightCycler 2.0 Instrument from Roche (Roche, Germany). Each 10-μl reaction mixture consisted of 2 μl TaqMan Master 5x, 0.1 μl of specific Universal Probe Library Probe (10 μM), 0.2 μl of each primer (10 μM) and 3 μl template cDNA. Primer sequences were: left EAG1 5’-cctgagaggtatcaagagt-3’, right EAG1 5’-ccaaacagctcctcccttc-3’ with probe number 49; left VDR 5’-gtgagacccacagagagca-3’, right VDR 5’-cattgcctcctccgtga-3’ with probe number 68; left CYP24A1 5’-catagccagaacaaac-3’, right 5’-gcagctcgaagttg-3’ with probe number 88; left (glyceraldehyde-3-phosphate dehydrogenase) GAPDH 5’-gacaacagctgcagac-3’, right GAPDH 5’-gccaactcagcactaccc-3’ with probe number 60. Thermal cycling consisted of an initial denaturation step at 95°C for 10 min, followed by 45 cycles at 94°C for 10 s, 60°C for 30 s, and 72°C for 1 s. Expression levels were calculated after normalisation to the housekeeping gene GAPDH and results were expressed relative to values from vehicle-treated cells, which were set at 1. HeLa, MCF-7, SiHa cells and syncytiotrophoblast primary cultures have been already reported to express endogenous EAG1 mRNA (1, 7, 10).

Statistical analysis. Data are expressed as mean±standard deviation (SD). Statistical analysis was carried out using one way ANOVA, and significant differences among groups were determined by Fisher’s protected least-square difference test. Differences were considered significant at p<0.05.

Results

Calcitriol is known to inhibit EAG1 gene expression in primary cultures from breast cancer cells. Therefore, it was first investigated if calcitriol regulates EAG1 expression in different cancer cells as well as in non-tumour cells. Figure 1A shows that EAG1 gene expression is down-regulated by calcitriol in cancer cells from cervix, prostate and mammary gland (HeLa, PC-3 and MCF-7 cells, respectively). This inhibition was also observed in non-tumour cells, namely, syncytiotrophoblast primary cultures from normal term placenta (SC, Figure 1A). Next, since VDR, the anabolic and catabolic vitamin D hydroxylases and EAG1 are highly expressed in cervical cancers (4, 16), SiHa cervical cancer cells were investigated. Calcitriol down-regulated EAG1 gene expression in a concentration-dependent manner (Figure 1B). The mean inhibitory concentration (IC₅₀) was 2.4×10⁻⁸ M (assessed by non-linear regression analysis using sigmoidal fitting with a sigmoidal dose-response curve). EAG1 expression inhibition by calcitriol was also observed at different incubation times. Figure 1C shows that EAG1 gene expression increased after 4 and 6 days of culture in untreated cells. Calcitriol treatment (refreshed daily) resulted in a significant reduction of EAG1 gene expression at days 2, 4 and 6 of treatment. It was investigated whether calcitriol also decreased EAG1 protein expression by performing Western blot experiments in SiHa cells incubated with calcitriol during different culture conditions. EAG1 protein expression
Figure 1. EAG1 expression is inhibited by calcitriol. Calcitriol (1×10^{-7} M, 24 h) down-regulated EAG1 gene expression in the cancer cell lines HeLa, PC-3 and MCF7 as well as in syncytiotrophoblasts (ST) from normal human placenta (A). Calcitriol decreased EAG1 mRNA levels in a concentration-dependent manner in SiHa cells incubated for 24 h in the presence of calcitriol or its vehicle (vh) (B). Time-dependent effect of calcitriol (1×10^{-7} M) was also evaluated on EAG1 mRNA and protein expression (C, D). SiHa cells were treated daily with either vehicle (vh) or calcitriol at the indicated times and then subjected to qPCR analysis for EAG1 (C) and Western blot for EAG1, VDR and tubulin expression (D). Day 0 corresponded to the time when calcitriol was added 24 h after plating. Bars in A, B, and C represent the normalized mRNA levels relative to values from vehicle-treated cells which were set at 1. Data are presented as the mean±SD from three independent experiments. *p<0.05 vs. vh, **p<0.05 vs. vh day 0.

Figure 2. Calcitriol precursor 25OHD₃ inhibits EAG1 expression in CYP27B1-transfected cells. Calcitriol-synthesising enzyme CYP27B1 was overexpressed in SiHa cells. Cells were transfected with either 3 μg of 1α-hydroxylase (CYP27B1) expression vector or the empty control vector (control). As expected, transfected cells expressed a higher CYP27B1 protein expression (A), and a significant increase in both CYP24A1 gene expression and endogenous calcitriol production (B and C, respectively), when incubated in the presence of 25OHD₃. Calcitriol precursor decreased EAG1 gene expression only in transfected cells (D). Data are presented as mean±SD from three independent experiments. * p<0.05 vs. vh.
remained constant during the period studied in untreated cells (vehicle, Figure 1D). However, calcitriol produced a clear decrease in EAG1 protein level at the different incubation times. In all cases, tubulin expression (used as a constitutive protein) was constant. Interestingly, VDR expression increased in a time-dependent manner after calcitriol exposure, but this effect on VDR was not observed at the mRNA level (data not shown).

Then, the participation of different components of the calcitriol pathway on EAG1 regulation, namely, the calcitriol-synthesising enzyme CYP27B1 and the VDR was studied.

SiHa cells were transfected with the gene for the calcitriol-synthesising enzyme CYP27B1. As expected, transfected cells displayed a higher CYP27B1 protein expression in comparison to control cells transfected with the empty vector (Figure 2A). The presence of the calcitriol precursor 25OHD3 did not affect CYP27B1 protein expression. However, in transfected cells, 25OHD3 significantly increased gene expression of the calcitriol catabolising enzyme CYP24A1, and the endogenous production of calcitriol (Figure 2B-C). Interestingly, the calcitriol precursor decreased EAG1 gene expression only in cells overexpressing the calcitriol-synthesising enzyme (Figure 2D), probably because of the elevated endogenous production of calcitriol.

Since most biological actions elicited by calcitriol involve VDR function, it was next investigated if this transcription factor participates in the repression of EAG1 mediated by calcitriol. This issue was approached by using the specific VDR antagonist ZK 159222 (19) and a VDR-overexpression strategy. Calcitriol did not reduce EAG1 gene expression in the presence of its antagonist (Figure 3A). The effects of calcitriol on the CYP24A1 gene were attenuated by 63% when co-incubated with ZK 159222 (data not shown). These results suggest that VDR participates in the regulation of EAG1 gene expression by calcitriol in cervical cancer cells. In addition, SiHa cells were transfected with an expression vector for human VDR. Figure 3B shows that VDR-transfected cells express higher levels of VDR in comparison to control cells, as expected. Noteworthy, down-regulation of
EAG1 gene expression by calcitriol was more pronounced in VDR-transfected cells (Figure 3C), emphasising the participation of VDR in such regulation. Finally, cell proliferation was studied in the absence or presence of calcitriol in SiHa cells. Figure 4 shows that calcitriol significantly inhibits cell proliferation after 2 days of treatment, reaching a 37% reduction on day 6 of treatment.

Discussion

EAG1 channels are considered both as potential tumour markers and therapeutic targets for many types of cancer (1, 4-11). Therefore, finding new EAG1 inhibitors is of great interest in oncology. EAG1 gene expression and VDR activity are regulated by several factors including insulin-like growth factor I (20), Ca\(^{2+}\)/calmodulin (13), high-risk HPV-16 E6 and E7 oncogenes (10) and ligands for transcription factors of the superfamily of nuclear receptors including estradiol through the oestrogen receptor α (10) and retinoic acid, probably through the retinoic acid receptor (12). It has been recently shown that calcitriol, another ligand of a nuclear receptor, inhibits EAG1 expression through a mechanism involving VDR function in breast cancer primary cultures (11). Here it is shown that calcitriol also inhibits EAG1 in other types of cancer cells, namely cervical, prostate, and mammary gland derived cancer cell lines. Interestingly, EAG1 gene expression was also inhibited in syncytiotrophoblasts primary cultures from normal placenta, suggesting calcitriol as a potential natural mechanism that keeps EAG1 at low levels in most normal tissues. Actually, vitamin D deficiency occurs worldwide (14), and has been associated with an increased risk of developing several types of cancer including colorectal, prostate and breast cancer (21). Whether such vitamin D deficiency is related to an increase in oncogenic EAG1 channels, leading to cancer, remains elusive.

The current experiments of overexpression of the calcitriol synthesising enzyme CYP27B1, strongly suggest that enhanced calcitriol synthesis from its precursor 25OHD\(_3\) inhibited EAG1 gene expression. Thus, diet or sun exposure producing more calcitriol precursors may help to reduce EAG1 expression. A VDR antagonist and the overexpression of VDR significantly affected the regulation of EAG1 gene expression by calcitriol. These results suggest that EAG1 gene promoter contains cis-acting elements responsible of the negative regulation by calcitriol or that calcitriol regulates a repressive factor of EAG1 expression.

Whether EAG1 genomic region contains E-box type elements similar to those mapped in the promoters of CYP27B1 and parathyroid hormone genes (22, 23) remains unknown. Another possibility on the potential mechanism of action by which calcitriol reduces EAG1 expression is via retinoblastoma. EAG1 gene expression is up-regulated by the HPV 16 E7 oncogene (10). The HPV16 E7 protein associates with the active hypophosphorylated form of retinoblastoma tumour suppressor protein pRB, targeting it for proteosomal degradation (24). Calcitriol increases the levels of hypophosphorylated pRB through mechanisms involving a reduction of global cyclin-dependent kinase activity as a result of transcriptional repression of several cyclin genes and induction of cyclin-dependent kinase inhibitors (15, 21). Thus, probably calcitriol regulates EAG1 gene expression via retinoblastoma, however, this deserves further investigation.

In this study, an increase in VDR expression was observed at the protein level but not at the mRNA level in transfected SiHa cells treated with calcitriol. This is probably explained by the ligand-induced stabilisation of VDR, or by a block of the ubiquitin/proteosome-mediated degradation as has been already reported (25, 26). In some cases, the increase of VDR protein is associated with elevated mRNA expression (27), whereas in other cell types calcitriol increases VDR protein without altering VDR mRNA levels (25).

It was recently demonstrated that inhibition of EAG1 by calcitriol also leads to reduction of cell proliferation in breast cancer primary cultures (11). Although most of the antitumoural effects of calcitriol have been studied in colorectal, breast and prostate cancer models (15, 21), cervical cancer is a very suitable malignancy to be targeted by calcitriol. VDR and the anabolic and catabolic vitamin D hydroxylases are expressed in higher proportion in cervical carcinomas in comparison to healthy cervical tissue (16). In addition, EAG1 is overexpressed in cervical cancer (4) and EAG1 gene expression is up-regulated by cervical cancer aetiological factors, namely, oestrogens and HPV oncogenes (10). Incubation of CYP27B1-transfected SiHa cells with the calcitriol precursor 25OHD\(_3\) led to an elevated production of endogenous calcitriol and resembled the inhibitory effect of exogenously administrated calcitriol on EAG1 gene expression. Such calcitriol production was sufficient to induce the expression of the 24-hydroxylase mRNA, the most vitamin D-responsive gene (17). Since proliferation of SiHa cells is inhibited by calcitriol, VDR may be a new target for cervical cancer prevention and treatment.

The current results suggest EAG1 as a molecular target of calcitriol in normal and cancer cells including those from cervical cancer. In addition, since VDR, the anabolic and catabolic vitamin D hydroxylases, and EAG1 are highly expressed in cervical carcinomas, these data propose vitamin D as a therapeutic option for cervical cancer.

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