Epigenetic regulation of the 1,25-dihydroxyvitamin D₃ 24-hydroxylase (CYP24A1) in colon cancer cells

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A R T I C L E   I N F O

Article history:
Received 9 July 2012
Received in revised form 31 July 2012
Accepted 9 August 2012

Keywords:
CYP24A1
Histone deacetylase inhibitors
Methylation
Epigenetics
5-Aza-2'-deoxycytidine
Trichostatin A

A B S T R A C T

Calcitriol is the hormonally active form of vitamin D and has anti-proliferative and pro-apoptotic effects. Calcitriol and its precursor calcidiol (25(OH)D₃) are degraded by the 1,25-dihydroxyvitamin D₃ 24-hydroxylase (CYP24A1). This enzyme is overexpressed in colorectal tumors, however, the mechanisms of this overexpression remain to be elucidated. CYP24A1 mRNA level differs among colorectal cancer cell lines and range from almost undetectable to high. Since DNA methylation and histone acetylation regulate CYP24A1 gene expression in prostate cancer cell lines, we investigated whether epigenetic mechanisms could explain the differences in basal expression of CYP24A1 in colon cancer cells. Methyltransferase inhibitor 5-aza-2'-deoxycytidine (DAC) treatment resulted in an over 50-fold induction of CYP24A1 mRNA expression in Coga1A and HT-29 cells but in no response in Caco2/AQ and Coga13 cells. This finding is supported by a strong increase in CYP24A1 activity after DAC treatment in Coga1A (35%). In addition, calcitriol and DAC had synergistic effects on CYP24A1 gene transcription. Interestingly, the CYP24A1 promoter was not methylated in Coga1A and HT-29 (<5%), while in Caco2/AQ it was 62% methylated. This suggests that DNA demethylation must activate genes upstream of CYP24A1 rather than act on the gene itself. However, transcriptional regulators of CYP24A1 such as vitamin D receptor (VDR), retinoid X receptor (RXR), specificity protein 1 (SP1), or mediator complex subunit 1 (MED1) were not upregulated. We conclude that in colon cancer cells, CYP24A1 gene expression is inducible by methyltransferase and some histone deacetylase inhibitors in a cell line-dependent manner. This effect does not correlate with the methylation state of the promoter and therefore must affect genes upstream of CYP24A1.

This article is part of a Special Issue ‘Vitamin D Workshop’.

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1. Introduction

The most active vitamin D metabolite 1,25-dihydroxyvitamin D₃ (calcitriol) is a pleiotropic secosteroid hormone that seems to have anti-tumorigenic effects in several cancer types [1,2]. High serum levels of its precursor 25-hydroxyvitamin D₃ (calcidiol) correlate with reduced risk of colorectal cancer [3]. The calcidiol and calcitriol degrading enzyme 1,25-dihydroxyvitamin D₃ 24-hydroxylase (CYP24A1) is overexpressed in colorectal tumors [4]. The causes of this deregulation are poorly understood but would most likely reduce the anti-tumorigenic effects of calcitriol. In prostate cancer cell lines, CYP24A1 expression was shown to be regulated by promoter DNA methylation and histone acetylation [5]. In the present study we investigated the involvement of epigenetic mechanisms as the causes of different CYP24A1 expression levels and sensitivity to calcitriol in colorectal cancer cell lines.

2. Materials and methods

2.1. Cell culture

At 30% confluency Coga1A, Caco2/AQ, HT-29, and Coga13 cells were treated with either 5-aza-2’-deoxycytidine (DAC) or trichostatin A (TSA) or a combination of both. Cells were treated with DAC (1 μM in PBS) every 24 h for 3 consecutive days followed by a 24 h treatment with TSA (100 nM in DMSO) after which cells were harvested. For calcitriol treatment, 10 nM calcitriol was added 5 h before the end of the experiment. Controls were treated with 0.01% ETOH and 0.01% DMSO. For long term effect of DAC, Caco2/AQ, Coga1A and HT-29 cells were treated with 1 μM DAC every 24 h for 3 days and total RNA was isolated 8 days after last treatment.
2.2. RNA isolation and reverse transcription (RT) and quantitative RT-PCR

RNA was extracted with Trizol (LifeTechnologies, Vienna, Austria) and reverse transcribed with RevertAid H Minus Reverse Transcriptase (Fermentas, St. Leon-Rot, Germany) using random hexamer primers.

We screened five reference genes for stable expression after TSA and DAC treatments and selected beta-2-microglobulin (B2M) as the reference gene since it was not affected by the drugs. Quantitative RT-PCR was performed in duplicates with POWER SYBR GREEN Mastermix (LifeTechnologies) on a StepOnePlus Real Time PCR system (LifeTechnologies). ΔΔCt was calculated relative to the reference gene B2M and total human RNA (Clontech, Mountain View, CA, USA) was used as calibrator. Graphs were made using GraphPad Prism software v5. Primer sequences of CYP24A1 and VDR have been described before [6], RXRalpha (fwd: GGACATGCAGATGGAGAG, rev: CCTTGGAGTCAGGGTTAA-AGAG), MED1 (fwd: CTTCTCTCTCTCTCCACCT), and SP1 (fwd: TAACTTCTCCTCAGGAG, rev: TTCTCTTTCTCCTCCA). DNA was phenol/chloroform extracted and bisulfite-converted with the EpiTect Bisulfite Kit (Qiagen, Hilden, Germany). PCR amplification was performed using HotStarTaq DNA Polymerase (Qiagen) and gel-purified with PureLink Quick Gel Extraction Kit (LifeTechnologies). Cloning was performed with the Topo TA Cloning Kit for Subcloning with chemically competent bacteria (LifeTechnologies). Miniprep and DNA-Sequencing was performed by Microsynth AG (Balgach, Switzerland). Sequencing results were analyzed with the BiQAnalyzer software (60).

2.4. High pressure liquid chromatography (HPLC)

HPLC was performed as described before [7].

3. Results

3.1. Induction of CYP24A1 expression and activity by inhibition of methyltransferases

To assess basal CYP24A1 mRNA expression and inducibility by calcitriol, we treated the colon cancer cell lines Caco2/AQ, Coga1A, Coga13, and HT-29 with 10 nM calcitriol for 5 h and assessed mRNA expression. All cell lines showed low basal CYP24A1 expression which was highly inducible by calcitriol with the exception of Coga13 cells where the high basal CYP24A1 levels could not be further elevated by calcitriol (Fig. 1A). To evaluate whether differences in promoter DNA methylation and histone acetylation could explain the differences in CYP24A1 gene expression among cell

![Graphs showing CYP24A1 expression and activity](image-url)

**Fig. 1.** Treatments with the methyltransferase inhibitor, 5-aza-2’-deoxycytidine (DAC) and the histone deacetylase inhibitor trichostatin A (TSA) alter CYP24A1 mRNA expression and activity in a cell line dependent manner. (A) CYP24A1 mRNA expression in cells treated with DAC and TSA followed by calcitriol. Each experiment was set relative to the vehicle treated control of each run. Columns represent mean of 3 experiments; bars indicate SEM. Asterisks above bars indicate statistical significant changes compared with vehicle treated control, asterisk above brackets compared with calcitriol treated control (*p < 0.05, **p < 0.001). (B) CYP24A1 mRNA expression 8 days after final DAC treatment. (C) HPLC detection of CYP24A1 metabolites in cell lines after DAC treatment and control, enzymatic activity evaluated from areas under the curve.
lines, we treated cells with the DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine (DAC) and the histone deacetylase inhibitor trichostatin A (TSA) in the presence and absence of calcitriol. None of the investigated cell lines upregulated CYP24A1 expression after TSA treatment, however, the histone deacetylase inhibitor sodium butyrate but not suberoylanilide hydroxamic acid (SAHA) increased CYP24A1 expression in HT-29 cells but not Caco2/AQ (data not shown). In Coga1A and HT-29 cells DAC resulted in a strong increase of basal CYP24A1 transcription which was still observed 8 days after treatment was terminated (Fig. 1A and B). The combination of DAC and calcitriol had a synergistic effect on these cells, while Coga13 and Caco2/AQ cells showed no response to DAC. As a control, we assessed CYP24A1 activity in Coga1A and Coga13 cells after treatment with DAC. While activity increased only marginally in Coga13, we detected a 35% increase of CYP24A1 activity in Coga1A cells (Fig. 1C).

3.2. Promoter methylation of CYP24A1

We performed bisulfite genomic sequencing of the promoter region 1 (−949 to +3) and region 2 (−18 to +609) of the CYP24A1 gene locus (Fig. 2B). Region 1 is located upstream to the transcription start site and showed less than 5% methylation in Coga1A, Coga13, and HT-29 and 62% methylation in Caco2/AQ. Region 2 is located after the transcription start site and showed high overall methylation (Table 1). As a control, we bisulfite sequenced promoter region 2 after DAC treatment and observed a 65% decrease in methylation in Caco2/AQ cells and a 10% decrease in Coga1A (Table 2).

**Table 1**

Promoter methylation of CYP24A1 in colon cancer cell lines.

| Cell line | CYP24A1 mRNA expression | DNA methylation (%) |
|-----------|-------------------------|---------------------|
|           |                         | Region 1 | Region 2 |
| Caco2/AQ  | 0.02                    | 62       | 89       |
| Coga1A    | 0.03                    | 1        | 60       |
| Coga13    | 3.75                    | 3        | 60       |
| HT-29     | 0.005                   | 3        | n.d.     |
Table 2
Promoter methylation of CYP24A1 after 3 days of treatment with 1 μM 5-Aza-2’-deoxycytidine.

| Cell line | Increase in CYP24A1 mRNA expression | DNA methylation (% Region 2) |
|-----------|------------------------------------|-----------------------------|
| Caco2/AQ  | 0.57-fold                          | 31 (65% reduction)          |
| Coga1A    | 54-fold                            | 54 (10% reduction)          |

3.3. Effect of DAC treatment on transcription factors and co-activators regulating CYP24A1 expression

To assess whether the DAC treatment upregulated CYP24A1 gene transcription through an indirect mechanism, we determined the expression of several upstream transcription factors and co-activators of CYP24A1 which contain CpG islands close to their transcription start sites. Since DAC treatment and calcitriol had synergistic effects, we assessed the expression of vitamin D receptor (VDR) and retinoid X receptor (RXR), the main transcription factors mediating CYP24A1 transcription. In addition, we assessed expression of mediator complex subunit 1 (MED1) which is thought to act as a bridge between transcription factors and polymerase II [8] and specificity protein 1 (SP1) which plays a role in both basal and vitamin D induced transcription of CYP24A1. Surprisingly, none of these genes were up regulated after DAC treatment (Fig. 2A).

4. Discussion

In this study, we showed that in colon cancer cell lines the promoter methylation status of CYP24A1 does not correspond to basal and vitamin D induced expression of CYP24A1. Further, we observed a cell line specific strong up regulation of CYP24A1 expression after treatment with the methyltransferase DAC which showed synergistic effects in combination with calcitriol. However, this up regulation did not correlate with CYP24A1 promoter methylation status.

The mechanisms behind the over expression of CYP24A1 in colorectal cancer are not clearly understood but may include gene amplification and epigenetic modifications. Multiple transcription factor binding sites are located upstream of the CYP24A1 transcription start site, including two vitamin D response elements and SP1 binding sites. A large CpG island spans the CYP24A1 promoter and therefore, DNA methylation of this region could lead to gene silencing. In prostate cancer cell lines, the CYP24A1 promoter methylation state correlated with gene expression [5,9]. Treatment with demethylating agents increased mRNA expression only in cell lines that showed promoter methylation before treatment. We hypothesized that in colon cancer cell lines the differences in basal and calcitriol induced transcription of CYP24A1 could also be due to differences of promoter methylation and acetylation status. It is interesting that in colon cancer cell lines the histone deacetylase inhibitor TSA did not affect CYP24A1 expression while it affected gene transcription in prostate cancer cell lines [5,9]. Interestingly, natrium butyrate, another histone deacetylase inhibitor, amplified basal CYP24A1 expression, again in a cell line-dependent manner.

Although inhibition of methyltransferases strongly induced CYP24A1 expression in two of the tested cell lines, this effect cannot be attributed to demethylation of CYP24A1 promoter, since a 65% reduction of methylation achieved less than 4-fold transcriptional increase in Caco2/AQ, while a methylation decrease by 10% was accompanied by a 42-fold increase in Coga1A. Several distant regulatory elements have been described in addition to the ones proximal to the transcription start site. [10,11] however, these elements are not located within CpG islands and therefore it is unlikely that DAC treatment would influence their activity. The synergistic effect of DAC and calcitriol suggested that genes involved in regulation of calcitriol-induced CYP24A1 transcription might be possible candidates of genes that could be induced by DAC, however neither VDR and RXR, the classical mediators of calcitriol signaling nor MED1 and SP1, both required for basal and calcitriol induced CYP24A1 transcription, responded to DAC treatment. In conclusion, the induction of CYP24A1 transcription after methyltransferase inhibitor treatment seems to be due to the altered expression of unidentified regulators of CYP24A1 expression rather than demethylation of the gene itself.

Acknowledgement

This work was funded by the Austrian Science Fund FWF (Grant P22200-B11) and Herzfelder’sche Familienstiftung (Grant #AP00422OFF).

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