Research Article

Nutritional calcium modulates colonic expression of vitamin D receptor and pregnane X receptor target genes

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Low nutritional calcium contributes to disruption of the intestinal epithelial barrier function, to hyperproliferation of colonocytes and increased occurrence of aggressive secondary bile acids in the gut lumen. These mechanisms are also known to be involved in the etiology of colonic inflammation and cancer. We studied in mice and human adenocarcinoma-derived Caco-2 cells the impact of low calcium on markers of inflammation (cyclooxygenase-2; COX-2), of detoxification (pregnane and xenobiotic receptor (PXR)/steroid and xenobiotic receptor (SXR), cytochrome P450 steroid-inducible 3a11 (CYP3A11)), and on expression of the vitamin D system as a protection against tumorigenesis. Caco-2 cells express high COX-2 and low SXR mRNA levels when subconfluent. During differentiation this is reversed, while low calcium enhanced COX-2 protein expression. In vivo low dietary calcium significantly increased the expression of the PXR target gene CYP3A11 in the proximal colon, suggesting compensatory defense mechanisms. In comparison with males, low nutritional calcium elicits a better protective response in females: both the vitamin D synthesizing 25-hydroxyvitamin D_3 1α hydroxylase (CYP27B1) mRNA and the detoxifying CYP3A11 mRNA are augmented more. While it is recognized that colonic vitamin D synthesis may prevent tumor progression, low dietary calcium also elevates the 1,25-$(OH)_2$-D_3 catabolic 25-hydroxyvitamin D_3 24 hydroxylase (CYP24) expression primarily in the proximal colon. Our data suggest the proximal colon as the primary site of response to insufficient calcium intake.

Keywords: Caco-2 cells / CYP3A11 / Gender specificity / Proximal colon / Vitamin D hydroxylases

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1 Introduction

Inflammatory bowel disease (IBD), which manifests as either ulcerative colitis (UC) or Crohn's disease (CD) is associated with chronic inflammation of the intestinal tract. IBD is thought to be the result of an altered intestinal epithelial barrier function in combination with dysregulation of the mucosal immune system. While the precise etiology of UC is still unclear, its importance as a precursor lesion for colorectal cancer (CRC) is well recognized: risk is increased at least two-fold compared with the normal population. While up to 14% of UC patients develop colorectal tumors, the incidence in CD patients is less than 1% [1].

CRC is potentially one of the most preventable malignancies. Nutritional awareness (low fat, low red meat, high fruit, and vegetable intake) and physical activity have good potential for primary prevention. Chemoprevention with calcium supplements, aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs) all have potential to reduce both the formation of premalignant lesions as well as that of CRC.

Dairy products contain high levels of calcium, however a nutritional calcium deficiency in European and American populations is evident (see, *e.g.*, [2]). It has been suggested previously [3] that high calcium intake *in vivo* ameliorates the hyperproliferative effect of toxic secondary bile acids by sequestering them and inhibiting contact with the colonic epithelium. However, we have demonstrated, that the antiproliferative effect of calcium is not observed only *in vivo*, but also *in vitro* in those colonic cell lines that

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Abbreviations: COX-2, cyclooxygenase-2; **CRC,** colorectal cancer; **CYP24,** 25-hydroxyvitamin D_3 24 hydroxylase; **CYP27B1,** 25-hydroxyvitamin D_3 1α hydroxylase; **CYP3A11,** cytochrome P450 steroid inducible 3a11; **FCS,** fetal calf serum; **IBD,** inflammatory bowel disease; **PXR,** pregnane and xenobiotic receptor; **SXR,** steroid and xenobiotic receptor; **UC,** ulcerative colitis; **VDR,** vitamin D receptor

express the calcium sensing receptor [4, 5]. While the molecular mechanism for a cancer-preventive effect of calcium is not completely clear yet, it has been demonstrated that low dietary calcium also increases severity of IBD in IL-10 KO mice [6].

The steroid and xenobiotic receptor (SXR) and its rodent ortholog pregnane and xenobiotic receptor (PXR) are members of the nuclear receptor superfamily [7]. PXR behaves as a xeno-sensor, regulates expression of detoxification genes and has recently been suggested to play a role in IBD pathogenesis as well [8]. This prompted us to investigate in a mouse model for nutritional deficiencies (see, *e.g.*, [9]) in parallel to the human colon adenocarcinoma-derived cell line Caco-2 the effect of normal and low levels of calcium on expression of selected markers of detoxification and inflammation, and of colon cancer prevention such as the vitamin D hydroxylases and the vitamin D receptor (VDR) [10].

2 Materials and methods

2.1 Mouse studies

C57BL/6 mice were housed at the Centre for Laboratory Animal Care at the University of Vienna in a contained environment. Mice were weaned when 3 wk of age, were then fed *ad libitum* either a standard diet (basis was AIN 76A) containing high (0.9%) or low levels (0.04%) of calcium (Harlan Teklad Europe, Borchen, Germany). The calcium levels chosen represent the equivalent of high and low calcium intake in humans [11]. Mice were killed by cervical dislocation and tissue samples were collected. All animals were sacrificed when 13–14 wk old. Treatment groups consisted of at least five animals. The colon (without coecum) was divided into proximal (from end of coecum to middle of transversum) and distal (from middle of transversum to rectum) parts, the lumen was cut open, rinsed with PBS, the mucosa was scraped off on ice, and was immediately frozen in liquid nitrogen. Study protocols were reviewed and approved by the Institutional Committee of Animal Experimentation of the Medical University of Vienna and by the Austrian Ministry of Science and Education.

2.2 Cell culture

For comparison with the normal mouse colon mucosa we selected the human cell line Caco-2, subclone AQ. While Caco-2 cells are colon adenocarcinoma-derived, they still are a valuable model for the normal colon, since they differentiate spontaneously after confluence. From the original cell line we cloned a cell type, the Caco-2/AQ, that has low 25-hydroxyvitamin D_3 24 hydroxylase (CYP24) activity [12] and a higher proliferation rate and reduced alkaline phosphatase activity compared with the original cell line [13]. Cells were routinely cultured at 37°C in a humidified atmosphere of 95% air and 5% CO_2 in DMEM. Media were supplemented with 10% fetal calf serum (FCS), 10 mM HEPES, 100 IU penicillin, 100 μg/mL streptomycin, and 2 mM glutamine (all from Gibco Life Technologies, Gaithersburg, MD). Media were changed every second day. For low calcium treatment cells were transferred to "control TS" medium: DMEM containing only 0.74% FCS and supplemented with 10 μg/mL transferrin (Sigma) and 5 ng/mL sodium selenite (Merck, Darmstadt, Germany) 7 days after confluency. The final Ca^{2+} concentration in this medium was 1.8 mM. After 48 h accommodation to the low serum condition, the cells were treated for further 48 h with a TS medium containing 0.025 mM calcium. This concentration was reached by adding 0.74% FCS to nominally Ca^{2+}-free DMEM.

2.3 Methods

Total RNA was prepared with TRIzol™ reagent (Invitrogen, Lofer, Austria) according to the manufacturer's instructions. RNA (2 μg) was reverse-transcribed (RevertAid™ H Minus M-MuLV Reverse Transcriptase; Fermentas) using random hexamer primer at 42°C for 60 min, 45°C for 10 min, followed by 72°C for 15 min.

Semiquantitative RT-PCR was performed on MyCycler™ (BioRad, Hercules, CA). RT-PCR conditions were: 94°C for 15 s, 58°C for 30 s (18S rRNA, cyclooxygenase-2 (COX-2), 25-hydroxyvitamin D_3 1α hydroxylase (CYP27B1), VDR) or 65°C for 30 s (PXR), 72°C for 1 min for 35 cycles with a final extension at 70°C for 10 min.

The primer sets were:

18S rRNA forward: 5′-CCCGGGGAGGTAGTGACGAAA-AAT-3′;
18S rRNA reverse: 5′-CGCCCGCTCCCAAGATCCAAC-TAC-3′;
COX-2 forward: 5′-TTCAAATGAGATTGTGGGAAAAT-3′;
COX-2 reverse: 5′-AGATCATCTCTGCCGAGATCTT-3′;
CYP27B1 forward: 5′-CAGAGGCAGCCATGAGGAAC-3′;
CYP27B1 reverse: 5′-GGGTCCCTTGAAGTGGCATAG-3′;
PXR forward: 5′-CAAGCGGAAGAAAAGTGAACG-3′;
PXR reverse: 5′-CACAGATCTTTCCGGACC TG′-3′;
VDR forward: 5′-CGCTCCAATGAGTCCTTCACC-3′;
VDR reverse: 5′-GCTTCATGCTGCACTCAGGC-3′

PCR products were separated on a 2% agarose gel containing ethidium bromide and bands were analyzed with a video camera imaging system under UV light (Herolab, Wiesloch, Germany). For each experimental sample, the relative abundance value obtained by densitometric analysis was normalized to the value derived from the control sequence (18S rRNA) in the corresponding sample.

2.4 Quantitative real time RT-PCR

Basal levels of vitamin D hydroxylases are at least ten-fold lower in normal mucosal cells than in tumor cells. Therefore, in order to assess mRNA levels accurately in the

mouse colon, we quantified VDR, CYP24, and CYP27B1 mRNA levels by the comparative $\Delta\Delta C_T$ method. Reliability of data was improved by including an invariant endogenous control: 18S rRNA. For each experimental sample, the relative abundance value obtained was normalized to the value derived from the control sequence (18S rRNA) in the corresponding sample. A pool of C57BL/6 mouse colon mucosa (containing proximal and distal parts) cDNAs was designated as the "calibrator", and the relative expression levels of all other samples were assessed relative to the calibrator. The real-time PCR was performed on an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). Triplicates were set up for each sample and transcript under investigation. PCR conditions were: 50°C for 2 min, 94°C for 2 min, which was followed by 40 cycles of 94°C for 15 s and 60°C for 30 s. Primers and internal probes were designed using Primer Express (Applied Biosystems) and are located on different exons to prevent amplification of contaminating genomic DNA. For detection of 18S rRNA we used the VIC-labeled "Assays-on-Demand™ Gene Expression" kit (Applied Biosystems).

Additional primers and internal probes were:

mCOX-2 forward: 5′-GCTCAGCCAGGCAGCAAA-3′;
mCOX-2 reverse: 5′-ATCCTGTGCTCATACATTCCCC-3′;
mCOX-2 probe: FAM-TTGCTGTTCCAATCCATGTCA-AAACCG-TAMRA;
mCYP3A11 forward: 5′-GCCACTCACCCTGATATCCAGA-3′;
mCYP3A11 reverse: 5′-ATACGTGGGAGGTGCCTTGTT-3′;
mCYP3A11 probe: FAM-AAACTGCAGGATGAGATCGA-TGAGGCTCT-TAMRA;
mPXR forward: 5′-ACACCTGGCCGATGTGTCA-3′;
mPXR reverse: 5′-GGCAGGTCCCTAAAGTAGGATATGA-3′;
mPXR probe: FAM-TACATGTTCAAGGGCGTCATCAA-CTTCG-TAMRA;

The sequences of CYP27B1, CYP24, and VDR were described earlier [14].

2.5 Immunoblotting

Western blot analysis was performed as described previously. Briefly, total proteins extracted from snap frozen, ascending, and descending colon of mice were separated by 10% SDS-PAGE and subsequently blotted to a nitrocellulose membrane. The membranes were incubated with a goat anti-COX-2 antibody (Santa Cruz, Santa Cruz, CA) respectively with an anti-actin antibody as internal loading control (Sigma, Saint Louis, MI). To evaluate specificity of the COX-2 antibody we also exposed the membrane to the COX-2 antibody preincubated for 2 h with a five-fold excess of the peptide against which the antibody was raised. Horseradish peroxidase-conjugated secondary antibodies (Amersham Life Sciences, Buckinghamshire, UK) were used and subsequent detection was performed with the SuperSignal CL-HRP Substrate system (Pierce, Rockford, IL). Bands were evaluated by densitometry with a video camera imaging system (Herolab).

2.6 Statistical evaluation

Statistical analyses were performed with the SPSS software (version 12.0.1., SPSS, Chicago, IL). We assessed whether our data were normally distributed using the one-sample Kolmogorov-Smirnov goodness of fit-test for continuous variables. Bivariate comparisons for normally distributed data were calculated with the Student's *t*-test (two tailed). Data that were not normally distributed were analyzed non-parametrically with the Mann–Whitney–Wilcoxon test. A confidence level of 0.95 was applied throughout. Results are expressed as mean ± SEM. Values of $p \leq 0.05$ were considered statistically significant and were marked with an asterisk (*).

3 Results

3.1 *In vivo* regulation of VDR/ PXR target genes by low dietary calcium in the mouse colon

In the normal mouse colon vitamin D hydroxylases are expressed at very low levels, barely detectable by semi-quantitative RT-PCR. We have previously demonstrated that only in hyperproliferative colonic mucosa and in tumors these mRNA species have an at least eight-fold higher expression [15, 16].

We therefore quantified mRNA levels of CYP27B1, the 1,25-$(OH)_2$-D_3 – synthesizing hydroxylase, of CYP24, the 1,25-$(OH)_2$-D_3 – catabolizing hydroxylase, and of the VDR by quantitative real-time RT-PCR separately in male and female mice both in the proximal and distal colon.

There were no gender-related differences in basal expression patterns of the vitamin D system in mice fed AIN76 chow containing 0.9% calcium, with the one important exception of VDR mRNA: females have significantly higher expression ($p = 0.026$) than males but only in the distal colon (see Fig. 1A). Interestingly, feeding 0.04% calcium led to an at least four-fold induction of CYP24 mRNA in male mice and a three-fold induction in female mice, but in the proximal colon only (Fig. 1B). In the distal colon the low calcium diet did not change CYP24 expression. Feeding 0.04% calcium elevated VDR as well as CYP27B1 expression in the proximal colon, and significantly reduced VDR expression in the distal colon ($p = 0.04$) however only in female mice (Figs. 1A and C).

3.2 COX-2 and PXR mRNA expression in mouse colon

Highest COX-2 expression and lowest PXR mRNA were observed in the proximal colon of male mice, whereas in female mice there are no site-dependent differences in

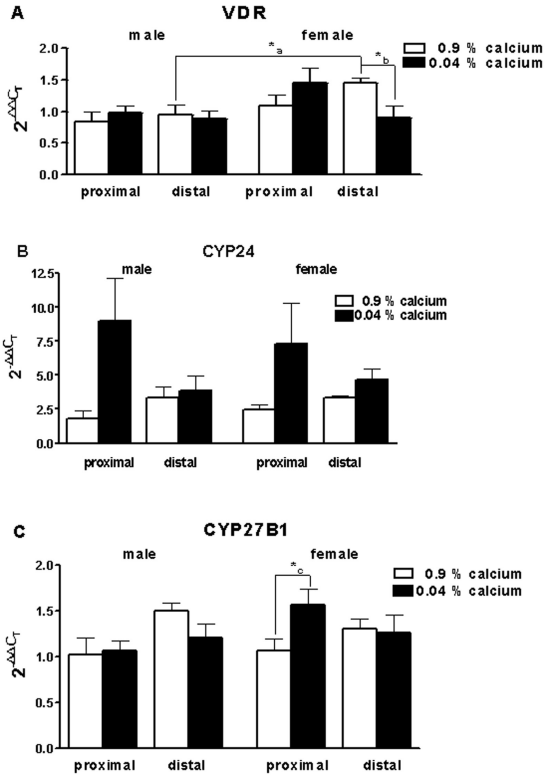

Figure 1. Quantitative Real-Time PCR analysis of VDR (A), CYP24 (B), and CYP27B1 (C) mRNA levels in colonic mucosa of C57BL/6 mice. Amount of mRNA species were compared in proximal and distal colon of female and male animals fed either with 0.9% (white bars) or 0.04% Ca^{2+} (black bars). Data were quantified by the comparative $\Delta\Delta C_T$ method and expressed as $2^{-\Delta\Delta C_T}$ with mean ± SEM ($n = 5$ mice/group). *p*-value: *a = 0.027, *b = 0.042, *c = 0.043.

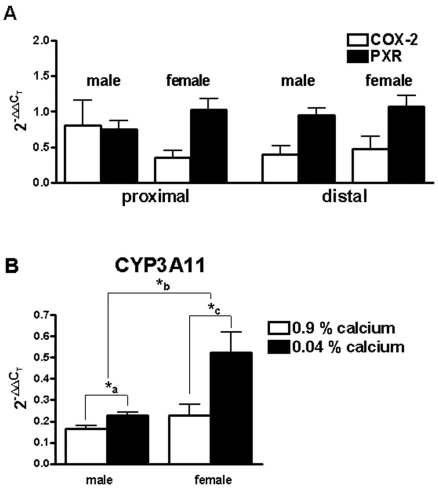

Figure 2. (A) COX-2 and PXR expression in the colonic mucosa assessed by Quantitative Real-Time PCR analysis for proximal and distal colon of both male and female C57BL/6 mice. (B) Gender-related effect of mice fed either 0.9% (white bars) or 0.04% Ca^{2+} (black bars) on CYP3A11 mRNA expression in proximal colon. Data were quantified by the comparative $\Delta\Delta C_T$ method and expressed as $2^{-\Delta\Delta C_T}$ with mean ± SEM ($n = 5$ mice/group). *p*-value: *a = 0.014, *b = 0.049,*c = 0.025.

COX-2 or PXR expression (Fig. 2A). None of the changes in COX-2 and PXR mRNA expression elicited by low calcium were significantly different from values obtained by feeding high dietary calcium (not shown). Cytochrome P450 steroid inducible 3a11 (CYP3A11) however, the enzyme responsible for detoxification and a target gene of PXR, was significantly elevated in the colon of mice receiving a low calcium diet (males $p = 0.014$; females $p = 0.025$) (Fig. 2B). In comparison with male mice, expression was significantly augmented in female mice and only in the proximal colon (female *vs.* male mice, $p = 0.04$), while the distal colon was not found to be positive for CYP3A11 expression (not shown).

3.3 Caco-2/AQ cells

3.3.1 Basal expression of the VDR and of vitamin D hydroxylases CYP27B1 and CYP24 mRNA during differentiation

Caco-2 is the most "normal" human colon cancer cell line available since it differentiates spontaneously during culture and expresses high levels of cytokeratin 8, a marker of normal epithelial cells, of p27, the cyclin-dependent kinase inhibitor WAF-1, and low levels of vimentin and of cyclin D1 [12]. During increasing differentiation of Caco-2/AQ cells, *i.e.*, from subconfluent highly proliferative growth until 7 days after confluency in nonproliferative cultures, expression and activity of alkaline phosphatase, a marker for intestinal differentiation, is enhanced [17]. Also the VDR was suggested to be a marker of differentiation in colonic cells [18]. In Caco-2 cells, mRNA expression of both VDR and CYP27B1 was augmented with differentiation, however this increase was statistically not significant due to high variability of data (Fig. 3A). Basal, non-1,25-$(OH)_2$-D_3-induced expression of CYP24 was barely detectable independent of the degree of differentiation (not shown).

Treatment of confluent Caco-2/AQ cells with low concentrations of calcium (0.025 mM) in the culture medium doubled VDR mRNA expression while CYP27B1 mRNA levels remained similar relative to basal levels found in cells cultured in medium containing 1.8 mM calcium (Fig. 3B).

3.3.2 Basal COX-2 and SXR mRNA expression during differentiation of Caco-2/AQ cells

We measured COX-2 and SXR mRNA levels by semiquantitative RT-PCR. In subconfluent, highly proliferative Caco-2/AQ cells, COX-2 mRNA was high whereas SXR mRNA was barely detectable (Fig. 4A). During culture of Caco-2 cells up to 21 days after confluency this expression pattern was reversed in a time-dependent manner: in highly differentiated Caco-2 cells COX-2 was reduced to barely

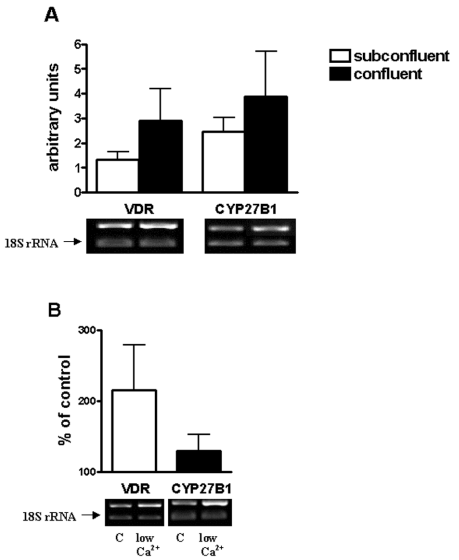

Figure 3. Semiquantitative RT-PCR analysis of VDR and CYP27B1 mRNA in human adenocarcinoma-derived Caco-2 cells. (A) Differences in expression levels of VDR and CYP27B1 in subconfluent (white bars) and confluent (black bars) Caco-2 cells grown in DMEM containing baseline (1.8 mM) concentrations of calcium. (B) Quantification of VDR (white bar) and CYP27B1 (black bar) transcription levels in well differentiated Caco-2 cells treated with 0.025 mM Ca^{2+} for 2 days (% of 1.8 mM calcium control). Lower panels show representative agarose gel analysis of VDR and CYP27B1 expression compared to 18S rRNA (C = 1.8 mM Ca^{2+}, low Ca^{2+} = 0.025 mM Ca^{2+}). Data are mean ± SEM (n = 3).

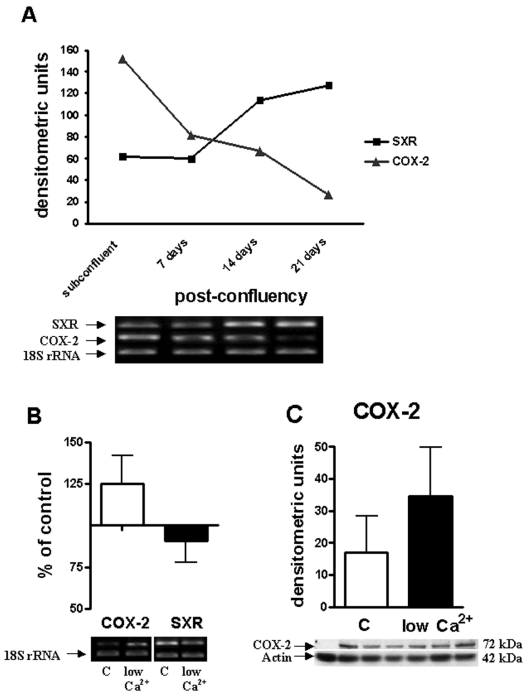

Figure 4. Evaluation of COX-2 and SXR expression levels in Caco-2 cells by semiquantitative RT-PCR and immunoblotting. (A) Time-dependent evaluation of COX-2 and SXR mRNA expression patterns in Caco-2 cells during differentiation. (B) Assessment of COX-2 (white bar) and SXR (black bar) mRNA levels in confluent (7 days) Caco-2 cells treated with 0.025 mM Ca^{2+} for 2 days (% of 1.8 mM calcium control). Lower panel shows representative agarose gel analysis of SXR and COX-2 expression pattern compared to 18S rRNA. (C = 1.8 mM Ca^{2+}, low Ca^{2+} = 0.025 mM Ca^{2+}) (C) Evaluation of COX-2 protein in confluent (7 days) Caco-2 cells treated with 0.025 mM Ca^{2+} for 2 days. β-Actin was used as internal loading control (C = 1.8 mM Ca^{2+}/white bar, low Ca^{2+} = 0.025 mM Ca^{2+}/black bar). Original Blot is shown below. Lane 1: specificity control of the COX-2 antibody (preincubation with the immunogenic peptide); lanes 2–4: 1.8 mM Ca^{2+}; lanes 5–7: 0.025 mM Ca^{2+}. Data are mean ± SEM (n = 3).

detectable levels and SXR mRNA became highly expressed (Fig. 4A).

3.3.3 *In vitro* regulation of COX-2 expression by low calcium

Treating differentiated (confluent) Caco-2/AQ cells with a medium containing 0.025 mM calcium, COX-2 mRNA expression was elevated with a parallel decrease in SXR levels (Fig. 4B). When we evaluated COX-2 regulation at the protein level by immunoblotting, 0.025 mM calcium clearly elevated also COX-2 protein expression, however results just missed being significant (p = 0.07) (Fig. 4C).

4 Discussion

CRC is one of the most preventable forms of visceral cancer. While only about 5% of colon cancers are associated with high-risk, inherited colon cancer syndromes, there is weaker familial clustering in about 30% of all CRC cases [19]. Average risk patients include persons 50 years and older and risk is enhanced in the male population. Risk is at least doubled if individuals have IBD. COX-2 expression is low or undetectable in normal colonic mucosa, but is found in approximately half of adenomas and 90% of CRCs [20]. Nonsteroidal anti-inflammatory drugs like celecoxib that specifically target and block COX-2 are effective inhibitors of inflammation and may also reduce risk of CRC [21]. However, considerable side effects are known. Therefore, it would be preferable to prevent the disease, if possible by nutritional means. Increasing dietary calcium as well as vitamin D intake could be such factors [22].

Calcium shows an inverse association (risk reductions in the range of 15–40% for the highest *versus* the lowest intake categories, see, *e.g.*, [23]) with CRC incidence, whereas it has been estimated that increasing 25-OH-D_3 levels to 40 ng/mL serum would result in a 50% decrease of CRC incidence [24]: it is assumed that this enhancement would provide sufficient precursor for elevation of colonic 1,25-

$(OH)_2$-D_3 synthesis to prevent CRC progression locally [10]. Recently it has been demonstrated in a mouse model that both calcium and 1,25-$(OH)_2$-D_3 target the TNF-α pathway and thus might be able to suppress onset of IBD as a risk factor for CRC [6].

We previously demonstrated the importance of the VDR for prevention of colonic hyperproliferation and of potential tumorigenesis in the VDR-knockout mouse [25]. Complete loss of the VDR resulted in colonic hyperproliferation as well as cyclin D1 elevation, suggesting that proper functioning of the vitamin D system may be essential for normal growth of mucosal cells. It is interesting to observe in our present mouse model a gender-related modulation of the VDR: female mice have significantly higher expression of the VDR than male mice (male *vs.* female, $p = 0.027$) in the distal colon only (Fig. 1A). This suggests (i) that the VDR is potentially increased by female sex hormones as protection against colonic hyperproliferation (see also [26]), and (ii) that this protection extends mainly to the distal colon. Low dietary calcium decreases significantly ($p = 0.04$) VDR expression in the distal colon of female mice whereby VDR levels become similar to those found in the male mouse. This regulation of VDR expression primarily in the distal colon again is highly suggestive of its role there: we have previously demonstrated in the VDR knockout mouse that lack of VDR activity resulted in hyperproliferation of primarily distal colon crypts and a concomitant increased positivity for 8-hydroxy-2′-deoxyguanosine, a marker of oxidative DNA damage [25].

We also provide evidence in our mouse model that mRNA of the catabolic CYP24 hydroxylase is present in both the proximal and distal colon at approximately equal levels. While, due to individual variabilities, differences do not quite reach significance ($p = 0.066$) there is a four-fold enhancement by low nutritional calcium in the proximal colon of male mice, whereas in female mice enhancement is only about three-fold (Fig. 1B). This suggests that the female gender has a higher potential for accumulation of 1,25-$(OH)_2$-D_3 also in the proximal colon due to lower degradation of the steroid hormone, and this in turn could protect against premalignant lesions. A reduced incidence of CRC in women is well known: women age-matched to men have a significantly decreased risk [27] and this may be due (at least in part) to improved activity of the vitamin D protective system in women.

In the intestine specifically PXR may be instrumental in degradation of secondary bile acids known to be irritants and potential mutagens [28]. Interestingly, it has been suggested that PXR can interact with the vitamin D system by modifying expression of CYP24, the catabolic vitamin D hydroxylase [29]. Although low dietary calcium had no significant effect on COX-2 and PXR mRNA expression (not shown), it did increase expression of the PXR target gene CYP3A11, the enzyme involved in detoxification processes, significantly but only in the proximal colon (Fig. 2B). This is especially apparent in the female gender (female *vs.* male, $p = 0.049$). It is also interesting that in female mice there is a tendency for lower COX-2 expression and higher PXR expression in the proximal colon than in male mice though these differences are not statistically significant (Fig. 2A). Taken together this suggests that also enhanced detoxification processes could contribute to the reduced CRC incidence found in the female gender.

The colon adenocarcinoma-derived cell line Caco-2/AQ is obviously an excellent model to investigate regulation of COX-2 and SXR expression. With decreasing proliferation and increasing differentiation we observed a consistent decrease of COX-2 and an increase of SXR mRNA expression (Fig. 4A). During enhanced differentiation and reduced proliferation also the VDR as well as CYP27B1 are increasingly expressed (Fig. 3A).

Low (0.025 mM) calcium in the culture medium not only led to enhanced proliferation of Caco-2 cells [5] similar to that observed in mice on low dietary calcium [14], but also increased COX-2 expression and lowered that of SXR (Fig. 4B). We also evaluated COX-2 protein expression by immunoblotting under identical culture conditions: low calcium again augments expression though just missing significance (Fig. 4C). This suggests, at least in part, a direct effect of low calcium on colonocytes, potentially *via* the calcium sensing receptor (CaSR) that has been described in Caco-2 cells [4]. Interestingly, with hyperproliferation of cells mainly VDR mRNA expression, but also that of CYP27B1 was elevated (Fig. 3B). This response is reminiscent of the upregulation of the VDR and of CYP27B1 expression we observed in human colon tissue during hyperproliferation and premalignancy, which we interpret as a physiological defense mechanism to prevent further disease progression [10].

Our present results suggest, both *in vivo* and *in vitro*, that low calcium, on the one hand, contributes to colon pathogenesis by increasing vitamin D degradation, and, on the other hand, induces defense mechanisms such as detoxification processes and potentially enhanced vitamin D synthesis. These effects are site-specific in the proximal colon only and could contribute to disease etiology. We observed better defense mechanisms in female mice: though the protective effect of 17β-estradiol on UC is controversial [30], it has been documented that in UC patients progressing to colorectal neoplasia the estrogen receptor gene is methylated, *i.e.*, inactivated [31].

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The authors have declared no conflict of interest.

5 References

[1] Pohl, C., Hombach, A., Kruis, W., Chronic inflammatory bowel disease and cancer, *Hepatogastroenterology* 2000, *47*, 57 – 70.

[2] Kudlacek, S., Schneider, B., Peterlik, M., Leb, G. *et al*., Assessment of vitamin D and calcium status in healthy adult Austrians, *Eur. J. Clin. Invest.* 2003, *33*, 323 – 331.

[3] Wargovich, M. J., Eng, V. W., Newmark, H. L., Bruce, W. R., Calcium ameliorates the toxic effect of deoxycholic acid on colonic epithelium, *Carcinogenesis* 1983, *4*, 1205 – 1207.

[4] Kallay, E., Bajna, E., Wrba, F., Kriwanek, S. *et al.*, Dietary calcium and growth modulation of human colon cancer cells: Role of the extracellular calcium-sensing receptor, *Cancer Detect. Prev.* 2000, *24*, 127 – 136.

[5] Kallay, E., Kifor, O., Chattopadhyay, N., Brown, E. M. *et al*., Calcium-dependent c-myc proto-oncogene expression and proliferation of Caco-2 cells: A role for a luminal extracellular calcium-sensing receptor, *Biochem. Biophys. Res. Commun.* 1997, *232*, 80 – 83.

[6] Zhu, Y., Mahon, B. D., Froicu, M., Cantorna, M. T., Calcium and 1 alpha,25-dihydroxyvitamin D3 target the TNF-alpha pathway to suppress experimental inflammatory bowel disease, *Eur. J. Immunol.* 2005, *35*, 217 – 224.

[7] Kliewer, S. A., Goodwin, B., Willson, T. M., The nuclear pregnane X receptor: A key regulator of xenobiotic metabolism, *Endocr. Rev.* 2002, *23*, 687 – 702.

[8] Langmann, T., Moehle, C., Mauerer, R., Scharl, M. *et al*., Loss of detoxification in inflammatory bowel disease: Dysregulation of pregnane X receptor target genes, *Gastroenterology* 2004, *127*, 26 – 40.

[9] Cross, H. S., Lipkin, M., Kallay, E., Nutrients regulate the colonic vitamin D system in mice: Relevance for human colon malignancy, *J. Nutr.* 2006, *136*, 561 – 564.

[10] Cross, H. S., Bises, G., Lechner, D., Manhardt, T., Kallay, E., The Vitamin D endocrine system of the gut – its possible role in colorectal cancer prevention, *J. Steroid Biochem. Mol. Biol*. 2005, *97*, 121 – 128.

[11] Newmark, H. L., Yang, K., Lipkin, M., Kopelovich, L. *et al*., A Western-style diet induces benign and malignant neoplasms in the colon of normal C57Bl/6 mice, *Carcinogenesis* 2001, *22*, 1871 – 1875.

[12] Lechner, D., Kallay, E., Cross, H. S., 1alpha,25-dihydroxyvitamin D3 downregulates CYP27B1 and induces CYP24A1 in colon cells, *Mol. Cell. Endocrinol.* 2007, *263*, 55 – 64.

[13] Brozek, W., Bises, G., Girsch, T., Cross, H. S. *et al*., Differentiation-dependent expression and mitogenic action of interleukin-6 in human colon carcinoma cells: Relevance for tumour progression, *Eur. J. Cancer* 2005, *41*, 2347 – 2354.

[14] Kallay, E., Bises, G., Bajna, E., Bieglmayer, C. *et al*., Colon-specific regulation of vitamin D hydroxylases – a possible approach for tumor prevention, *Carcinogenesis* 2005, *26*, 1581 – 1589.

[15] Bareis, P., Bises, G., Bischof, M. G., Cross, H. S., Peterlik, M., 25-hydroxy-vitamin d metabolism in human colon cancer cells during tumor progression, *Biochem. Biophys. Res. Commun.* 2001, *285*, 1012 – 1017.

[16] Cross, H. S., Bareis, P., Hofer, H., Bischof, M. G. *et al*., 25-Hydroxyvitamin D(3)-1alpha-hydroxylase and vitamin D receptor gene expression in human colonic mucosa is elevated during early cancerogenesis, *Steroids* 2001, *66*, 287 – 292.

[17] Hofer, H., Ho, G., Peterlik, M., Uskokovic, M. R. *et al*., Biological effects of 1alpha-hydroxy- and 1beta-(hydroxymethyl)-vitamin D compounds relevant for potential colorectal cancer therapy, *J. Pharmacol. Exp. Ther.* 1999, *291*, 450 – 455.

[18] Evans, S. R., Nolla, J., Hanfelt, J., Shabahang, M. *et al*., Vitamin D receptor expression as a predictive marker of biological behavior in human colorectal cancer, *Clin. Cancer Res.* 1998, *4*, 1591 – 1595.

[19] Kaz, A. M., Brentnall, T. A., Genetic testing for colon cancer, *Nat. Clin. Pract. Gastroenterol. Hepatol.* 2006, *3*, 670 – 679.

[20] Eberhart, C. E., Coffey, R. J., Radhika, A., Giardiello, F. M. *et al*., Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas, *Gastroenterology* 1994, *107*, 1183 – 1188.

[21] Reddy, B. S., Hirose, Y., Lubet, R., Steele, V. *et al*., Chemoprevention of colon cancer by specific cyclooxygenase-2 inhibitor, celecoxib, administered during different stages of carcinogenesis, *Cancer Res.* 2000, *60*, 293 – 297.

[22] Lamprecht, S. A., Lipkin, M., Chemoprevention of colon cancer by calcium, vitamin D and folate: Molecular mechanisms, *Nat. Rev. Cancer* 2003, *3*, 601 – 614.

[23] Martinez, M. E., Marshall, J. R., Sampliner, R., Wilkinson, J., Alberts, D. S., Calcium, vitamin D, and risk of adenoma recurrence (United States), *Cancer Causes Control* 2002, *13*, 213 – 220.

[24] Feskanich, D., Ma, J., Fuchs, C. S., Kirkner, G. J. *et al*., Plasma vitamin D metabolites and risk of colorectal cancer in women, *Cancer Epidemiol. Biomarkers Prev.* 2004, *13*, 1502 – 1508.

[25] Kallay, E., Pietschmann, P., Toyokuni, S., Bajna, E. *et al*., Characterization of a vitamin D receptor knockout mouse as a model of colorectal hyperproliferation and DNA damage, *Carcinogenesis* 2001, *22*, 1429 – 1435.

[26] Schwartz, B., Smirnoff, P., Shany, S., Liel, Y., Estrogen controls expression and bioresponse of 1,25-dihydroxyvitamin D receptors in the rat colon, *Mol. Cell. Biochem.* 2000, *203*, 87 – 93.

[27] Beral, V., Banks, E., Reeves, G., Evidence from randomised trials on the long-term effects of hormone replacement therapy, *Lancet* 2002, *360*, 942 – 944.

[28] Bernstein, H., Bernstein, C., Payne, C. M., Dvorakova, K., Garewal, H., Bile acids as carcinogens in human gastrointestinal cancers, *Mutat. Res.* 2005, *589*, 47 – 65.

[29] Pascussi, J. M., Robert, A., Nguyen, M., Walrant-Debray, O. *et al*., Possible involvement of pregnane X receptor-enhanced CYP24 expression in drug-induced osteomalacia, *J. Clin. Invest.* 2005, *115*, 177 – 186.

[30] Verdu, E. F., Deng, Y., Bercik, P., Collins, S. M., Modulatory effects of estrogen in two murine models of experimental colitis, *Am. J. Physiol. Gastrointest. Liver Physiol.* 2002, *283*, G27 – G36.

[31] Fujii, S., Tominaga, K., Kitajima, K., Takeda, J. *et al*., Methylation of the oestrogen receptor gene in nonneoplastic epithelium as a marker of colorectal neoplasia risk in longstanding and extensive ulcerative colitis, *Gut* 2005, *54*, 1287 – 1292.

