ORIGINAL ARTICLE

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1,25-Dihydroxyvitamin D_3 Influences Cellular Homocysteine Levels in Murine Preosteoblastic MC3T3-E1 Cells by Direct Regulation of Cystathionine β-Synthase

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ABSTRACT

High homocysteine (HCY) levels are a risk factor for osteoporotic fracture. Furthermore, bone quality and strength are compromised by elevated HCY owing to its negative impact on collagen maturation. HCY is cleared by cystathionine β-synthase (CBS), the first enzyme in the transsulfuration pathway. CBS converts HCY to cystathionine, thereby committing it to cysteine synthesis. A microarray experiment on MC3T3-E1 murine preosteoblasts treated with 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] revealed a cluster of genes including the *cbs* gene, of which the transcription was rapidly and strongly induced by 1,25$(OH)_2D_3$. Quantitative real-time PCR and Western blot analysis confirmed higher levels of *cbs* mRNA and protein after 1,25$(OH)_2D_3$ treatment in murine and human cells. Moreover, measurement of CBS enzyme activity and quantitative measurements of HCY, cystathionine, and cysteine concentrations were consistent with elevated transsulfuration activity in 1,25$(OH)_2D_3$-treated cells. The importance of a functional vitamin D receptor (VDR) for transcriptional regulation of *cbs* was shown in primary murine VDR knockout osteoblasts, in which upregulation of *cbs* in response to 1,25$(OH)_2D_3$ was abolished. Chromatin immunoprecipitation on chip and transfection studies revealed a functional vitamin D response element in the second intron of *cbs*. To further explore the potential clinical relevance of our ex vivo findings, human data from the Longitudinal Aging Study Amsterdam suggested a correlation between vitamin D status [25$(OH)D_3$ levels] and HCY levels. In conclusion, this study showed that *cbs* is a primary 1,25$(OH)_2D_3$ target gene which renders HCY metabolism responsive to 1,25$(OH)_2D_3$. © 2011 American Society for Bone and Mineral Research.

KEY WORDS: 1,25$(OH)_2D_3$; HOMOCYSTEINE (HCY); CYSTATHIONINE B-SYNTHASE (CBS); VITAMIN D RECEPTOR (VDR); OSTEOPOROSIS

Introduction

The secosteroid prohormone vitamin D was discovered as an essential nutrient for the prevention of rickets.[1] Vitamin D is indispensable to sustain calcium and phosphorus homeostasis within the body. Classically, vitamin D is a potent facilitator of calcium absorption in the small intestine and thereby necessary for bone mineralization. 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], the active metabolite of vitamin D, is formed after two sequential hydroxylations: 25-hydroxylation in the liver followed by a second hydroxylation step in the kidney by the key activating enzyme, CYP27B1. The combined presence of CYP27B1 and the vitamin D receptor (VDR) in different tissues introduced the idea of a paracrine function for 1,25$(OH)_2D_3$.[2] The genomic actions of 1,25$(OH)_2D_3$ are mediated through the VDR, which acts as a ligand-activated transcription factor. The heterodimer between VDR and the retinoid X receptor (RXR) binds to vitamin D responsive elements (VDREs) in the promoter region of target

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genes and therewith affects transcription. To unravel the diverse actions of $1,25(OH)_2D_3$ on different cell types, microarray experiments have been performed in classic and nonclassic target cells.[3] These results suggested that $1,25(OH)_2D_3$ regulates transcription of ~3% of the mouse and human genome.[4] The regulated genes are involved in mineral and bone homeostasis but also in a number of other processes like cell cycle control and differentiation.

The classic role of vitamin D in calcium absorption and bone metabolism introduced the important role of vitamin D for the treatment of rickets in infants and for prevention of weak bones in the elderly. Combined supplements of vitamin D and calcium are widely used to prevent and treat osteoporosis.[5] This disease is a major public health problem, especially in older women. Nutritional, lifestyle, hormonal, and genetic factors are involved in the development of osteoporosis.[6] Among other factors, high circulating levels of the nonproteinogenic, sulfur-containing amino acid homocysteine (HCY) may contribute to osteoporotic fracture risk.[7,8] With aging, the concentration of HCY increases and easily exceeds the upper limit of 15 μM, giving rise to hyperhomocysteinemia. Bone quality and strength are compromised by aggravated hyperhomocysteinemia as a result of the negative impact of HCY on collagen cross-link formation and maturation.[9,10] Moreover, high HCY levels change bone homeostasis toward bone resorption, which further exacerbates decreased bone quality.[11]

The transsulfuration pathway is necessary for HCY disposal and culminates in the synthesis of cysteine (Fig. 1). The initial step of this metabolic pathway is the condensation of HCY and serine catalyzed by the vitamin B_6-dependent enzyme, cystathionine β-synthase (CBS). In the next step, cystathionine is cleaved by the action of cystathionine gamma-lyase to cysteine, which can be incorporated into proteins or used for the synthesis of the antioxidant, glutathione (GSH).[12] Further, cysteine can be oxidized to sulfate and excreted in the urine. Additionally, the gaseous transmitter H_2S is formed in the transsulfuration pathway by enzymatic reactions catalyzed by cystathionine gamma-lyase and CBS.[13,14] Mice deficient in *cbs* show severe hyperhomocysteinemia accompanied by elevated levels of reactive oxygen species (ROS) and a bone phenotype characterized by continuous progression of scoliosis and kyphosis.[15,16] Humans suffering from hyperhomocysteinemia caused by mutations in *cbs* or in genes of the remethylation cycle, such as methylenetetrahydrofolate reductase (MTHFR) and methionine synthase, or deficiencies in vitamins B_6, B_{12}, and folate show vascular complications, connective tissue abnormalities, and marfanoid symptoms and are also prone to osteoporotic fractures.[17] The detrimental effects caused by elevated HCY levels emphasize the necessity for a functional CBS enzyme. Microarray analysis of MC3T3-E1 preosteoblast cells treated with $1,25(OH)_2D_3$ or vehicle[18] revealed transcriptional activation of a cluster of genes in which *cbs* exhibited one of the strongest responses. This observation prompted us to investigate a possible link between HCY metabolism and $1,25(OH)_2D_3$ action via the upregulation of the *cbs* gene in bone.

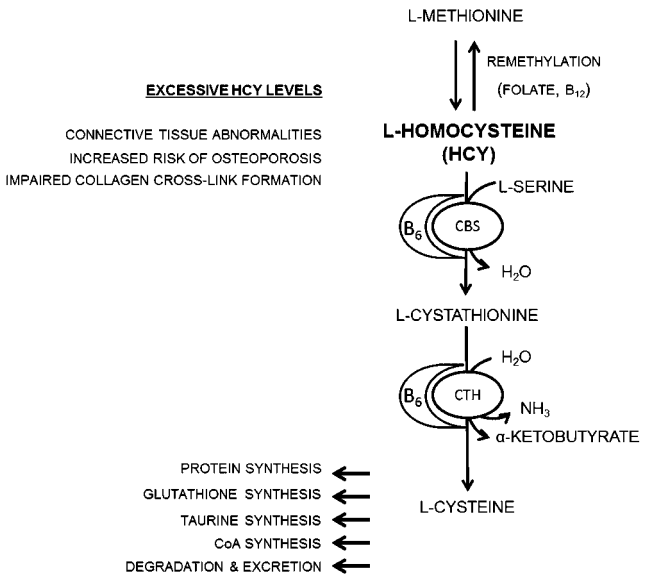

Fig. 1. Transsulfuration pathway. CBS, cystathionine β-synthase; CTH, cystathionine gamma-lyase the cofactor is vitamin B_6. Mutations in *cbs* or *cth* hamper the transsulfuration pathway and cause hyperhomocysteinemia. Also, a failure in remethylation (dependent on folate, vitamin B_{12}) or deficiencies in vitamins B_6, B_{12}, and folate cause hyperhomocysteinemia, which is associated with impaired cross-link formation, connective tissue abnormalities and increased risk of osteoporosis.

Materials and Methods

Cell culture

MC3T3-E1 cells are preosteoblasts derived from C57BL/6 mice (Riken Cell Bank, Ibaraki, Japan). The cells were maintained in α modified essential medium (αMEM) with 2 mM glutaMAX-I supplemented with 10% heat inactivated fetal bovine serum (FBS) (Biochrom AG, Berlin, Germany) and 100 units/mL penicillin and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA, USA). The human osteosarcoma SaOS-2 cell line (Riken Cell Bank) is a model for human osteoblasts. The cells were maintained in Dulbecco's modified eagle medium (DMEM) with L-glutamine, pyruvate and 4.5 g/L glucose supplemented with 10% heat-inactivated FBS (Biochrom AG) and 100 units/mL penicillin and 100 μg/mL streptomycin (Invitrogen). We isolated primary osteoblasts from newborn VDR wild-type (wt) and VDR knockout (ko) mice from calvaria by sequential digestion with 0.1% collagenase A and 0.2% dispase (Invitrogen) as described previously.[19] Cells obtained from the second to fifth fraction were pooled and cultured in αMEM with 2 mM glutaMAX-I supplemented with 10% FBS and 100 units/mL penicillin and 100 μg/mL streptomycin. We seeded the cells at a density of 11,000 cells per cm^2. The next day, we treated the cells with $1,25(OH)_2D_3$ (10^{-8} M) (Sigma-Aldrich, St. Louis, MO, USA) dissolved in ethanol or with ethanol (vehicle) for distinct time intervals. The final concentration of ethanol in medium was <0.01%.

Quantitative real-time polymerase chain reaction

We isolated total RNA for quantitative real-time polymerase chain reaction (qRT-PCR) analysis with the High Pure RNA Isolation Kit (Roche, Mannheim, Germany) following the manufacturer's instructions. One microgram RNA was reverse

transcribed using SuperScriptII (Invitrogen). We diluted the resulting cDNA 1/10 and used it as a template in PCR reactions, applying the 7500 Fast Real Time PCR System (Applied Biosystems, Inc., Foster City, CA, USA). We purchased PCR primers and fluorogenic probe for mouse *cbs* from Eurogentec (Seraing, Belgium). The gene-specific primers and probe we used are as follows: *cbs*-forward: 5′-GCAGCGCTGTGTGGTCAT-3′, *cbs*-reverse: 5′-CCATTTGTCACTCAGGAACTTG-3′, *cbs*-probe: 5′-CCTGCCTGACTCTGTGCGGAACTA-3′. In order to deduce the absolute copy number within the examined sample, we used a defined standard clone in a serial dilution. We used these data to calculate a standard curve and to assure assay specificity and efficiency. The expression of the housekeeping gene β-actin served as internal control and was used to normalize gene expression.

Western blot analysis

We isolated total protein from MC3T3-E1 cells treated with $1,25(OH)_2D_3$ (10^{-8} M) or vehicle. Cells were lysed with a buffer containing 50 mM Tris HCl, pH 8 (Sigma-Aldrich); 150 mM NaCl (Thermo Fisher Scientific, Pittsburgh, PA, USA); 0.1% sodium dodecyl sulfate (SDS) (Thermo Fisher Scientific); 1% IGEPAL CA-630 (Sigma-Aldrich); and 0.5% sodium deoxycholate (Merck, Darmstadt, Germany). We sonicated the cell lysate for 2×10 seconds at cycle 1 and amplitude 80 with the UP50H Ultrasonic Processor (Hielscher Ultrasound Technology, Teltow, Germany). Thereafter, we centrifuged the material for 10 minutes at 14,000 rpm and used the supernatant for Western blotting. Twenty-five micrograms (μg) of protein was boiled in sample buffer and reducing agent (Invitrogen) and subsequently separated by SDS-PAGE using 4–12% polyacrylamide gels (Invitrogen) and transferred to a nitrocellulose membrane (GE Healthcare, Piscataway, NJ, USA). The membrane was blocked for 1 hour with TBS (10 mM Tris-HCl; pH 7.6; 150 mM NaCl) containing 1% casein (Sigma-Aldrich) and 1% Tris (1 M). After blocking, we incubated the membrane with slight agitation overnight at 4 °C with the primary antibody (rabbit anti-human CBS 1/500, developed in the lab of Prof. R. Banerjee, Ann Arbor, MI, USA). Incubation with a horseradish peroxidase (HPR)-conjugated secondary antibody (Dako, Glostrup, Denmark) was performed for 1 hour at room temperature. All blots were developed by enhanced chemiluminescence (Perkin Elmer, Waltham, MA, USA).

Cystathionine β-synthase activity assay

CBS activity was measured by the ninhydrin assay as described by Vitvitsky and colleagues.[20] In brief, cells were homogenized using a mortar cooled with liquid nitrogen and subsequently 150 μg of the obtained cell powder was mixed with 350 μL 1.15% KCl solution (Merck). After centrifugation (15,000 *g* for 20 minutes at 4°C) the supernatant was aspirated. For the assay, 100 μL sample supernatant, 10 μL Tris-HCl (1 M), 2 μL pyridoxal phosphate (1.2 mM in 0.1 M Tris, pH 8.3) (Sigma-Aldrich) and 20 μL propargylglycine (25 mM in 0.1 M Tris, pH 8.3) (Sigma-Aldrich) were mixed and incubated for 15 minutes at 37°C. Subsequently, 20 μL serine (1 M in 0.1 M Tris, pH 8.3) (Fluka by Sigma-Aldrich, Steinheim, Germany) and 40 μL HCY (0.75 M in water) (Sigma-Aldrich) solutions were added, mixed and incubated for 60 minutes at 37°C. We stopped the reaction with 20 μL of 50% trichloroacetic acid (Merck). We centrifuged the samples and discarded the pellets. We mixed the supernatant (100 μL) with 825 μL ninhydrin reagent (1 g ninhydrin [ACROS Organics by Thermo Fisher Scientific], dissolved in 100 mL glacial acetic acid [Sigma-Aldrich] mixed with 33 mL molten crystal phosphoric acid [Sigma-Aldrich]). The mixture of sample and reagent was incubated for 5 minutes in boiling water followed by 2 minutes on ice and 20 minutes at room temperature. We measured the optical density of the resulting solution (455 nm) against a blank sample. We assayed all samples in duplicate and calculated CBS activity using a calibration curve generated with cystathionine (2–10 mM) (Sigma-Aldrich).

Chromatin immunoprecipitation on chip

Data obtained for the *cbs* gene locus in the MC3T3-E1 cell line were derived from a genome-wide chromatin immunoprecipitation (ChIP)-on-chip analysis previously reported by Meyer and colleagues.[21] In brief, we performed ChIP-on-chip analyses using HD2-formatted mouse whole genome tiling arrays (Roche-NimbleGen Inc.). The data were extracted using the NimbleScan software (version 2.5) and normalized using locally weighted scatter-plot smoothing (LOWESS) normalization in R. The $\log_2$ ratio of test versus experimental data were calculated for each point and peaks were called using CMARRT algorithms. Data shown are representative of two or more ChIP-on-chip analyses performed for each experimental set. All data were visualized using Gbrowse (www.gmod.org/wiki/Gbrowse). Classical ChIP experiments for VDR, RXR, and acetylated histone 4 (H4-Ac) were performed to confirm the ChIP-on-chip results. These experiments were done as previously described.[21,22] We treated MC3T3-E1 cells with $1,25(OH)_2D_3$ (10^{-7} M). The cell lysates used in these ChIP and ChIP-on-chip experiments were from independent experiments.

Transfections

We transfected MC3T3-E1 cells either with 200 ng empty pGL3basic luciferase reporter vector (Promega, Madison, WI, USA) or pGL3basic vector, in which luciferase expression is under control of a wild-type 1456-bp fragment. This fragment originates from the second *cbs* intron present on the murine chromosome 17 between nucleotide 31,359,592 and 31,361,047. We retrieved all the murine genome data from the University of California, Santa Cruz (UCSC) genome browser and based on the Build 36 "essentially complete" assembly (MM8) by the National Center for Biotechnology Information (NCBI) and the Mouse Genome Sequencing Consortium. Furthermore, we used a 1456-bp fragment with a mutation in the direct repeat 3 (DR3)-type VDRE. We performed mutations with the QuickChange II Site-Directed Mutagenesis kit (Stratagene, Santa Clara, CA, USA). Additionally, we co-transfected cells with 20 ng of the β-galactosidase expression vector pcDNA3.1(−)/Myc-His/lacZ (Invitrogen) to assess transfection efficiency. We performed the transfection using Fugene 6 transfection reagent (Roche) following the manufacturer's instructions. After transfection, we

cultured the cells overnight and treated them the following day with $1,25(OH)_2D_3$ (10^{-8} M) or vehicle. After 24 hours, we rinsed the cells in PBS and lysed them with 100 μL of the Reporter Gene Lysis Buffer (Roche). We used cell extracts to measure luciferase activity with the Luciferase Assay System (Promega); the obtained values were normalized for the efficiency of transfection using β-galactosidase activity detected by the Tropix® Galacto-Light Plus System (Applied Biosystems). We measured luciferase and β-galactosidase activity by the Luminoskan Ascent luminometer (Thermo Fisher Scientific). We performed all experiments in triplicate and repeated them three to five times.

Metabolite analysis

For the measurement of HCY, cystathionine, and cysteine in cell lysates and growth medium, we seeded MC3T3-E1 cells in 60-cm^2 dishes (11,000 cells per cm^2). Cells were left overnight to attach. The next day, we treated the cells with $1,25(OH)_2D_3$ (10^{-8} M) or vehicle. To determine baseline levels, we prepared cell lysates from cells that were not treated and measured metabolite concentrations. After 48 hours or 72 hours incubation, we scraped cells and centrifuged the cell-medium suspension for 10 minutes at 1500 *g*. Subsequently, we aspirated the medium and used it for further analysis. Additionally, we froze an aliquot of fresh medium and used it to determine the standard amino acid content. We resuspended the cell pellet in 500 μL distilled water and subjected it to a freeze-thaw cycle and to 5 minutes sonication with the Bioruptor Sonicator (Diagenode, Liège, Belgium). Finally, we measured the concentrations of the cellular metabolites HCY, cysteine, and cystathionine by gas chromatography–mass spectrometry (Dr. Sally Stabler, Colorado University Medical Center, Aurora, CO, USA) as described before.[23] In order to normalize obtained measurements, cell samples that had been treated with vehicle or $1,25(OH)_2D_3$ (10^{-8} M) were analyzed for protein content. After washing with PBS, we lysed cells and determined protein content as described before (Western blot analysis). For GSH measurements, we rinsed MC3T3-E1 cells twice with cold PBS and subsequently scraped them in 100 μL PBS. The cell suspension was subject to GSH measurement by HPLC as described before.[24]

Longitudinal Aging Study Amsterdam cohort

The Longitudinal Aging Study Amsterdam (LASA) is a cohort study of elderly people aged 55 to 85 years at the start in 1992. Persons recruited were a random sample stratified by age, sex, and expected 5-year mortality rate drawn from the population registers of 11 municipalities in three regions of the Netherlands. In total, 3107 persons were enrolled in the baseline examination in 1992–1993. Serum 25-hydroxyvitamin D_3 [$25(OH)D_3$] was measured in samples obtained in 1995–1996 in all participants of 65 years and older as of January 1, 1996. The blood samples were immediately centrifuged and kept frozen at −20 °C until analysis in 1999. Serum $25(OH)D_3$ was measured with a competitive protein binding assay (Nichols Diagnostics, San Juan Capistrano, CA, USA) with an interassay coefficient of variation of 10%. For the present study, we selected 1264 subjects with normal renal function; ie, with creatinine levels between 50 and 150 μM. We applied a regression model to evaluate the relation between $25(OH)D_3$ and HCY in 1264 subjects. The multivariable model contained age (linear), gender, creatinine level (linear), and $25(OH)D_3$ level (nonlinear) as predictors. In the analysis, no interactions were included. We performed all analyses using the statistical package SAS (version 9.2; SAS Institute, Cary, NC, USA). Values of p smaller than 0.05 were considered significant.

Results

Effects of $1,25(OH)_2D_3$ on *cbs* gene expression

In order to study the effects of $1,25(OH)_2D_3$ on the expression of *cbs*, we cultured MC3T3-E1 mouse preosteoblasts in the presence of $1,25(OH)_2D_3$ (10^{-8} M) and monitored transcript levels of *cbs* at different time points. *Cbs* mRNA levels increased as early as 3 hours after treatment and showed a 350-fold

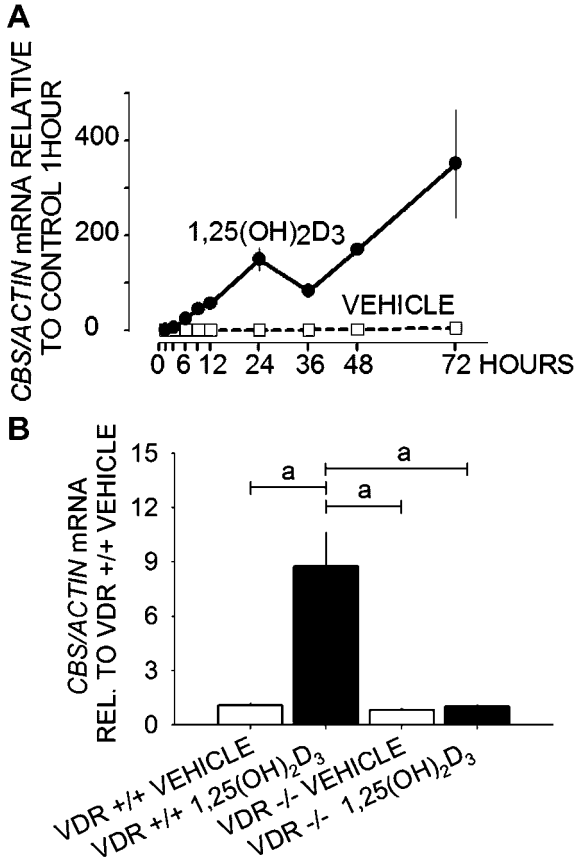

Fig. 2. Transcript levels of *cbs* are upregulated by $1,25(OH)_2D_3$ in murine MC3T3-E1 and primary osteoblasts. (*A*) Transcript levels were determined by qRT-PCR at different time points after treatment with $1,25(OH)_2D_3$ (10^{-8} M). Each data point represents the ratio of *cbs* levels normalized over β-actin, relative to the normalized *cbs* levels of vehicle-treated cells (1 hour). The data are the mean ± SEM of four independent experiments. The overall upregulation of *cbs* expression by $1,25(OH)_2D_3$ was found to be significant according to ANOVA ($p < 0.05$). (*B*) *Cbs* expression measured by qRT-PCR in primary osteoblasts treated for 24 hours with vehicle or $1,25(OH)_2D_3$ (10^{-8} M). Osteoblasts originate from VDR wild-type (VDR wt; $n = 6$) and VDR knock out (VDR ko; $n = 5$) mice. Bars represent the mean ± SEM of each group. The expression of *cbs* in $1,25(OH)_2D_3$-treated VDR wt osteoblasts is significantly different from the other groups displayed, according to ANOVA followed by a Bonferroni multiple-comparison test ($^a p < 0.05$).

induction at 72 hours compared to control conditions at 1 hour (Fig. 2*A*). *Cbs* levels did not change in vehicle-treated cultures. Further, to investigate whether a functional VDR is required for the induction of *cbs* transcript levels by $1,25(OH)_2D_3$, we measured *cbs* cDNA levels in VDR ko murine primary osteoblasts treated for 24 hours with $1,25(OH)_2D_3$ (10^{-8} M) or with vehicle. The *cbs* cDNA levels in VDR ko osteoblasts did not change after incubation with $1,25(OH)_2D_3$, whereas in osteoblasts from VDR wt mice *cbs* expression was strongly enhanced by $1,25(OH)_2D_3$ (more than eightfold) (Fig. 2*B*).

Transcriptional activation of *cbs* by vitamin D receptor

Next, we used a ChIP-on-chip approach to identify a VDR binding site within the *cbs* gene. $1,25(OH)_2D_3$-treated MC3T3-E1 cells showed robust binding of VDR, RXR, and the presence of H4-Ac at a 1-kilobase (kb) region approximately 6 kb downstream of the *cbs* transcription start site (chromosome 17 between nucleotide 31,359,000 and 31,361,000) (Fig. 3*A*). The detected receptor binding suggested the presence of a VDRE in the second intron of the *cbs* gene. In silico screening revealed a putative VDRE with a classical DR3 motif containing two hexameric sequences separated by a 3-bp spacer within the second *cbs* intron located at +5983 to 5998 relative to the *cbs* transcription start site (ENSMUST00000067801). Subsequently, we applied the classical ChIP approach to confirm the ChIP-on-chip data for VDR, RXR, and H4-Ac (Fig. 3*A*). Next, a 1456-bp fragment of the *cbs* gene (position +4862 to +6318 relative to transcription start site or nucleotide 31,359,592 and 31,361,047 on chromosome 17), which contained the putative VDRE, was cloned into a pGL3basic luciferase reporter plasmid to investigate the transactivating activity of $1,25(OH)_2D_3$ in transient transfection experiments (Supporting Fig. S1). Relative to vehicle-treated cells, incubation with 10^{-8} M $1,25(OH)_2D_3$ resulted in an eightfold increase in

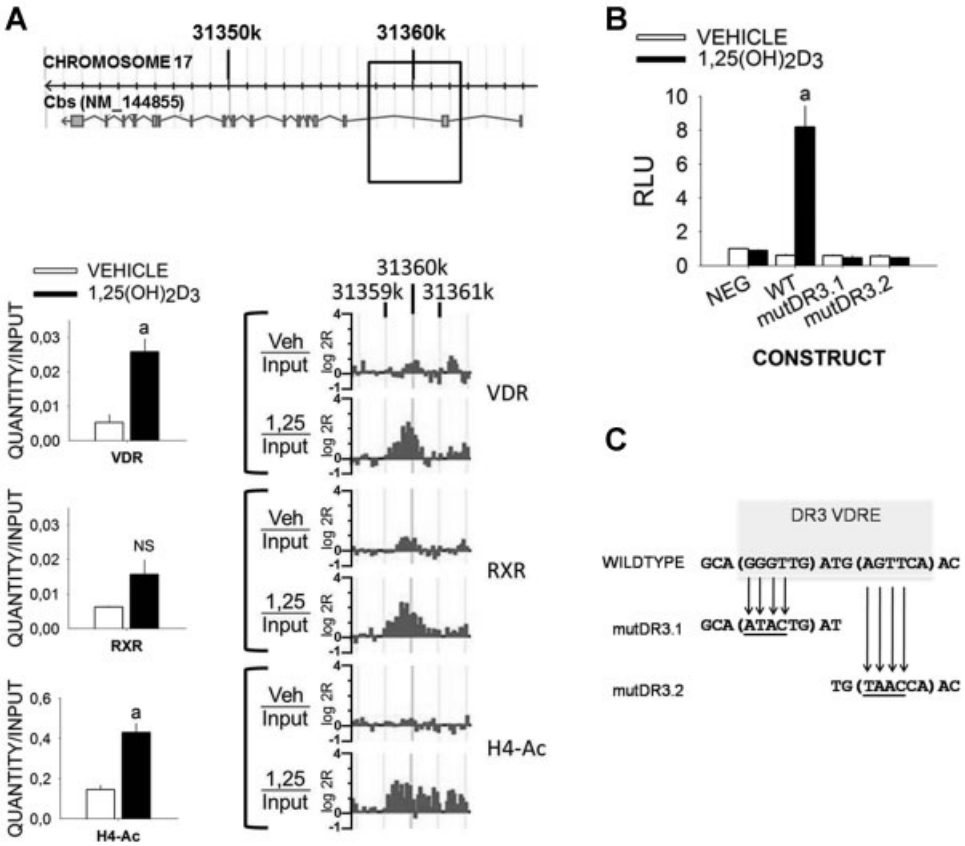

Fig. 3. Transcriptional activation of the *cbs* gene by VDR bound to a classical DR3-type VDRE. (*A*) ChIP-on-chip was used to determine transcription factor binding. The genomic location for the *cbs* gene is shown for chromosome 17 with genomic base pairs given in kilobases (k). Increased binding is indicated by enhanced readout (peaks) at the murine DNA sequence at chromosome 17 between nucleotide 31,359,000 and 31,361,000. The assay was performed on chromatin from vehicle- (Veh/Input) and $1,25(OH)_2D_3$ (1.25/Input)-treated MC3T3-E1 cells with antibodies against VDR, RXR, and H4-Ac. Data are displayed as log2 ratios (log2 R). Next to the murine DNA sequence, the results of classical ChIP experiments confirm the ChIP-on-chip data. Bars are the mean ± SEM of three independent experiments. $^a p < 0.05$ according to Student's *t* test). (*B*) MC3T3-E1 cells were transfected with empty pGL3basic (neg) or pGL3basic containing the wild-type (wt) or mutated (mutDR3.1 or mutDR3.2) fragment of the second *cbs* intron (murine chromosome 17 between nucleotide 31,359,592 and 31,361,047) and with a β-galactosidase-expressing construct. After 24 hours, cells were treated with $1,25(OH)_2D_3$ (10^{-8} M) or vehicle and after another 24 hours luciferase and β-galactosidase were measured. Luciferase activities were normalized to β-galactosidase activities. RLU = relative luciferase units. Bars are the mean ± SEM of at least three independent experiments. (*C*) Mutated fragments were obtained by substitution of 4 nucleotides (GGGTTG to ATACTG) within the 3′ hexamer of the VDRE (mutDR3.1) and the 5′ hexamer of the VDRE (AGTTCA to TAACCA (mutDR3.2). The relative luciferase activity (RLU) in $1,25(OH)_2D_3$-treated cells transfected with the wt construct is significantly different from the other measurements displayed, according to ANOVA followed by a Bonferroni multiple-comparison test ($^a p < 0.05$).

luciferase activity in MC3T3-E1 cells transfected with the *cbs* DR3-type VDRE-containing reporter construct (Fig. 3*B*). To confirm that the transactivation was driven by the putative VDRE, we made two reporter constructs, which contained mutations within the DR3-type VDRE (Fig. 3*C*). In one mutant, the 3′ hexamer sequence GGGTTG was mutated to ATACTG (mutDR3.1), whereas in the second, the 5′ hexamer AGTTCA was altered to TAACCA (mutDR3.2). As shown in Figure 3*B*, mutation of the VDRE within the *cbs* promoter construct (mutDR3.1 or mutDR3.2) completely abolished the responsiveness to $1,25(OH)_2D_3$.

Effect of $1,25(OH)_2D_3$ on CBS protein and enzymatic function

Western blot analysis showed a strong increase in CBS levels at 24 hours after 10^{-8} M $1,25(OH)_2D_3$ treatment and at later time points (Fig. 4*A*). The induction of CBS by $1,25(OH)_2D_3$ was observed in both a murine (MC3T3-E1) and a human (SaOS-2) cell line. To investigate whether $1,25(OH)_2D_3$ treatment resulted in increased CBS activity, we measured the synthesis of cystathionine from the substrates, HCY and serine, in MC3T3-E1 cell lysates at baseline (0 hours) and after 48 hours and 72 hours after treatment. CBS activity was 2.6- and 1.9-fold higher at 48 hours and 72 hours, respectively, in cell lysates derived from $1,25(OH)_2D_3$-treated cells compared to vehicle-treated controls (Fig. 4*B*).

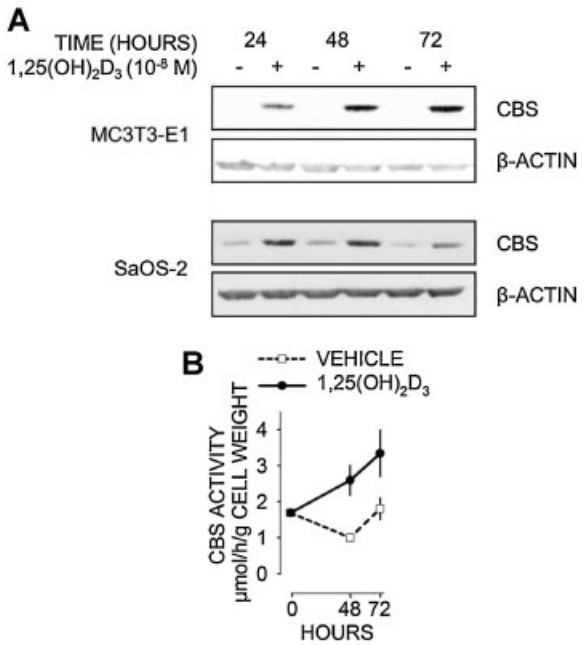

Fig. 4. (*A*) Western blot analysis of CBS expression on total lysates of vehicle- and $1,25(OH)_2D_3$-treated murine preosteoblasts (MC3T3-E1) and human osteosarcoma cells (SaOS-2) at different time points; β-actin was used as loading control. (*B*) The enzymatic activity of CBS is depicted at 0 hours and after 48 hours and 72 hours in vehicle- and $1,25(OH)_2D_3$-treated cells. The mean ± SEM from six independent experiments is shown. The overall changes in CBS enzymatic activity by $1,25(OH)_2D_3$ were found to be significant according to ANOVA ($p < 0.05$).

Effect of $1,25(OH)_2D_3$ on the amino acids of the transsulfuration pathway

Next, we investigated targeted metabolite changes by determining the concentrations of HCY, cystathionine, cysteine, and GSH in MC3T3-E1 cell lysates (Fig. 5). The HCY concentration was lower in $1,25(OH)_2D_3$-treated cells compared to vehicle-treated controls (−30% and −26% versus control after 48 hours and 72 hours, respectively; Fig. 5*A*). In contrast, the concentration of cystathionine (112% and 74% versus control after 48 hours and 72 hours, respectively; Fig. 5*B*) and cysteine (23% and 24% versus control after 48 hours and 72 hours; Fig. 5*C*) increased in $1,25(OH)_2D_3$-treated cells compared to control conditions, whereas GSH decreased in $1,25(OH)_2D_3$-treated cells (−28% and −44% versus control after 48 hours and 72 hours, respectively; Fig. 5*D*). We further investigated the concentrations of amino acids in the culture medium (Fig. 6). Whereas the concentration of HCY (−39% and −43% versus control after 48 hours and 72 hours; Fig. 6*A*) was decreased significantly, the concentration of cystathionine (115% and 152% versus control after 48 hours and 72 hours, respectively; Fig. 6*B*) and cysteine (91% and 171% versus control after 48 hours and 72 hours, respectively; Fig. 6*C*) was significantly higher after $1,25(OH)_2D_3$ treatment. Metabolite concentrations in cell lysates and medium

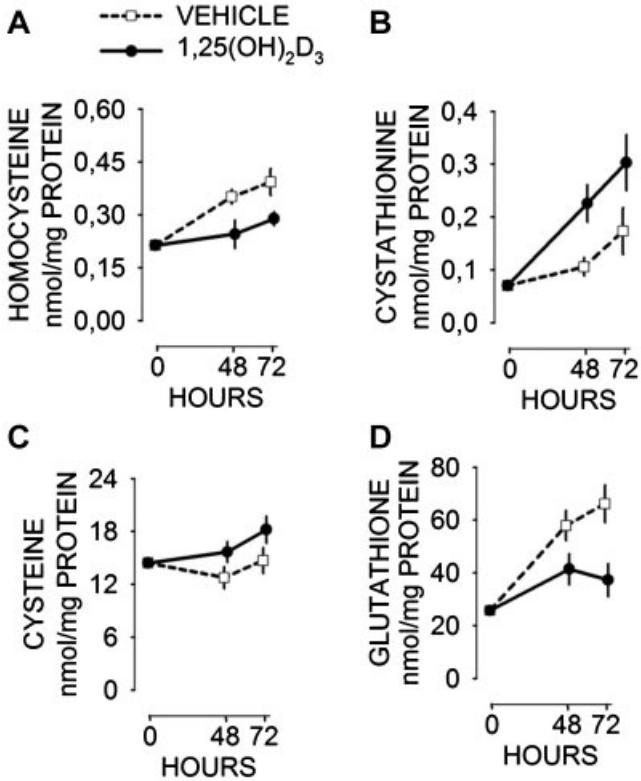

Fig. 5. Changes in metabolite composition in $1,25(OH)_2D_3$-treated MC3T3-E1 preosteoblasts. The concentrations of (*A*) HCY, (*B*) cystathionine, (*C*) cysteine, and (*D*) GSH are depicted at 0 hours and after 48 hours and 72 hours in vehicle- and $1,25(OH)_2D_3$-treated cells. Metabolites were measured in cell lysates. Each data point represents the mean ± SEM of three or four duplicate measurements. The overall changes in HCY, cystathionine, cysteine, and GSH levels by $1,25(OH)_2D_3$ were found to be significant according to ANOVA ($p < 0.05$).

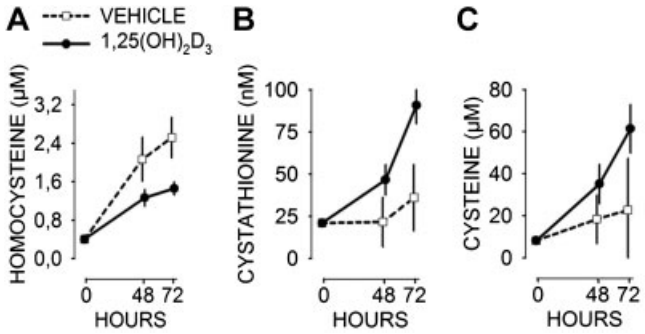

Fig. 6. Changes of amino acid content in growth medium of vehicle- or $1,25(OH)_2D_3$-treated cells. The concentrations of (*A*) HCY, (*B*) cystathionine, and (*C*) cysteine are depicted at baseline and after 48 hours and 72 hours of vehicle or $1,25(OH)_2D_3$ treatment. Measurements were normalized by subtraction of the amino acid concentration present in fresh medium. Each data point represents the mean ± SEM of four duplicate measurements. The overall decrease of HCY levels and increase of cystathionine and cysteine levels by $1,25(OH)_2D_3$ were found to be significant according to ANOVA ($p < 0.05$).

from $1,25(OH)_2D_3$-treated cultures were always significantly different from those in vehicle-treated cultures according to analysis of variance (ANOVA) ($p < 0.05$).

Vitamin D status and HCY levels

In order to assess whether vitamin D status is correlated with HCY levels in vivo, we investigated the LASA cohort, a human population-based study. We analyzed HCY, $25(OH)D_3$, and creatinine (creatinine levels between 50 and 150 μM) levels in 1264 individuals between 65 and 88 years of age. We used a flexible regression model that allows for nonlinearity to analyze the relation between HCY and $25(OH)D_3$ levels. A significant correlation ($p = 0.0015$) between HCY and $25(OH)D_3$ levels, the marker of choice for vitamin D status, was observed and the relation was U-shaped (Fig. 7), with lowest HCY levels when $25(OH)D_3$ status was between 50 and 60 nM (20–24 ng/mL). To estimate this transition point, we applied a two-phase linear model (in a multivariable setting correcting for age, gender, and creatinine), resulting in an estimated cut-point of 51.8 nM (20.72 ng/mL) (95% confidence interval, 40.6–62.9). The Spearman correlation equalled −0.103 ($p = 0.009$) before the cut-point and 0.078 ($p = 0.054$) beyond the cut-point, respectively. A statistically significant difference in HCY levels between males and females was not shown ($p = 0.57$); a significant correlation between HCY and creatinine levels ($p < 0.0001$) was found.

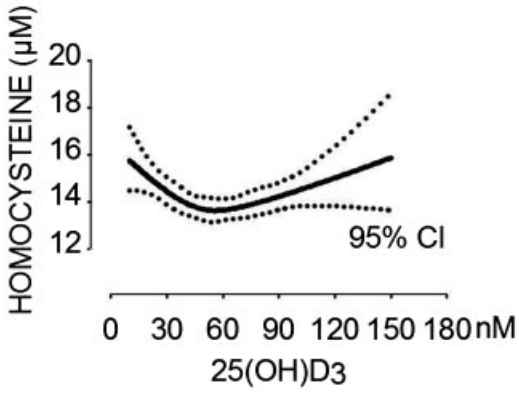

Fig. 7. Relation between $25(OH)D_3$ and homocysteine obtained from a regression model on 1264 subjects with creatinine levels between 50 and 150 μM. Dotted lines represent pointwise 95% confidence intervals (CI) for the mean relation, which is depicted for an "average" subject; ie, having a creatinine level of 100 μM and being 75 years of age.

Discussion

This study provides evidence that *cbs* is a direct target of VDR regulation and suggests that a functional transsulfuration pathway is present in osteoblasts. Our analysis showed that basal levels of *cbs* mRNA and protein in MC3T3-E1 murine preosteoblasts were low and increased strongly after incubation with $1,25(OH)_2D_3$. A functional VDR was required for the induction of *cbs* mRNA levels, since the expression of *cbs* was not increased in VDR ko primary osteoblasts after $1,25(OH)_2D_3$ treatment. The direct regulation of *cbs* by $1,25(OH)_2D_3$ was further established by ChIP-on-chip and classic ChIP experiments and additionally by ChIP-sequencing (data not shown). All these experiments show VDR binding together with RXR and acetylated histone H4 in the intergenic region of the *cbs* gene. This observation led to the identification of a functional VDRE in the second *cbs* intron. The induction of *cbs* expression by $1,25(OH)_2D_3$ was not limited to MC3T3-E1 cells but was also shown in a number of different murine cell lines like ATDC5 chondrocytes, bone marrow stromal ST-2 cells, in GR mammary carcinoma cells, but also in primary murine osteoblasts, bone marrow stromal, and kidney cells (data not shown). The liver expresses the highest CBS protein levels and activity.[25] Nevertheless, we could not detect an effect of $1,25(OH)_2D_3$ on the expression of *cbs* in hepatocytes (data not shown); however, the basal *cbs* expression in these cells is comparable to *cbs* expression in $1,25(OH)_2D_3$-treated osteoblasts. The absence of $1,25(OH)_2D_3$-driven *cbs* regulation in liver is likely as a result of the lack of sufficient VDR expression in this tissue.[26] In liver, regulation of *cbs* relies on different transcription factors and regulators. Among these is the allosteric activator S-adenosylmethionine, which can increase CBS activity twofold to threefold; it also stabilizes the protein against degradation.[27,28] In addition, the binding of a number of different transcription factors within the *cbs* promoter, such as the constitutively active specificity protein 1 and factor NF-Y, was reported.[29] Taken together, this suggests that the effects of $1,25(OH)_2D_3$ on the HCY metabolism are local, affecting osteoblasts and other VDR-expressing cells directly. This assumption was further supported by experiments performed in VDR wt and VDR ko mice (data not shown), in which no differences in the total plasma HCY levels were detected. This is probably attributed to the before mentioned natural lack of VDR expression in the liver[26] and an unaffected hepatic HCY metabolism. For MC3T3-E1 preosteoblasts we could conclusively show that *cbs* expression was directly driven by $1,25(OH)_2D_3$ activating VDR bound to a classical DR3-type VDRE. The $1,25(OH)_2D_3$-induced increase in

cbs expression in osteoblasts was accompanied by an increase in CBS protein levels and enzymatic activity. The changes suggest an increased production of transsulfuration products in $1,25(OH)_2D_3$-stimulated osteoblasts. Furthermore, the lower level of CBS substrate (HCY) and higher concentrations of transsulfuration pathway products (cystathionine and cysteine) in $1,25(OH)_2D_3$-treated MC3T3-E1 preosteoblasts and in the culture medium are consistent with an enhanced flux through the transsulfuration pathway. These changes may have several implications, since increased cysteine levels after $1,25(OH)_2D_3$ treatment could be used for the increased production of GSH, H_2S, or taurine, or used in protein synthesis.[12] However, we found decreased intracellular GSH levels in $1,25(OH)_2D_3$-treated MC3T3-E1 cells. This finding is in line with the observation that the concentration of GSH is unchanged and the level of the GSH breakdown product, cysteinyl-glycine, is decreased in transgenic mice with higher enzymatic CBS activity.[30] On the contrary, increased GSH levels have been shown in primary rat astrocytes treated with lipopolysaccharide (LPS) and $1,25(OH)_2D_3$ owing to the stimulating effect of $1,25(OH)_2D_3$ on the expression and activity of the enzyme gamma-glutamyl transpeptidase.[31] Moreover, increased CBS activity is also linked to enhanced HCY elimination and may have implications for the maintenance of bone quality and strength. It has been shown that HCY accumulates specifically in bone by binding to collagen.[32] Vitamin D may counteract the effects of high HCY levels on bone by increased CBS synthesis and enzymatic activity. The effects of $1,25(OH)_2D_3$ on CBS were not limited to murine bone cells but were also observed in human osteosarcoma SaOS-2 cells, a cell line with osteoblastic features.[33]

To further explore the potential clinical relevance of the ex vivo data, and to evaluate the relation between serum $25(OH)D_3$ and HCY levels without making any a priori assumptions about the type of relation, we used a flexible regression modeling approach. We used data from the LASA study, which contains a random population-based sample of older men and women aged 65 to 88 years, for analysis. Serum $25(OH)D_3$ is the best indicator of overall vitamin D status, because this measurement reflects total vitamin D from dietary intake and sunlight exposure, as well as the conversion of vitamin D from adipose stores in the liver.[34] The aged human population is generally characterized by elevated HCY, low vitamin D levels and an increased susceptibility to osteoporotic fracture, especially in women. Based on the relationship between serum $25(OH)D_3$, bone mineral density, bone turnover, lower extremity function, and falls, 50 nM (20 ng/ml) is considered an appropriate serum $25(OH)D_3$ threshold to define vitamin D insufficiency.[35] In line with this 50-nM threshold, several studies have suggested that vitamin D supplementation may be most effective in reducing fractures and falls in institutionalized elderly persons, in whom serum levels of $25(OH)D_3$ are often below 50 nM.[36] In our analysis of LASA samples, we found a significant, creatinine-adjusted relationship between HCY and serum $25(OH)D_3$ levels. In particular, levels below 50 nM were associated with an increase in circulating HCY (Spearman correlation of -0.013 before the cut-point of 51.8 nM), consistent with the assumption that vitamin D insufficiency may potentially contribute to the amount of HCY in serum. To further unravel the $25(OH)D_3$–HCY relationship, more data are needed on the effects of vitamin D supplementation on serum HCY. The data showed no difference in HCY levels between males and females but confirmed the significant positive relationship between HCY and creatinine levels, which is in line with previously reported evidence that HCY levels increase with the degree of renal insufficiency.[37] Elevated HCY levels are correlated with a number of different diseases and detrimental health implications, such as osteoporotic fractures or rising risk of stroke.[38] In osteoblast cells, it was shown that high HCY levels inhibit the expression of enzymes necessary for enzymatic collagen cross-link.[39,40] Moreover, HCY binds to cross-link precursors and might result in weaker bone and reduced bone quality.[41] Therefore, high HCY levels have negative effects on collagen cross-linking and affect bone in a negative way. The possibility to lower HCY levels systemically or locally should impact bone health in a positive manner.

From our data, we can conclude that there is an association between the $25(OH)D_3$ status and the amount of HCY in serum. This effect is relatively small compared to the HCY lowering effects of other vitamins, such as the B vitamins but $1,25(OH)_2D_3$ may support the action of folic acid, vitamin B_6, and B_{12} in lowering high HCY levels.[42] Nevertheless, this beneficial effect of $1,25(OH)_2D_3$ on HCY levels adds to the multitude of positive effects linked to an optimal vitamin D status.

Disclosures

All authors state that they have no conflicts of interest.

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