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Short Communication

Dietary vitamin E deficiency does not affect global and specific DNA methylation patterns in rat liver

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The aim of the present study was to determine the effects of a 6-month dietary vitamin E (VE) deficiency on DNA methylation and gene expression in rat liver. Two enzymes, 5-α-steroid reductase type 1 (SRD5A1) and the regulatory subunit of γ-glutamylcysteinyl synthetase (GCLM), which are differentially expressed on the mRNA level, were analysed for promoter methylation in putative cytosine-phospho-guanine (CpG) island regions located at the 5′ end using base-specific cleavage and matrix-assisted laser desorption ionisation time-of-flight MS. A twofold increase in the mRNA level of SRD5A1 gene and a twofold decrease in the mRNA level of GCLM gene in VE-deficient animals were not associated with different CpG methylation of the analysed promoter region. Furthermore, global DNA methylation was not significantly different in these two groups. Thus, the present results indicate that the VE-induced regulation of SRD5A1 and GCLM in rat liver is not directly mediated by changes in promoter DNA methylation.

Tocopherol: Rat liver: DNA methylation: Epigenetics

Vitamin E (VE), particularly its major congener α-tocopherol (αT), has been shown to regulate gene expression[1–3]. We have previously demonstrated that long-term dietary VE deficiency resulted in an increase in 5-α-steroid reductase type 1 (SRD5A1) mRNA levels, which was accompanied by changes in the plasma 5-dihydrotestosterone:testosterone ratio[4]. Furthermore, VE deficiency significantly lowered mRNA levels of γ-glutamylcysteinyl synthetase (GCLM), which is the rate-limiting enzyme of glutathione synthesis. Rats fed the diets supplemented with αT exhibited 20 % higher hepatic GSH levels than the αT-depleted animals. Cellular GSH levels may influence epigenetic processes including DNA and histone methylation by limiting the availability of *S*-adenosylmethionine, which is the cofactor utilised during epigenetic control of gene expression by DNA and histone methyltransferases[5]. Furthermore, demethylases require oxygen as a cofactor which links the cellular redox state to the epigenotype. DNA methylation consists of the addition of a methyl group to the 5′ position of cytosine in a cytosine-phospho-guanine (CpG) dinucleotide. Whereas most genomic DNA in mammals is deficient in CpG sites, clusters of CpG dinucleotides (CpG islands) are located in the promoter regions of >70 % of known rat genes[6]. Thus, it seems likely that DNA methylation at CpG islands may constitute one means by which gene expression in laboratory rats is affected.

As αT influences redox-regulated gene expression, we have investigated whether diet-induced VE deficiency in rat liver is associated with alterations in global and specific DNA methylation.

Experimental methods

Experimental animal and diets

Two groups of eight male Fischer 344 rats each (mean body weight, 51 (SD 5) g, Charles River Laboratories, Sulzfeld, Germany) were randomly assigned to a VE-containing (VE^+) or a VE-deficient (VE^-) semi-synthetic diet (ssniff Spezialdiaeten GmbH, Soest, Germany). All VE in the diets originated from native or antioxidant-stripped rapeseed oils, respectively, and were as follows (mg/kg diet; analysed by HPLC): VE^-:

Abbreviations: αT, α-tocopherol; CpG, cytosine-phospho-guanine; GCLM, γ-glutamylcysteinyl synthetase; SRD5A1, 5-α-steroid reductase type 1; VE, vitamin E; VE^-, VE-deficient; VE^+, VE-sufficient.

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αT, <1; VE^+: αT, 12. Tocopherols were quantified by HPLC with fluorescence detection[7].

The rats had free access to tap water and the experimental diets throughout the experiment, and were housed in pairs in a conditioned room (temperature, 22 ± 2°C; relative humidity, 55 %; 12 h light–12 h dark cycle). The animal experiment was conducted in accordance with the German regulations on animal care and with the permission of the responsible authority. After 6 months, the rats were fasted for 12 h before CO_2 anaesthesia and decapitation. The liver was excised and dissected: one part was stored in RNAlater (Qiagen, Hilden, Germany), and the remainder was snap-frozen in liquid nitrogen and stored at −80°C until used.

Real-time quantitative real time-PCR

Total RNA was isolated from rat liver according to the RNeasy Lipid-tissue Protocol (Qiagen) with primer pairs as described[8]. One-step quantitative real time-PCR was carried out using the QuantiTect SYBR Green RT-PCR kit (Qiagen), and measured in a Rotor-Gene 3000 thermocycler (Corbett Research, Sydney, Australia). Relative mRNA concentrations were normalised to the housekeeping gene β-actin.

Promoter DNA methylation analysis

The presence of CpG islands within the SRD5A1 and GCLM genes was predicted using The European Molecular Biology Open Software Suite CpGplot. Quantitative methylation analysis of the two genes was performed with the MassARRAY® system (Sequenom, Hamburg, Germany) at BioGlobe (Hamburg, Germany). The MassCLEAVE™ biochemistry was applied after bisulphite treatment of DNA samples and matrix-assisted laser desorption ionisation time-of-flight MS for analyte detection according to the standard protocols recommended by the supplier. Genomic DNA was extracted from rat liver using the DNeasy Kit (Qiagen). One microgram of DNA was treated with sodium bisulfite (DNA Bisulfite Treatment Kit, Sequenom), and target regions of the modified nucleic acid were amplified by PCR using methylation-independent primers, which were designed by the MassARRAY platform-specific EpiDesigner software (Table 1). The PCR products were then subjected to *in vitro* transcription, with the RNase A cleavage being used for the T-reverse reaction (Sequenom). The generated fragments were displayed based on their molecular weight in the mass spectrum, which was acquired after sample conditioning with a MassARRAY® Analyzer Compact. The resulting methylation calls were analysed with EpiTyper software (Sequenom) to generate quantitative results for each CpG site.

DNA global methylation analysis

Tissue DNA was extracted using the DNeasy Tissue Kit (Qiagen) including RNase treatment. The extracted DNA was quantified using Quant-iT™ PicoGreen® dsDNA kit (Invitrogen, Karlsruhe, Germany). For each sample, methylation analysis was performed in duplicate aliquots (100 ng DNA each) using anti-methylated cytosine antibody-based Methylamp™ Global DNA Methylation Quantification Kit (Epigentek, Brooklyn, NY, USA) as well as Imprint™ Methylated DNA Quantification Kit (Sigma Aldrich, Darmstadt, Germany). DNA methylation status was compared to an artificially fully methylated DNA standard supplied with each kit. The levels of methylated DNA were then proportional to the optical density intensity on a microplate reader at 450 nm (Spectra Max 190, Molecular Devices, Ismaning, Germany).

In different animal species, the spectrum of methylation levels and patterns is very broad. At the low extreme is the nematode worm *Caenorhabditis elegans*, whose genome lacks detectable methylated cytosine and does not encode a conventional DNA methyltransferase[9]. Thus, DNA from *C. elegans* was chosen to serve as a negative control for methylation pattern. In fact, the global DNA methylation of *C. elegans* was low (44·1 (SD 11·0) %), which was considered as the background level. Furthermore, we could confirm differences in the relative global DNA methylation patterns between different rat tissues such as brain (152·7 (SD 5·5) %, relative to rat liver) and testes (100·3 (SD 20·1) %, relative to rat liver). This finding is in accordance with previous data reported by Wallwork & Duerre[10].

Statistical analysis

Results are expressed as the means and standard deviations for each group of rats. Differences among groups were analysed by Student's unpaired two-tailed t test. P values <0·05 were considered significant.

Results

Liver vitamin E content, gene expression and promoter methylation of 5-α-steroid reductase type 1 and γ-glutamylcysteinyl synthetase

Neither symptoms of ataxia nor differences in feed intake and live weight gain were observed in rats fed VE^- or VE-sufficient (VE^+) diets for 24 weeks (data not shown), which is in agreement with previous results[4]. As anticipated, hepatic αT levels were significantly ($P<0·001$, n 4 rats) reduced inthe VE^- rats (0·312 (SD 0·045) nmol/g) than in the VE^+ rats (27·4 (SD 1·53) nmol/g). Furthermore, plasma αT levels were significantly ($P<0·001$) lower in VE^- (0·355 (SD 0·011) μmol/l) *v.* VE^+(17·8 (SD 0·93) μmol/l) rats.

Differences in αT in the liver of rats have been previously shown to modulate relative mRNA levels of several VE-sensitive genes[4,8]. Out of these, we have chosen to determine the mRNA levels of SRD5A1 and GCLM genes via quantitative real time-PCR, as they show putative CpG islands in the 5′ region, which could suggest regulatory functions through methylation in this area. In the present study, we have found a significant ($P=0·0038$) twofold induction in relative mRNA concentrations of SRD5A1 gene from 0·85 (SD 0·12) in VE^+ animals to 1·65 (SD 0·33) in VE^- animals. Contrarily, VE deficiency significantly ($P=0·0057$) reduced relative mRNA levels of GCLM from 1·58 (SD 0·35) to 0·74 (SD 0·2).

The effects of a VE^- diet on the methylation of CpG islands in the promoter regions of SRD5A1 and GCLM genes in rat liver were examined by quantitative analysis of DNA methylation based on matrix-assisted laser desorption ionisation time-of-flight MS. Such an analysis at 130 (sense orientation)

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Table 1. PCR primers for the analysis of the methylation status of (a) γ-glutamylcysteinyl synthetase (GCLM) and (b) 5-α-steroid reductase type 1 (SRD5A1) gene promoters*

Amplicon processing ID	Left primer	Right primer	Amplicon size	Number of CpG	Amplicon start	Amplicon end
(a) GCLM						
Forward analysis						
GCLM_001	ATTTGAAAATTAATTGTTTTGTGGT	CCAAAAATCCCTTCCTCTAAAACTT	351	9	−836	−486
GCLM_002	AAAGTTTTAGAGGAAGGGATTTTTG	ATTTTTAAAACCACCACTCCAACC	409	52	−511	−103
GCLM_003	TTGTTTAGTTATTGTTTTTTAGGAAATAG	11 ACCCCAATTAAACAAATTCCC	383	46	−173	+209
GCLM_004	GGGAATTTGTTTAATTGGGG	ACCTCCCAAAACTAAAATAAAAAAC	497	24	+189	+685
Reverse analysis						
GCLM_007	TTGTTTTTATTTTTTAGGAATAGATGTGA	TTTAATACAACACCAACAACAAACT	200	5	−522	−221
GCLM_008	GGAGTTGTTTTTTGGAAGATAATGA	ACAAAACAAAACAAAACAAAACAAA	500	55	−141	−640
GCLM_009	GGTTTGTAGGTGTAGGGTGTTGG	CAAAAAACAACTCCCTCTTTTCAAC	343	45	+188	−154
GCLM_010	TTAATATTTGGTTAAGGGAGAGGGA	AACCAACACCCTACACCTACAAAC	336	22	+499	+164
(b) SRD5A1						
Forward analysis						
SRD5A1_0p0	GTGAGTTTATGAATTGGTTTTATTTTTT	TTTCAAACATATTTCTTAACTACCCC	445	35	−998	−554
SRD5A1_001	GGGGTAGTTAAGAAATATGTTTGAAAG	AAAAATTACACATTAACCCAAAACTTC	431	27	−579	−149
SRD5A1_002	GAGGTTTTGGGATTTTGTTGTG	CATAAAACCTTCCAAATAAACCAAC	455	24	−241	+213
SRD5A1_003	TTATGGAGTTGGATGAGTTGTGTTT	CCACTATACCACTATCCCTAACCAA	437	29	+155	+591
Reverse analysis						
SRD5A1_007	GTTGTATTTTTTAGGTATATTTTTTGATTG	AAACCCATAAATTAATCCCATTCTC	451	35	−546	−996
SRD5A1_008	TTTTGAGAATTATATATTGGTTTAGAGTT	CAATCAAAAAATATACCTAAAAAATACAAC	431	27	−145	−575
SRD5A1_009	GAGTTTTATATAAGGTTAGGAGGAGGG	AACCCAAAAAACTCTAAACCAATA	296	17	+113	−182
SRD5A1_010	TGTATTTTGGAATTAAGTTTTTGAGT	CCTCCCCTAAACAAATACACACTAC	468	32	+508	+41

ID, identity; CpG, cytosine-phospho-guanine.
* All positions are relative to the transcription start.

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and 107 (antisense orientation) CpG units, spanning nucleotides −1500 to +674 for GCLM (Fig. 1(a)) and −1000 to +1796 for SRD5A1 (Fig. 1(b)), revealed that all samples showed a similar signal pattern. Thus, no (significant) differences in the promoter methylation of SRD5A1 and GCLM between VE^{+} and VE^{-} rats could be detected.

Global DNA methylation

Fig. 2 shows global cytosine methylation levels in DNA samples of controls and VE^{-} rat liver. DNA global methylation of 110·2 (SD 24·0) % was detected in VE^{-} rat liver as compared with controls (100·0 (SD 18·4) %). DNA methylation was determined by two independent global methylation assays, which turned out similar data. No significant differences in DNA global methylation levels in VE^{-} rat liver compared to VE^{+} rat liver were evident.

Discussion

Research on the role of essential micronutrients in the epigenetic regulation of gene expression is an area of increasingly recognised importance. Zn deficiency has been described to reduce the utilisation of methyl groups from *S*-adenosylmethionine in rat liver, which resulted in genomic DNA hypomethylation as well as in histone hypomethylation[11,12].

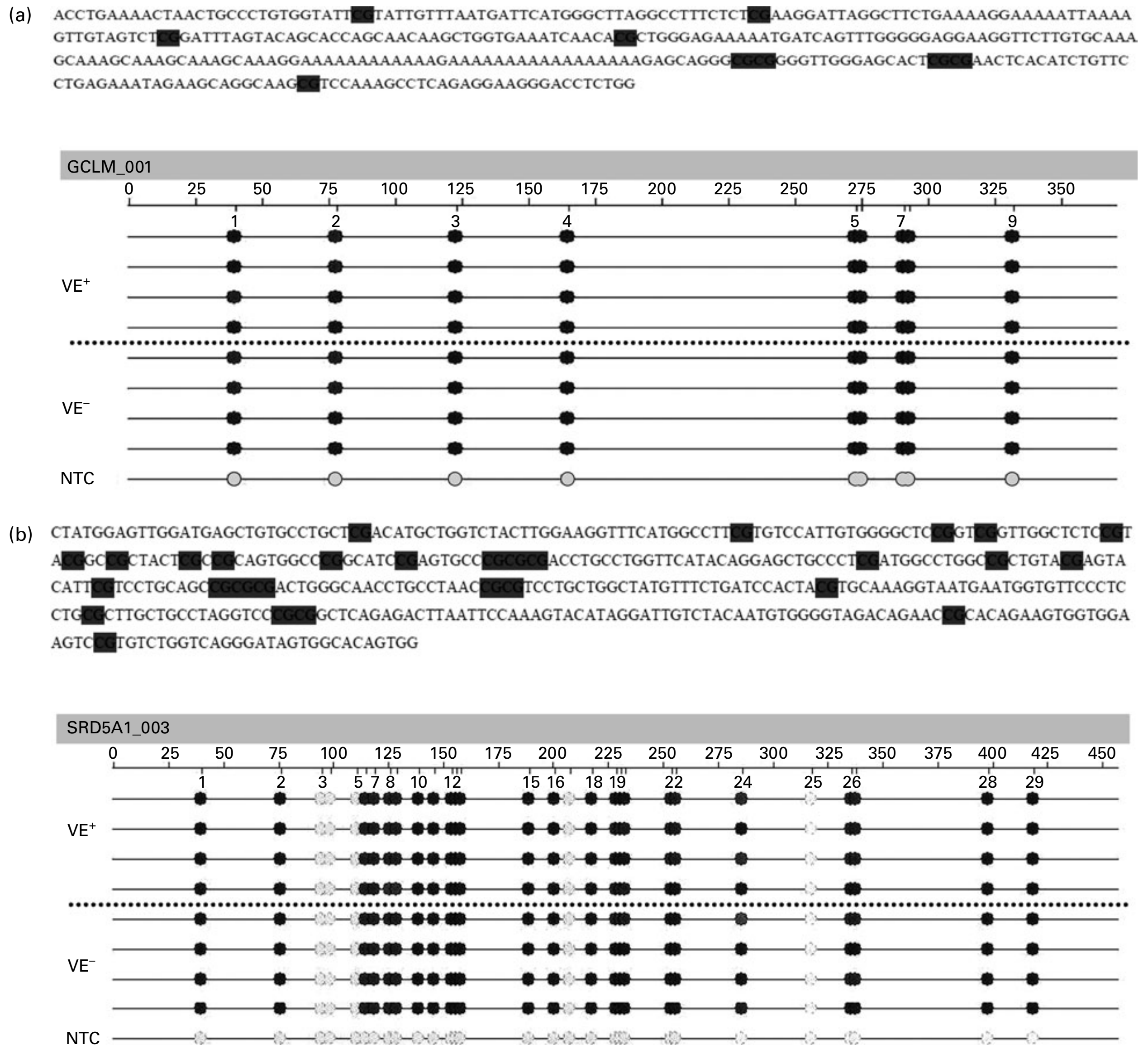

Fig. 1. Epigram of quantitative methylation analysis of promoter region. Genomic DNA isolated from the liver of vitamin E (VE)-sufficient (VE^{+}, *n* 4) and VE-deficient (VE^{-}, *n* 4) rats was analysed for methylation status of 237 (sense and antisense) cytosine-phospho-guanine (CpG) sites of the (a) γ-glutamylcysteinyl synthetase (GCLM) and (b) 5-α-steroid reductase type 1 (SRD5A1) gene promoters. Exemplary data for one amplicon for each gene are shown. The grey dots indicate the software-determined methylation ratio at each analysed CpG unit for each sample. The reference sequence above the epigram corresponds to the genomic sequence of the analysed strand. The sense orientation in 5′ → 3′ direction is displayed. Base numbering in the epigram refers to the analysed amplicon. NTC, non-template control. 0 % ●●●●●●●●●○ 100 %, ◯, not analysed.

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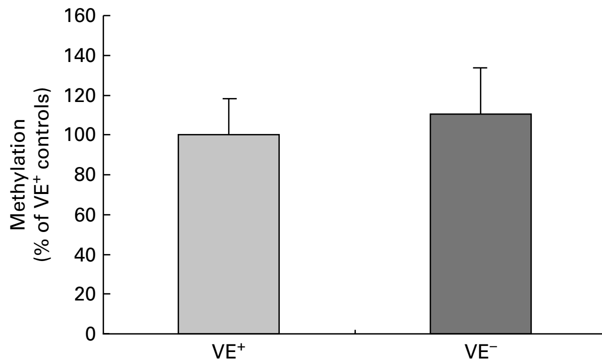

Fig. 2. Effects of vitamin E (VE) deficiency on global DNA methylation in rat liver. Global levels of cytosine methylation in DNA samples of controls VE-sufficient (VE^+) and VE-deficient (VE^-) rat liver, each expressed as percentage relative to the methylation signal in methylated DNA standard. Values are represented as means and standard deviations (*n* 8).

Dietary deficiency of selenium decreased genomic DNA methylation in Caco-2 cells and in the rat liver and colon[13,14], and vitamin C deficiency has been associated with DNA hypermethylation in lung cancer cells[15,16]. The role of VE in DNA methylation, however, is less clearly defined, although VE-dependent gene expression has been demonstrated[4,8,17].

Based on previous results, we have investigated the methylation-dependent epigenetic regulation of the SRD5A1 gene and the regulatory subunit GCLM gene as well as the total DNA methylation in VE^- and VE^+ rat liver. SRD5A1 and GCLM represent two out of four genes which were shown to be differentially regulated in the rat liver transcriptome in response to dietary VE deficiency[4]. Both the genes exhibit CpG islands, and were therefore selected for DNA methylation analysis. Since the other two VE-sensitive genes, namely coagulation factor IX and scavenger receptor CD36, exhibit no CpG islands, these transcripts were not further considered for DNA methylation analysis.

As transcriptional variation has been correlated with CpG island methylation in cells[18], we analysed for promoter methylation in putative CpG island regions located at the $5'$ end in these two genes using base-specific cleavage and matrix-assisted laser desorption ionisation time-of-flight MS. Overall, the present results indicate that DNA methylation does not contribute directly to the regulation of SRD5A1 and GCLM gene expression in VE^- rat liver.

In VE^- rats, Morante *et al.*[19] have shown that NF-κB directly regulates transcription of γ-glutamylcysteine synthetase (both subunits) through the binding of NF-κB to the corresponding gene promoters, which was enhanced in VE deficiency. Furthermore, it has been demonstrated that VE affects steroidogenesis, which in turn may affect SRD5A1 mRNA levels[20]. Thus, VE-induced changes in SRD5A1 and GCLM gene expression in hepatocytes seem to be regulated by mechanisms others than promoter DNA methylation. Furthermore, it is possible that differences in gene expression of SRD5A1 and GCLM may involve intragenic CpG islands rather than promoter CpG islands[21,22]. A recent study in human subjects[23] identified a considerable number of tissue-specific differentially methylated regions in CpG islands among the 873 genes analysed, but these were preferentially located several kb away from the transcription start site of the associated genes. Therefore, it cannot be ruled out that methylation other than that in the promoter region, as investigated in the present study, has led to differences in gene expression. Having this in mind, a global DNA methylation analysis was performed in rat liver. The analysis of global DNA methylation provides information on the degree of DNA methylation that occurs primarily in the repetitive, non-coding and non-regulatory sequences[24]. Nevertheless, we could not detect any significant differences in global methylation pattern between the two dietary groups. Thus, expression of the SRD5A1 and GCLM genes during VE deficiency does not appear to be directly regulated by DNA methylation in the promoter region. It is possible that epigenetic modification of histones by acetylation, methylation, phosphorylation, ubiquitination, sumoylation or isomerisation[25,26] contributes to the regulation of the expression of genes associated with VE deficiency.

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