DNA Methylation – Body Memory of Persistent Dietary Effects on Trait-associated Hepatic Gene Expression in Swine

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Introduction

Diet has been known for many years to play a key role as a risk factor for chronic diseases. In this context, nutritional factors may account for variable antioxidant defenses, which may be more or less effective at counteracting oxidative damage. We have recently demonstrated in juvenile pigs that a soy protein (SPI) based diet characterized by an imbalanced amino acid pattern causes long-term modification of hepatic expression of a variety of genes involved in oxidative stress defense mechanisms as the glutathione S-transferase gene (*GSTA1*) in comparison with a casein based diet (CAS) (Schwerin et al., 2008). In comparison with casein, SPI is characterized by a less than half concentration of methionine and about twice as much cystine and arginine. In regard to oxidative stress, limited dietary intake of methionine is significantly associated with increased antioxidant enzyme activities (Leclercq et al., 2000), and reduced synthesis rate and tissue concentration of glutathione (GSH), a major player in the organism's antioxidative defense system (Lyons et al., 2000, Taniguchi et al., 2000). In addition, methionine is the precursor for S-adenosyl methionine, the principal biological methyl donor for the methylation of newly synthesized DNA, regulating chromatin assembly and gene expression (Friso and Choi, 2005). Therefore, the present study was conducted to study DNA methylation as the potential mechanism "memorizing" the observed metabolic effects (persistent alterations of hepatic gene expression) of an early SPI diet on oxidative stress responsiveness.

Material and methods

Animals and diets. A total of 10 male castrated pigs (German Landrace; 50 days of age; 11.0 ± 0.3 kg body weight; BW) were individually housed in metabolic cages at a room temperature of 23.0 ± 1.0 °C. Animals were fed a semi-synthetic diet with casein as the sole protein source for four weeks. Subsequently, half of the pigs were given a similar semi-synthetic diet, but with SPI as the sole protein source for another four weeks. After this, for further four weeks the SPI group was switched back to the CAS diet. The control group continued on the CAS diet during all the three feeding periods until the end of the study. Diet composition was essentially as previously described (Schwerin et al., 2008). Pigs of both

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groups were killed at the age of 133 days, 18-24 h after the last food intake to minimize effects of direct nutrient intake. For Tissues were collected immediately and stored at -80 °C.

Quantitative RT-PCR. Quantitative analysis of PCR products was carried out essentially as described by Schwerin et al. (2008).

DNA methylation. Entire DNA methylation was studied by the 'cytosine extension method' (Pogribny et al., 1999) using the methylation sensitive restriction enzyme *Bss*HII. To study DNA methylation associated with oxidative stress response we have sequenced the [-1800 to -1] promoter region of the porcine *GSTA1* and quantified methylation rate (proportion of C residues of quantified T and C nucleotides) at 21 CpG sites identified within this promoter region by quantitative methyl-single nucleotide analysis with Pyrosequencing™ after bisulphite PCR (Uhlmann et al., 2002).

Statistical analyses. For all analyses the SAS/STAT package was used. Differences between the mean values were tested for statistical significance using Student's t-test. Significance was set at $P < 0.05$. Analyses of methylation rates of the individual CpG sites were carried out by means of variance analysis using 'false discovery rate' correction of *P* values (Storey et al., 2004). In order to prove joint effect of the CpG sites within the [-1800 to -1] *GSTA1* promoter region on *GSTA1* mRNA abundance, we introduced the following modelling scenario assuming that all CpG sites act together and that there is nothing known about a weighted response (methylation pattern) of some CpG sites:

- each CpG site – value are standardized over all animals to mean = 0 and SD = 1,
- for each animal we calculate S^+: the sum of all positive (standardized) values of CpG sites, that means the sum of all above-average methylation rates and S^-: the sum of all absolute negative (standardized) values of CpG sites, that means the sum of all below-average methylation rates,
- then we calculate the ratio $Q = S^+ / S^-$ as a new character of the animals, which involves the information about all measured CpG sites.

The dependence of *GSTA1* mRNA abundance (y) upon methylation rate of the CpG sites within the *GSTA1* promoter region was modelled by the non-linear function of Q: $y = a + b^* (1/Q^2)$.

Results and discussion

The entire hepatic DNA methylation of CpG islands studied by the 'cytosine extension method' was significant lower in SPI fed animals according to the significantly higher mean ^{3}H-dCTP incorporation in *Bss*HII digested DNA of pigs fed the SPI based diet in comparison with CAS fed animals (1,327.1 ± 169.75 cpm *vs.* 1,028.0 ± 81.51 cpm, $P < .0012$).

Statistical analyses of the individual CpG sites of the [-1800 to -1] promoter region of the porcine *GSTA1* by means of variance analysis using 'false discovery rate' correction of *P* values ($q < 0.05$) reveal no significantly different CpG methylation rates between both diet groups.

Interestingly, as shown in **Figure 1** mean methylation rate are quite different between the individual CpG sites showing values between 6.1 ± 3.79 % at the [-732] CpG site and 84.0 ± 1.67 % at the [-899] CpG site, whereas methylation rates of individual CpG site were of

corresponding ranges between animals of both feeding groups and of different age (not shown here). The homogeneity of animal DNA methylation pattern of *GSTA1* promoter indicates a high stability of somatic DNA methylation pattern in pigs, whereas different distribution of methylation may reflect different functions of corresponding promoter regions.

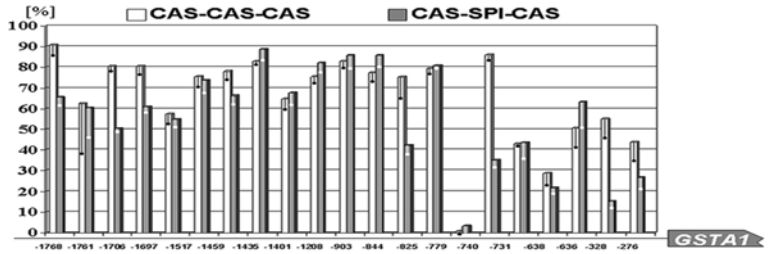

Figure 1: Methylation rates of C residues at 21 CpG dinucleotides identified within the [-1800 to –1] promoter region of the porcine glutathione-S-transferase gene (*GSTA1*) of each 5 pigs fed a chronically casein based and a casein with a temporary soy isolate based protein diet, respectively. Bars represent S.D.

However, in order to prove joint effect of the CpG sites on *GSTA1* mRNA abundance, we introduced the model described in 'Material and Methods' in detail. Four weeks after replacement of SPI by CAS, three of the five animals analysed exhibited still up to 1.5- to 2-fold increased hepatic *GSTA1* expression level in comparison with the pigs chronically fed CAS diet. Using the non-linear function of Q: $y = a + b^* (1/Q^2)$ (with y: *GSTA1* mRNA abundance, Q: S^+/ S^-) a coefficient of determination B = 0.65 was calculated. Calculated Q values indicated that the three temporary SPI fed animals showing increased hepatic *GSTA1* transcript level exhibited demethylation of the *GSTA1* promoter exceeding the CAS mean 3- to 5-fold, whereas the other two animals showed values comparable to chronically CAS fed animals.

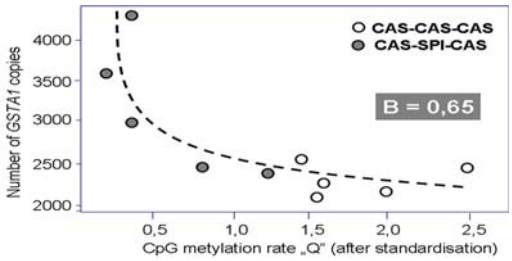

Figure 2: Non-linear correlation between methylation rates of the CpG sites of the porcine [-1800 to –1] *GSTA1* promoter and the hepatic *GSTA1*-mRNA abundance.

In addition, the present results indicate that DNA methylation is very likely the "memorizing" mechanism of persistent diet-affected changes of *GSTA1* expression. In addition, DNA methylation could explain the link between nutrition during early critical periods of development and later disease or growth related physiological and metabolic processes, a phenomenon referred to as 'metabolic programming' (Koletzko et al., 1998). The dependence of growth (body weight, y) upon methylation rate of the CpG sites within the [-1800 to -1] *GSTA1* promoter region was modelled by the same non-linear function of Q as used for GSTA1 transcript level and revealed a coefficient of determination of B = 0.66

suggesting a lasting diet related growth retardation in animals with decreased CpG methylation rates in the *GSTA1* gene that is associated with oxidative stress responsiveness.
The evidence of a direct link between DNA methylation changes and long-term consequences of early environmental influences in regard to elevated risk for adverse health outcome during the various stages of life is becoming increasingly stronger (Fenech, 2005). In the present study we have shown that DNA methylation is likely the "memorizing" mechanism of persistent changes of diet-associated expression of genes involved in oxidative stress responsiveness (Schwerin et al., 2008) a mechanism thought to be associated to the development and progression of neurodegenerative, as well as cardiovascular disease and cancer (Abrescia and Golino, 2005). We assume that the metabolic effects of both protein diets can be attributed mainly to the imbalanced amino acid pattern of SPI as compared to CAS feeding. The main differences between both protein diets are increased contents of methionine, proline, phenylalanine, and tyrosine in the CAS diet, and of cystine, glycine, aspartic acid, and arginine in the SPI diet. Effects of soy-associated constituents like trypsin inhibitors and lectins could be largely excluded because their levels were more than 200 times lower compared to other soy protein diets used.

Conclusion

The present results indicate (1) that protein diets with imbalanced amino acid pattern significantly modulate DNA methylation in swine and (2) that somatic epigenetic mechanisms referred to all modifications to genomes other than changes in the DNA sequence itself including chromatin and DNA modifications as addition of methyl groups to the DNA backbone, may long-lasting affect phenotype. Improved (standardised) keeping conditions will help to avoid or minimize such apparent effects of DNA sequence that reduce power of genomic approaches.

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