The influence of energy restriction and developmental state on DNA 5-methyldeoxycytidine in rat mammary and liver tissues

M.G. Baik, C.B. Choi, W.D. Slanger, and C.S. Park

Department of Animal and Range Sciences, North Dakota State University Fargo, ND, USA

Female rats were fed either ad libitum or 30% energy-restricted diets from 5 weeks through 25 weeks of age. Genomic DNA was extracted from mammary tissue and liver at 7, 9, 14 (mid-pregnancy), 16.5 (mid-lactation), and 25 weeks of age. The 5-methyldeoxycytidine content of DNA was determined by high pressure liquid chromatography. As animals aged from 7–25 weeks, 5-methyldeoxycytidine increased in mammary tissue, whereas in liver it decreased. This suggests that 5-methyldeoxycytidine exhibits tissue specific patterns. No changes in 5-methyldeoxycytidine content due to 30% energy restriction were observed in either mammary tissue or liver.

Keywords: energy restriction; liver and mammary development; 5-methyldeoxycytidine; rat

Introduction

Changes occur in DNA methylation during basic processes of cellular development including embryogenesis, differentiation, aging, and carcinogenesis.[1,2] DNA methylation is possibly related to the regulation of gene expression in mammalian cells. There is an inverse correlation between the degree of DNA methylation of certain genes and the extent to which those genes are expressed.[3] Studies contradict each other as to the DNA methylation state of various tissues during cellular development,[4,5] but methylation changes occurring as a function of time suggest hypomethylation may be developmentally important.[1,6] 5-Methyldeoxycytidine (5 mdC) constitutes 2–8% of the total cytidine in animal DNA. It is the modified base in DNA, and it occurs predominantly in the CG dinucleotides.[3] The 5 mdC levels have been shown to vary between species and within different tissues of the same organism.[1,7]

Energy restriction is known to reduce age-related loss of DNA integrity by reducing chromosomal aberrations or by altering DNA methylation.[8,9] Dietary energy restriction may cause a change in the 5 mdC level and possibly DNA methylation pattern. Thus, the present study was undertaken to examine the energy restriction-mediated changes in the degree of DNA methylation in mammary tissue as well as the liver of female rats throughout the different developmental stages.

Address reprint requests to Chung S. Park at the Department of Animal and Range Sciences, Hultz Hall, North Dakota State University, Fargo, ND 58105, USA
Received July 25, 1991; accepted April 24, 1992.

Methods and materials

Animal and diets

Thirty female Sprague-Dawley rats (4 weeks old) were fed commercial rat diet until 5 weeks of age. From 5–25 weeks of age, 15 animals were fed diet A ad libitum, and 15 animals (30% energy restriction group) were fed diet B *(Table 1)*. The concentrations of casein, DL-methionine, corn oil, mineral mix, vitamin mix, and choline bitartrate in diet B were modified to provide a daily intake that is identical to the mean intake of the rats fed diet A. The amount of food ingested by each ad libitum fed rat was measured biweekly, and average consumption per rat per day was calculated as described previously.[10] Each rat receiving a restricted diet was fed a daily feed allotment of 70% of the average ad libitum consumption.

The rats were housed singly in a barrier facility. The air pressure within the barrier was maintained at about 5 mmHg above the outside environment. The temperature was maintained at 22–24° C with relative humidity ranging between 50–60%. A 12-hour light/dark cycle was used. Water was provided ad libitum by an automatic watering system. At

Table 1 Composition of experimental diets

Ingredients	Diet A[a]	Diet B[b]
	%	
Casein	20.0	28.6
DL-methionine	0.3	0.43
Cornstarch	32.0	20.0
Sucrose	33.0	32.15
Fiber	5.0	5.0
Corn oil	5.0	7.1
AIN mineral mix	3.5	5.0
AIN vitamin mix	1.0	1.43
Choline bitartrate	0.2	0.2
Crude protein, %	18.0	25.7
Digestible energy, Kcal/kg	3,843	3,870

[a]Modified AIN-76 diet.
[b]Diet B was formulated to provide isonutrient intake as diet A except for energy content, and fed at 70% of ad libitum of diet A.

approximately 12 weeks of age, females were mated with proven males for a period of 10 days. Mating was confirmed by the presence of sperm in a vaginal smear and of vaginal plugs.[11] Mammary and liver tissues were obtained from three animals per treatment at 7, 9, 14 (mid-pregnancy), 16.5 (mid-lactation), and 25 weeks of age and utilized for DNA extraction.

5 mdC content of DNA

Genomic DNA was extracted from a crude nuclear fraction with a proteinase K and RNAase A digestion.[12] The DNA was digested with nuclease P1 and alkaline phosphatase to the deoxynucleosides.[13] The digests were injected onto a reversed phase HPLC column and eluted with KH_2PO_4 (pH 4.0) buffer and 2.5% methanol. Nucleosides were detected by the absorbance at 254 nm. The peaks were identified by comparison of elution times with those of standards. The relative portions of the individual nucleosides were calculated from the area under the peaks of the absorbance trace and the extinction coefficients of the nucleosides. The percentage of 5 mdC was calculated according to the following formula:

$$\% \ 5\ mdC = [5\ mdC/(5\ mdC + deoxycytidine)] \times 100$$

Statistics

The data were analyzed using the general linear models routine of SAS/STAT (SAS Institute, Cary, NC USA).[14] Treatment differences within periods were measured by one-way classification analysis. Treatment differences, differences between each pair of successive periods, and the treatment by period interaction were determined using 2 × 2 factorial analyses.

Results

To study the effect of energy restriction on DNA methylation, female rats were fed either ad libitum or 30% energy-restricted diets. Genomic DNA was extracted from mammary tissue and livers at various weeks of age. 5 mdC content of DNA was measured by high pressure liquid chromatography.

The effect of energy restriction on 5 mdC level was tested by one-way classification within each period. Energy restriction did not affect ($P > 0.05$) 5 mdC levels in either mammary or liver tissues. The 2 × 2 factorial analyses of the pairs of successive periods also strongly indicated that the 5 mdC levels in two dietary treatments were equal throughout the entire experimental period. There were no significant interactions ($P > 0.05$) between treatments and periods.

We tested differences between each pair of successive periods by 2 × 2 factorial analyses to examine changes in 5 mdC levels during various stages. The results reveal age-dependent changes in 5 mdC levels for both mammary *(Figure 1)* and liver *(Figure 2)* tissues. Increases ($P < 0.01$) in 5 mdC levels in mammary tissue were observed with advancing age from 7–25 weeks: increases were significant ($P < 0.01$) at 9 weeks compared with 7 weeks, at mid-lactation compared with mid-pregnancy, and at 25 weeks compared with mid-lactation. In contrast, the 5 mdC level in liver decreased ($P < 0.01$) as the animal aged from 7–25 weeks (i.e., hypomethylation).

Discussion

While studies have shown that DNA methylation varies between species and within different tissues of the same organism,[3,4,15,16] little is known on DNA methylation in mammary tissue. The present study demonstrated hypermethylation of DNA in developing mammary tissue. We observed increased 5 mdC levels in mammary tissue at mid-lactation compared with mid-pregnancy. An increase in 5 mdC in mammary tissue at 25 weeks of age was significant relative to that during pregnant or lactating states. However, the present study did not include non-breeding controls;

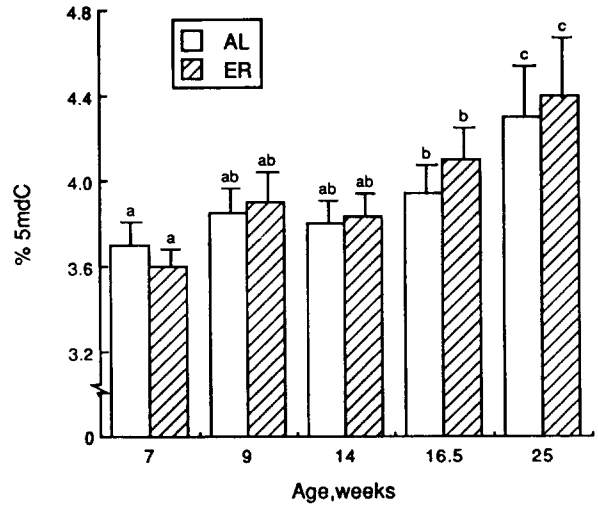

Figure 1 5-Methyldeoxycytidine (5 mdC) levels in mammary tissue of female rats fed either ad libitum (AL) or energy restricted (ER) diets at different physiological ages: 7 weeks, 9 weeks, 14 (mid-pregnancy), 16.5 (mid-lactation), and 25 weeks of age. Bars represent mean ± SE where SE = 0.11 and $n = 3$. Bars with different superscripts differ ($P < 0.05$). There were no significant ($P > 0.05$) differences between the two dietary groups at any age.

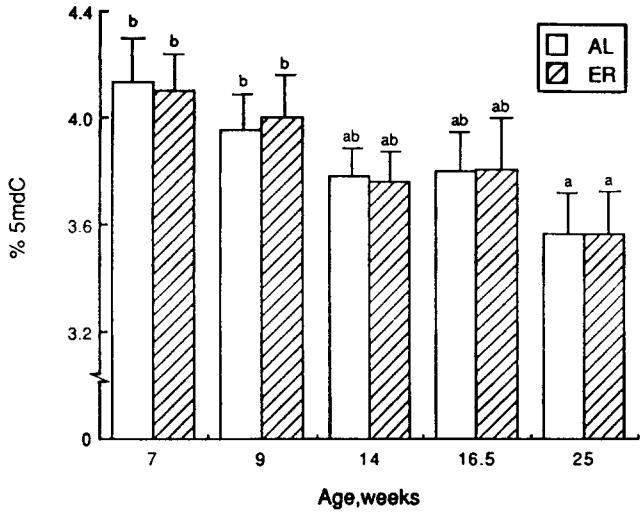

Figure 2 5-Methyldeoxycytidine (5 mdC) levels in liver of female rats fed either ad libitum (AL) or energy restricted (ER) diets at different physiological ages: 7 weeks, 9 weeks, 14 (mid-pregnancy), 16.5 (mid-lactation), and 25 weeks of age. Bars represent mean ± SE where SE = 0.08 and $n = 3$. Bars with different superscripts differ ($P < 0.05$). There were no significant ($P > 0.05$) differences between the two dietary groups at any age.

therefore, it is not clear whether the observed higher levels of 5 mdC at mid-lactation compared with mid-pregnancy reflect a difference in physiological stages or are simply due to animal age. Further studies that include non-breeding controls will be necessary to examine 5 mdC changes during pregnancy and lactation. Mammary tissue is a target organ for reproduction. Thus, we could not rule out that the hypermethylation in mammary tissue of female rats that had experienced pregnancy might reflect the effects of mating, pregnancy, tissue development, or combinations of these factors.

In contrast to the hypermethylation exhibited by mammary tissue, the present study showed hypomethylation of liver DNA with advancing age. Our findings are in accord with previous reports that show age-related hypomethylation in rat liver DNA through 25 weeks of age.[5,17] The slow turnover rate of hepatic cells may explain the decrease in DNA methylation with advancing age.[17] While our studies, as well as others, show a general trend towards demethylation of liver DNA with advancing age, hypermethylation detected in the α-fetoprotein gene from the liver of mature mice[18] suggests that overall 5 mdC levels do not necessarily correlate with DNA methylation levels of specific genes.

Dietary energy restriction represents adaptation whereby the physiological focus is redirected towards optimum cellular economy by reducing the maintenance requirements and minimizing (avoiding) futile pathways. This shift in cellular activity is mediated by up-regulation of specific transacting factors[19] and their subsequent interactions with respective DNA binding sites in the control region (promotors or enhancers) of various genes in the mammary gland. The nutrition-mediated transcriptional activation may alter DNA methylation and gene expression. In the present study, we did not observe any changes in 5 mdC content of DNA due to energy restriction in either mammary tissue or livers throughout the entire experimental period. A previous study[20] also demonstrated no changes in DNA methylation in the male rat liver due to a 10-day starvation. The lack of energy restriction-related changes in 5 mdC level is possibly due to the short duration of the trial period (5 months). Energy restriction-mediated changes in gene expression usually occur after 10 months in rats.[1,10,21]

The somatic mutation theory postulates the loss of DNA integrity as a cause of aging.[22] Age-related changes in DNA and the chromatin structure include increased chromosomal aberrations, strand breaks, and alterations in DNA methylation.[2] Some data indicate that the sequence CG, if present in the unmodified form, may be a site for specific cellular endonucleases.[23] Therefore, methylation of CG dinucleotides in DNA may be a fundamental process ensuring the safety of the genome and probably preventing chromosomes from breaking. It is proposed that a possible consequence of age-related demethylation may be an increased risk of chromosomal breakage.[2,23] The question remains if energy restriction compensates for age-dependent DNA methylation, thereby maintaining the longevity of tissue specific gene expression and function.

Acknowledgments

The authors thank Dr. J. Fleeker, Marsha Kapphahn, and Leonard Cook for assistance with 5-methyldeoxycytidine analysis.

References

1 Wilson, V.L., Smith, R.A., Ma, S., and Cutler, R.G. (1987). Genomic 5-methyldeoxycytidine decreases with age. *J. Biol. Chem.* **262,** 9948–9951

2 Mays-Hoopes, L.L. (1989). Age-related changes in DNA methylation: Do they represent continued developmental changes? *Int. Rev. Cytol.* **114,** 181–220

3 Doefler, W. (1983). DNA methylation and gene activity. *Ann. Rev. Biochem.* **52,** 93–124

4 Wilson, V.L. and Johnes, P.A. (1983). DNA methylation decreases in aging but not in immortal cells. *Science* **220,** 1055–1057

5 Gama-Sosa, M.A., Wang, R.Y-H., Kuo, K.C., Gehrke, C.W., and Ehrlich, M. (1983). The 5-methylcytosine content of highly repeated sequences in human DNA. *Nucleic Acids Res.* **11,** 3087–3095

6 Baik, M.G., Choi, C.B., Keller, W.L., and Park, C.S. (1991). Role of energy restriction in DNA methylation, ornithine decarboxylase gene and cellular oncogene expressions in rat mammary tissue. *J. Dairy Sci* **74,** 142

7 Vanyushin, B.F., Tkacheva, S.G., and Belozersky, A.N. (1970). Rare bases in animal DNA. *Nature* **225,** 948–949

8 Wiendruch, R. and Walford, R.L. (1988). *The retardation of aging and disease by dietary restriction,* Charles C. Thomas, Springfield, IL, USA

9 Turturro, A. and Hart, R.W. (1990). Caloric restriction and its effects on molecular parameters, especially DNA repair. In *Biological Effects of Dietary Restriction,* (L. Fishbein, ed), p. 185–190, Springer-Verlag, New York, NY, USA

10 Baik, M.G., Choi, C.B., Keller, W.L., and Park, C.S. (1992).

Developmental stages and energy restriction affect cellular oncogene expression in female rat tissues. *J. Nutr.* **122,** 1614–1620

11 Baik, M.G., Harrold, R.L., Choi, C.B., Slanger, W.D., and Park, C.S. (1991). Energy restriction and testosterone implants alter ornithine decarboxylase gene expression in kidney of female rats. *J. Nutr.* **122,** 1056–1061

12 Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual,* Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, NY, USA

13 Gehrke, C.W. (1984). Quantitative reversed-phase high-performance liquid chromatography of major and modified nucleosides in DNA. *J. Chromatogr.* **301,** 199–219

14 SAS Institute Inc. (1988). *SAS/STAT User's Guide,* Release 6.03 Edition. Cary, NC, USA

15 Ehrlich, M., Gama-Sosa, M.A., Huang, L-H., Midgett, R.H., Kuo, K.C., McCune, R.A., and Gehrke, C. (1982). Amount and distribution of 5-methylcytosine in human DNA from different types of tissues or cells. *Nucleic Acids Res.* **10,** 2709–2721

16 Tawa, R., Ono, T., Kurishita, A., Okada, S., and Shingo, H. (1990). Changes of DNA methylation level during pre- and postnatal period in mice. *Differentiation* **45,** 44–48

17 Hoal-Van Helden, E.G. and Van Helden, P.D. (1989). Age-related methylation changes in DNA may reflect the proliferative potential of organs. *Mutation Res.* **219,** 263–266

18 Papacostantinou, J. and Church, W.K. (1984). Methylation of α-fetoprotein gene DNA associated with repression and aging. *Age* **7,** 152–168

19 Mitchell, P.J. and Jjian, R. (1989). Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. *Science* **245,** 371–378

20 Vanyushin, B.F., Nemirovsky, L.E., Klimenko, V.V., Vasiliev, V.K., and Belozersky, A.N. (1973). The 5-methylcytosine in DNA of rats. Tissue and age specificity and the changes induced by hydrocortisone and other agents. *Gerontologia* **19,** 138–152

21 Richardson, A., Roberts, M.S., and Rutherford, M.S. (1985). Aging and gene expression. In *Review of Biological Research in Aging,* (M. Rothstein, ed.), Vol II, p. 395–419, Alan R. Liss, New York, NY, USA

22 Curtiss, H.J. (1963). Biological mechanisms underlying the aging processes. *Science* **141,** 688–691

23 Cato, A.C.B. and Burdon, R.H. (1979). Mammalian DNA methylation and a nuclear S-adenosylmethionine-dependent nuclease activity. *FEBS Lett* **99,** 33–38