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DNA Alkylation by Nitrosobis-(2-oxopropyl)amine in Rats of Different Ages

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We have examined the methylation of liver DNA (O^6- and N^7-methylguanine) by nitrosobis-(2-oxopropyl)amine (BOP) in male and female rats at various ages, following treatment with 2.5 mg of BOP; this dose given twice weekly for 30 weeks induces tumors in all animals. Except in young rats there was more methylation in female rat liver than in male rat liver, when adjusted for different sizes of the animals. There were differences in the extent of methylation between young (4 weeks) and older rats, but not between young adult (20 weeks) and old adult (65 weeks) males; the latter developed liver tumors when treated with BOP, and the former did not. There was no obvious relation between increased susceptibility to liver tumor induction by BOP and the extent of alkylation of liver DNA. Methylation of DNA was lower in the kidney than in the liver and, here, there was little difference between the sexes. In the testis there was N^7-methylation of guanine in DNA, but no O^6-methylguanine was detected.

Key words: Rats — DNA — Alkylation — Age — Nitrosobis-(2-oxopropyl)amine

In studying the effects of administering nitrosobis-(2-oxopropyl)amine (BOP) by gavage to rats of different ages, we reported recently that male rats more than a year old responded like female rats, with development of liver tumors, rather than like young male rats, which did not develop liver tumors. Furthermore, we found that feminization of male rats also made them susceptible to induction of liver tumors by BOP.[1] This change in susceptibility to liver tumor development was reflected in an increase in the extent of methylation of DNA in the liver of female rats, and of feminized male rats, compared with intact male rats. This effect of endogenous factors on the susceptibility to liver tumor induction by BOP was not shown by nitrosodimethylamine, for example, and seemed therefore not to be a general phenomenon in rats, although it is probably not restricted to BOP. Since BOP is not a directly acting carcinogen or mutagen (it is not activated to a bacterial mutagen by liver microsomes from male rats[2]), the effects appear to be on metabolic activation of BOP in rats. To explore whether these effects, as reflected in DNA alkylation, are also observed in older rats we compared the extent of DNA alkylation in the liver of older rats with that in 4-week-old rats, as well as with the young adults already examined. To make the study more complete, male and female rats were compared in all three age ranges.

MATERIALS AND METHODS

Nitrosobis-(2-oxopropyl)amine (BOP) was prepared as described previously,[3] as was this compound labeled with ^{14}C in the α-carbon of the 2-oxopropyl group.[1] The solution for treatment of the animals was prepared by appropriate dilution of the radiolabeled BOP in ethyl acetate/corn oil (1:2) with unlabeled BOP to a concentration of 12.5 mg and 25 μCi per ml; for an additional treatment of 4-week-old rats, another solution of radiolabeled BOP was prepared containing 3 mg and 12.5 μCi. The second solution was used for the 4-week-old animals, which were much smaller than the adults, to discover the difference in effect on nucleic acid alkylation between equal doses in rats of different ages, compared with similar dose per unit body weight.

Animals were F344 rats of the colony of the Frederick Cancer Research Facility, born and raised within a barrier. As reported previously,[1] a group of male rats were castrated shortly after birth, and of these a few were kept until more than a year old, as were several intact males and females of the same group. These animals were used in the

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present experiment when between 65 and 75 weeks of age. Several male and female rats were obtained shortly after weaning and were used in this experiment at age 4 weeks. Each animal was given by gavage 0.2 ml of a solution of radiolabeled BOP in ethyl acetate/corn oil, usually containing 12.5 mg of BOP per ml, but, in the case of two male and two female 4-week-old animals, the solution containing 3 mg of BOP per ml was also used. Each treatment group consisted of a pair of males or of females to which the solution was given and the animals were then housed singly in metabolism cages with facilities for collecting urine. Through the cage a gentle stream of air passed, and the effluent was collected in a tube containing 10 ml of ethanolamine to trap carbon dioxide. This trap was changed every hour for 6 hr, when the animals were sacrificed, and the liver, kidneys and, in one case, testes were dissected and frozen in liquid nitrogen until it was convenient to process them for extraction of nucleic acids. This extraction was carried out using a modified Kirby method, as previously described.[4] The isolated nucleic acids and soluble proteins were assayed by liquid scintillation counting, as were aliquots of the ethanolamine-trapping solutions (100 μl). Aliquots of the urine of each of the rats were also assayed by liquid scintillation counting.

The nucleic acids were hydrolyzed, DNA in 0.1N HCl at 75–80° for 1 hr and RNA in 1N HCl in boiling water for 1 hr, followed by neutralization with a few milligrams of sodium bicarbonate. Aliquots, 250 μl, of each hydrolysate were chromatographed on a PSX Partisil ion-exchange column, with 0.2M phosphate buffer (pH 3.5) at 0.8 ml per min, as described previously.[5] Beginning at 2 min, fractions were collected at 15 sec intervals until 15 min, and were counted after addition of scintillator solution. Under these chromatographic conditions, N^7-methylguanine elutes at 7.5 min and O^6-methylguanine at 12 min. The fractions in each peak of radioactivity corresponding to the methylguanines were combined. The results of the analysis are expressed as picomoles of methylguanine per milligram of nucleic acid, in Table I. Because of the difference in size between young and old rats the results for O^6-methylguanine are also shown adjusted for differences in dose of BOP-^{14}C per unit body weight, as suggested by Belinsky *et al.*[6]; it would be expected that 2.5 mg of BOP would produce more methylation of nucleic acids of younger animals than in adult males, simply because of their smaller size. The extent of methylation of RNA is not shown but was invariably greater (as N^7-methylguanine) than in DNA.

Results and Discussion

The radioactivity in the DNA and RNA of the liver of the 4-week-old rats was considerably higher in both males and females than in the young adults and the older rats. There was not a large difference between males and

Table I. Methylation of DNA of Rats by BOP-^{14}C[a] 6 Hours after Gavage Treatment

Sex and Age (weeks)	Average weight (g)	Organ	Excretion (% of dose)		Alkylation of DNA (pmol/mg DNA)		
			% CO_2	% in urine	N^7-MeG	O^6-MeG	O^6-MeG/dose[b]
♂ 4[c]	44	Liver	30	8	350	46	3.4
♂ 4	54	Liver	23	13	960	83	1.8
♀ 4[c]	48	Liver	30	9	320	19	1.5
♀ 4	52	Liver	26	10	500	49	1.0
♂ 20	369	Liver	17	24	43	5.7	0.84
♀ 20	173	Liver	17	19	181	14	1.0
♂ 65	430	Liver	33	9	40	4.3	0.74
♀ 75	280	Liver	29	11	126	13	1.5
♂ 75 Castrated	360	Liver	37	12	106	10	1.4
♂ 65		Kidney			19	2.1	0.36
♂ 65		Testis			7	ND	—
♀ 75		Kidney			21	3.3	0.37
♂ 75 Castrated		Kidney			26	4.0	0.58

a) 2.5 mg and 5 μCi per rat.
b) The level of O^6-methylation was adjusted to constant dose of BOP in mg/kg body weight.
c) These rats were given 0.6 mg of BOP-^{14}C containing 2.5 μCi.

females, nor between 4-week-olds given 2.5 mg of BOP and those given 0.6 mg. Neither was there a significant difference in the rate of excretion of CO_2 between these rats given the two different doses of BOP. Therefore, the availability of the metabolizing and activating enzymes did not appear to be a factor limiting metabolism of the higher dose; urinary excretion was also similar at both doses.

There was a considerable difference in the extent of labeling of cellular nucleic acids and proteins between very young rats and mature rats, but there was little difference between 20-week-old rats and 65-week-old rats. Of course, the very young rats were smaller and, therefore, the dose per unit body weight was greater than in the older rats. Similarly, adult male rats were larger than adult females, although in the very young rats there was no appreciable difference in weight. Nevertheless, even after correction for the body weight differences, the extent of reaction of the labeled BOP with liver macromolecules of 4-week-old rats was considerably greater than in the adult rats. It is not known whether very young rats are more susceptible to carcinogenesis by BOP than are adult rats, although experience with other nitrosamines suggests that that is probably the case.

One finding of this study is that the difference in the extent of methylation of DNA between male and female rats is not constant with age. In adult rats, there is more methylation in females than in males, whether the rats were young adults or old adults. In 4-week-old rats, on the other hand, there was more extensive methylation, measured as N^7- and O^6-methylguanines, in male rats than in females. The reason for this difference in response between old and young rats is not apparent.

In the older rats, castrated males had methylation levels induced by BOP approaching those in old females, exactly as was found in young castrated adults,[1] but intact old males did not. However, these results do not correlate with the tendency of intact old male rats to develop liver tumors primarily when treated with BOP, whereas in young adult males there have been no liver tumors.[1,7] It seems that the extent of methylation of DNA is dependent only on the rate of metabolism of BOP and is almost constant in adult rats of whatever age, even though the larger 65-week-old rats, male or female, metabolize the nitrosamine (as determined by rate of excretion of CO_2) faster, 33 and 29% respectively in 6 hr, than the 20-week-old rats, both 17% in 6 hr. The 20-week-old rats excrete a larger proportion of the dose in the urine. It must be concluded that other factors related to the treatment with BOP are responsible for the appearance of liver tumors in the older male rats, but not in younger males.

The castrated rats behaved like the adult female rats in response to BOP treatment. The predominant tumors induced were liver tumors, and DNA methylation in castrated males resembled in extent and in pattern that in female rats at both 20[1] weeks and 75 weeks of age, and was greater than that in male rats of the same age.

DNA alkylation in the kidneys of the older rats was determined for comparison with the alkylation in the livers. There was considerably less methylation in the kidney DNA than in liver DNA, although it was readily measurable. There was no great difference in the kidney DNA methylation between male, female or castrated male rats. Whether the low extent of DNA methylation in the kidneys is the reason for the absence of kidney tumors in rats given multiple doses of BOP cannot be decided, although it might be one of several factors. The single determination of alkylation of testicular DNA in the older male rats showed a lesser degree of methylation of DNA in the testis than in kidney, and O^6-guanine methylation, if present, was below the level of detectability. This strain of rat develops spontaneous interstitial cell tumors of the testis, and there is no evidence that carcinogen treatment of any kind affects the development or incidence of these tumors. Therefore, this negative finding of insignificant alkylation of DNA in the testis corresponds with the lack of carcinogenic action of BOP in that organ.

When corrected for different doses of BOP per unit body weight, as suggested by Belinsky *et al.*,[6] the differences between male and female rats of different ages in the extent of methylation of DNA by BOP are not large. In particular, there is almost no difference between 20-week-old rats — about midway in age between the 8 weeks at the beginning and 35 weeks at the end of BOP treatment used to induce tumors — and 65-week-old rats. Yet,

old male rats treated with repeated doses of BOP developed liver tumors, while young male rats did not.[7] However, the older male rats were considerably less susceptible to induction by BOP of tumors of other types, such as follicular cell tumors of the thyroid, lung tumors and bladder tumors, which were commonly induced in young male rats. Kidney tumors were never seen in rats, male or female, treated with BOP, although the extent of O^6-methylation of guanine in kidney DNA was not much smaller than that in the liver, and seemingly adequate to produce the needed mutations.

The results of these biochemical studies have shown no dramatic differences between male and female rats, or between rats of different ages, in the course or magnitude of metabolism of BOP leading to methylation of DNA — which is the principal alkylated base[8] formed from this nitrosamine *in vivo*. Our conclusion is the same as in our previous studies of the effect of feminization of male rats on carcinogenesis and DNA alkylation by BOP,[1] namely that the nature and extent of methylation of DNA by this carcinogen is not the determinant of induction and progression of tumors. Other factors of which we know little or nothing, physiological and hormonal included, must be considerably more important. It is significant that, while there is a large difference between young adult male and female rats in the response of their liver to carcinogenesis by BOP,[1] but no corresponding difference in liver DNA methylation, there is no such sex difference in the response of rat liver to carcinogenesis or DNA alkylation[9] by nitrosodimethylamine and a number of other methylating carcinogens. Differences in dose rate and simple pharmacokinetics appear not to be important in explaining the difference in response of male and female rats to BOP, since male rats given this carcinogen in drinking water also do not produce liver tumors, while females have a 100% incidence of liver tumors under these conditions.[10]

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