EFFECT OF CALORIC RESTRICTION ON AFLATOXIN B₁-DNA ADDUCT FORMATION AND ASSOCIATED FACTORS IN FISCHER 344 RATS: PRELIMINARY FINDINGS

REX A. PEGRAM, WILLIAM T. ALLABEN and MING W. CHOU*

National Center for Toxicological Research, HFT-140, Jefferson, AR 72079 (U.S.A.)

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SUMMARY

In this first report of an effect of caloric restriction on in vivo DNA binding by a chemical carcinogen in rats, hepatic nuclear binding by aflatoxin B₁ (AFB) (pmol/mg DNA) in ad libitum-fed (AL) animals was 2.1 times greater than in rats restricted to 60% of AL consumption for 6 weeks. Data indicating more rapid plasma clearance, increased urinary excretion of the toxin, and less microsome-mediated epoxidation of AFB by the restricted group suggest that decreased macromolecular binding may be attributable in part to metabolic alterations. Moreover, various levels of dietary restriction, initiated at different ages, significantly inhibited hepatic DNA synthesis, thus indicating that effects on cell proliferation could also be involved mechanistically. Finally, circulating levels of the lysosomal enzyme, ß-glucuronidase (ßG), were significantly reduced in the restricted rats, and the implications of this finding regarding potential relationships to aging and carcinogenesis are discussed.

Key words: Caloric/dietary restriction; Aflatoxin B₁; Carcinogenesis; DNA binding; DNA synthesis; Aging; Lysosomal enzymes

INTRODUCTION

Numerous investigators have demonstrated that caloric restriction of laboratory animals very effectively reduces the incidence of spontaneous and chemically-induced neoplastic disease [1,2]. These data have inspired a variety of hypotheses, but the mechanism(s) by which caloric restriction mediates its protective effect have yet to be elucidated. The promotional and hormonal aspects of a possible mecha-

*To whom all correspondence and reprint requests should be addressed.

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nism have seemingly received a majority of the attention given to this issue [1,3], but studies of the effects of restriction at the initiation level of carcinogenesis are also warranted. Recent reports of potentially beneficial modulation of xenobiotic metabolizing enzyme activities and free radical scavenging enzymes [4—6] indicate that initiation by both chemical (environmental) carcinogens and endogenous sources could be reduced in calorically restricted animals. Moreover, Pashko and Schwartz [7] suggested that modifications of metabolic activation pathways were responsible for decreased levels of DMBA binding to dermal DNA in food-restricted mice. Leakey et al. [8,9] most recently demonstrated alterations in cytochrome P-450 isozyme profiles and in conjugating enzyme activities which could enhance carcinogen detoxication processes in calorically restricted animals. The lack of an effect of dietary restriction on the incidence of intestinal tumors induced by a direct-acting carcinogen (N-methylnitrosourea) [10] is also indicative of a metabolism-dependent mechanism which alters initiation. In addition, the fact that suppression of mammary tumorigenesis occurred in rats underfed only 1 week before and 1 week after 7,12-dimethylbenz[a]anthracene (DMBA) administration further substantiates the need to examine the role of caloric restriction in the initiation phase of cancer [11].

While metabolism may indeed be an important factor in the abatement of carcinogenic initiation by dietary restriction, the proliferative state of target tissues might also play a significant role. The importance of DNA replication in DMBA-induced mammary tumorigenesis and in the initiation of chemically-induced liver cancer is well established [12,13], and cell proliferation/DNA synthesis is inhibited in these tissues by caloric restriction [14,15]. Therefore, cell cycle alterations may also contribute mechanistically to the positive outcomes of restriction.

AFB, the highly mutagenic and carcinogenic mycotoxin which has been implicated epidemiologically as a causative agent in human liver cancer, was selected as a model carcinogen to study the relationships described above. Newberne and Rogers [16] recently reported that reduced caloric intake does, in fact, significantly decrease AFB-induced hepatic tumor incidence in male rats. A series of experiments have been designed and implemented in an attempt to investigate mechanisms (including molecular and metabolic aspects), as well as initiation/promotion relationships, involved in the effect of caloric restriction on AFB carcinogenesis. The initial results from the first of these studies concerning nuclear DNA-AFB binding and some relevant parameters are presented here.

MATERIALS AND METHODS

Materials

$[^{13}H]$Aflatoxin B$_1$ (spec. act. 15 Ci/mmol) was obtained from Moravek Biochemicals, Brea, CA, and unlabeled AFB was purchased from Sigma Chemical Co., St. Louis, MO. The radiochemical purities of the AFB preparations were confirmed by
HPLC and radiometric flow detection (Beckman Model 171). Proteinase K, ribonuclease A, calf thymus DNA, and phenolphthalein mono-beta-glucuronic acid were also procured from Sigma Chemical Co. Reduced nicotinamide-adenine dinucleotide phosphate (NADPH) was obtained from Pharmacia, Piscataway, NJ, [methyl-\(^3\)H]thymidine (THY) (spec. act. 5 Ci/mmol) from Amersham International plc, Buckinghamshire, and TS-1 tissue solubilizer from Research Products International, Mt. Prospect, IL. All reagents used were analytical grade.

Animals and diets

Male Fischer 344 rats from the NCTR breeding colony were utilized in these experiments. The rats were weaned at 21 days, fed the NIH-31 diet AL, and randomly allocated to individual cages (NCTR project on caloric restriction (PCR) modified mouse cages) 2 weeks prior to the beginning of each experiment. In the in vivo AFB-DNA binding experiment, AL feeding was maintained until 10 weeks of age. At this point, half of the rats were restricted to 60% of AL consumption using a vitamin-supplemented NIH-31 ration formulated to attain AL equivalent vitamin consumption. The restricted group received a single daily feeding immediately prior to the dark phase of a 12-h light-dark cycle.

Both the AL and restricted rats were dosed with AFB at 16 weeks of age. In the first DNA synthesis experiment, which was started at 6 weeks of age, dietary restriction was maintained for 2 weeks in 3 different groups (80%, 70%, and 60% of AL). The second DNA synthesis experiment included AL, 70%, and 60% groups restricted for either 2 or 4 weeks and sacrificed at 12 weeks of age.

In vivo AFB-DNA binding

Three hours after receiving a single oral dose of \([\text{H}]\)AFB (0.1 mg AFB, 66.7 \(\mu\)Ci/kg body wt dissolved in 75% DMSO/25% \(\text{H}_2\text{O}\)), rats were sacrificed using hypoxia induction with \(\text{CO}_2\) followed by exsanguination. The livers were rapidly excised, rinsed in cold 0.85% \(\text{NaCl}\), frozen in liquid \(\text{N}_2\), and stored at \(-80^\circ\text{C}\) until analyzed. Hepatic nuclei were isolated by differential centrifugation as reported previously [17], and nuclear DNA was extracted, purified, and assayed according to Beland et al. [18]. Binding levels of AFB to DNA were determined radiometrically using liquid scintillation counting.

Plasma and urine determinations

A 0.2-ml aliquot of plasma derived from blood taken 3 h after dosing (as described above) was added to 1.5 ml of TS-1 tissue solubilizer and digested at 50°C for 5 h. These samples were then decolorized by adding 0.2 ml of 30% \(\text{H}_2\text{O}_2\) and heating at 55°C for 30 min, and counted after the addition of 10 ml of scintillation cocktail and 0.5 ml of 3N HCl. An additional group of restricted and AL rats dosed as above was placed in metabolism cages, and concentrations of radioactivity were measured in urine samples collected at 9 h after dosing.
Enzyme assays

Hepatic microsomes were prepared by differential centrifugation as described previously [19] from 12 week old AL rats and the corresponding group restricted to 60% of AL consumption for 4 weeks. *In vitro* microsome-mediated binding of [³H]AFB to calf thymus DNA (an estimation of AFB-epoxidase activity) was determined using a 1.0 ml reaction mixture containing 0.5 mg DNA, 1.0 μCi [³H]AFB (0.5 Ci/m mole; 2 μM final concentration), 0.1 M Tris (pH 7.2), 0.65 mM NADPH, 5 mM MgCl₂, and 1 mg of microsomal protein [20]. After 30 min, the reaction was stopped by addition of cold CHCl₃, the DNA was isolated, assayed [18], and counted using liquid scintillation. Benzphetamine N-demethylase (BPND) activity was assayed [21] using the same microsomal preparations. βG activity was measured in plasma [22] from rats restricted and dosed as described for the *in vivo* binding experiment.

Hepatic DNA synthesis

Two hours before sacrifice, rats were injected intraperitoneally with 50 μCi [methyl-³H]thymidine/100 g body wt. Hepatic DNA was isolated [18], assayed using the diphenylamine reagent [23], and thymidine incorporation was determined by liquid scintillation counting.

Statistical analyses

All data were submitted to a one-way analysis of variance to determine significant differences between AL and restricted groups (RS/1 Release 3.0 Software, BBN Software Products Corp., Cambridge, MA).

RESULTS

The body weight of each calorically restricted group was significantly reduced compared to the corresponding AL control group (Table I). In the first DNA synthesis experiment, after 2 weeks of restriction (begun at 6 weeks of age), body wt was depressed by 16%, 22%, and 28% in the rats fed 80%, 70%, and 60% of AL consumption, respectively. In the second DNA synthesis experiment, body weights of the 70% and 60% groups were decreased by 17% and 21% after 2 weeks and by 24% and 29% after 4 weeks of feeding. A 30% reduction in body weight was noted in the 60% group used in the *in vivo* AFB binding study after 6 weeks of restriction.

Dietary restriction had a dramatic impact upon hepatic nuclear DNA-AFB binding determined at 3 h after administration of a single, relatively low dose of AFB (Fig. 1). At this point, nuclear binding (pmol AFB/mg DNA) in the AL animals was 2.1 times greater than in the calorically restricted group. Moreover, total nuclear AFB-DNA adduction per liver in the AL group was 2.5 times that of the restricted rats.

Initial *in vivo* metabolism determinations also revealed significant diet-related
TABLE I

EFFECT OF VARYING LEVELS AND TIME SPANS OF CALORIC RESTRICTION ON BODY WEIGHTS OF MALE FISCHER 344 RATS

<table>
<thead>
<tr>
<th>Time of restriction (weeks)</th>
<th>Level of restriction (% of ad lib.)</th>
<th>Body weight depression (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2*</td>
<td>80</td>
<td>16</td>
</tr>
<tr>
<td>2*</td>
<td>70</td>
<td>22</td>
</tr>
<tr>
<td>2*</td>
<td>60</td>
<td>28</td>
</tr>
<tr>
<td>2*</td>
<td>70</td>
<td>17</td>
</tr>
<tr>
<td>2*</td>
<td>60</td>
<td>21</td>
</tr>
<tr>
<td>4*</td>
<td>70</td>
<td>24</td>
</tr>
<tr>
<td>4*</td>
<td>60</td>
<td>29</td>
</tr>
<tr>
<td>6*</td>
<td>60</td>
<td>30</td>
</tr>
</tbody>
</table>

*Started at 6 weeks of age; DNA synthesis expt. 1.
*Started at 10 weeks of age; DNA synthesis expt. 2.
*Started at 8 weeks of age; DNA synthesis expt. 2.
*Started at 10 weeks of age; DNA binding expt.
*Compared with the corresponding ad libitum group.

differences (Table II). The concentration of radioactivity in plasma taken 3 h after dosing was 73% greater in the AL group than in the restricted group. In addition, the restricted rats excreted a portion of the dose via the urine during the first 9 h subsequent to AFB administration that was 40% greater than that eliminated by the control rats.

Plasma levels of the lysosomal enzyme, βGl, were measured in the 3-h samples described above and in plasma obtained at 3 additional points following dosing (Fig. 2). At each point, a significant dietary effect was noted. βGl activity in the

![Graph](image-url)  

Fig. 1. Hepatic nuclear DNA-aflatoxin B, adducts in ad libitum-fed and diet-restricted rats. Samples were taken 3 h after administration of a single oral dose of [14C]aflatoxin B, (0.1 mg/kg body wt). Bars (mean ± S.E.M., N = 4) with different letter superscripts differ significantly (P < 0.01).
TABLE II
PLASMA CONCENTRATION AND URINARY EXCRETION OF RADIOACTIVITY IN AD
LIBITUM-FED AND DIET-RESTRICTED RATS FOLLOWING ADMINISTRATION
OF [3H]AFLATOXIN B.*

<table>
<thead>
<tr>
<th>Diet</th>
<th>3 h Plasma concentration of radioactivity (nCi/ml)</th>
<th>9 h Urinary excretion of radioactivity (% of dose)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad libitum</td>
<td>216 ± 15*</td>
<td>7.05 ± 0.44*</td>
</tr>
<tr>
<td>Restricted</td>
<td>125 ± 6*</td>
<td>9.90 ± 0.44*</td>
</tr>
</tbody>
</table>

*[^3H]Aflatoxin B was given as a single oral dose (100 μg/kg body wt; 208 mCi/mmol).
**Values (mean ± S.E.M., N = 4) in the same column with different letter superscripts differ significantly (P < 0.01).

restricted animals averaged 31% lower than in the AL group. No trend indicative of an AFB-related increase in activity was observed.

The results of the in vitro AFB-DNA binding experiment are shown in Table III. Compared with the AL group, microsome-mediated binding of AFB to calf thymus DNA was significantly less (10%) when preparations from rats restricted (60% of AL) for 4 weeks were used. Although not statistically significant, a mean decrease (15%) in hepatic microsomal BPND activity was also noted in the 4 week restricted group (Table III).

The effects of varying levels of dietary restriction on thymidine incorporation into hepatic DNA are depicted in Figs. 3 and 4. In rats restricted from 42 days of age for 2 weeks, DNA synthesis was markedly inhibited (66% average mean reduction)

![Plasma β-glucuronidase activity in ad libitum-fed and diet-restricted rats following aflatoxin B1 dosing](image)

**Fig. 2.** Plasma β-glucuronidase activity in ad libitum-fed and diet-restricted rats following aflatoxin B1 dosing (single oral dose, 0.1 mg/kg body wt). Activity = phenolphthalein (μg) liberated from phenolphthalein mono-β-glucuronate per h/ml plasma. The ad lib. and restricted group values (mean ± S.E.M., N = 4) differed significantly (P < 0.05) at each time point.
TABLE III

EFFECT OF CALORIC RESTRICTION ON HEPATIC MICROSOME-MEDIATED BINDING OF AFlATOXIN B1 TO DNA AND BENZPHETAMINE N-DEMETHYLASE ACTIVITY*

<table>
<thead>
<tr>
<th>Diet</th>
<th>Aflatoxin B1 DNA binding**</th>
<th>Benzphetamine N-demethylase***</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad libitum</td>
<td>36.9 ± 0.6*</td>
<td>15.2 ± 1.5*</td>
</tr>
<tr>
<td>Restricted</td>
<td>33.4 ± 0.4*</td>
<td>12.9 ± 0.5*</td>
</tr>
</tbody>
</table>

Rats were restricted (60% of ad lib.) for 4 weeks starting at 8 weeks of age.

**pmol AFB1/mg DNA/mg protein/min.

***nmol/mg protein/min.

*Values (mean ± S.E.M.) within each column which do not share common superscripts differ significantly (P < 0.05).

at each level of restriction (Fig. 3). Similar results were obtained at the 60% and 70% (of AL) levels in somewhat older animals restricted for either 2 or 4 weeks (Fig. 4). No significant variations were observed between the different levels of restriction with regard to inhibition of hepatic DNA synthesis.

DISCUSSION

To our knowledge, these data represent the first report of an effect of caloric or dietary restriction on in vivo DNA binding by a chemical carcinogen in rats. The inhibitory effect of restriction on adduct levels at 3 hr after dosing was dramatic.
Fig. 4. Effect of varying levels and lengths of caloric restriction on hepatic DNA synthesis (thymidine incorporation) in rats sacrificed at 12 weeks of age. 30% restricted = 70% of ad lib. 40% restricted = 60% of ad lib. Bars with different letter superscripts differ significantly ($P \leq 0.001$).

Previous work has shown that peak incorporation of AFB into hepatic DNA is observed by 2—3 hours after dosing [24,25], and therefore, we believe that our data approximate the maximal binding levels. In light of the fact that the hepatic concentration of DNA in the restricted rats was 40% greater than in the AL rats (thus providing more target per unit volume for the activated carcinogen), these results appear even more significant. The potential importance of the present findings is also enhanced when considered in conjunction with the prior report of decreased levels of DMBA binding to skin DNA in food-restricted mice [7]. Equivalent effects of dietary restriction on DNA binding have now been noted in different species, with different carcinogens, and in different tumor initiating models (skin cancer, topical application of agent; hepatocarcinoma, ingestion of carcinogen). Taken together, the observations of the two studies lend credence to a more general hypothesis addressing the beneficial effects of caloric restriction on cancer initiation in mammals. The binding of activated AFB to target tissue DNA is consistent with the belief that alteration of specific genomic sites by chemical carcinogens may initiate cell transformation [26], and our results therefore indicate that caloric restriction can beneficially modulate chemical carcinogenesis during the initiation phase. Furthermore, the stochastic genetic damages which theoretically contribute to the aging process [27] may be similarly reduced by dietary restriction. In this study, preliminary results indicate that restriction-related alterations in both metabolism and DNA synthetic rates may be mechanistically involved in the reduction of adduct levels.

Greater urinary excretion and lowered plasma levels (indicating more rapid plasma clearance) of AFB are suggestive of more efficient metabolism of AFB in the restricted animals. The single dose pharmacokinetics of AFB in rats (plasma
half-life = 29 min; peak biliary excretion at 20—30 min) [28,29] support our belief that the 3-h plasma and 9-h urine data reported here portray a more rapid detoxicating response of the restricted rats during the critical initial period of challenge. Increased urinary excretion may be indicative of greater production of hydrophilic conjugated metabolites in the restricted rats, which would be compatible with the restriction-related elevations in certain conjugating enzyme activities described recently [9]. Beneficial metabolic alterations by restriction are further evidenced by the reduction in in vitro microsome-mediated AFB-DNA binding (AFB-epoxidase activity) and the reductive trend in BPND activity. AFB epoxidation is believed to be catalyzed primarily by the cytochrome P-450 isozyme, P-450b, which also mediates benzphetamine N-demethylation [30].

Another striking effect of dietary restriction was the inhibition of hepatic DNA synthesis which was of approximately equivalent magnitude regardless of variation in restriction levels. Our results are in agreement with those of Merry and Holehan [15] who found that postweaning dietary restriction implemented before 65 days of age greatly inhibited DNA synthesis. Experiments are currently being conducted to analyze hepatocellular proliferative status and DNA synthesis rates in animals restricted from 70 days of age for 6 weeks to correlate with the in vivo DNA binding data. Should DNA synthesis be inhibited by this regimen, it is likely that the DNA-binding results reported here could be explained in part by increased repair opportunity and/or decreased fixation of genetic lesions [13].

The finding of lower plasma levels of the lysosomal enzyme βG in the restricted rats is also intriguing. AFB-induced liver damage in rats and birds results in a general release of lysosomal enzymes as reflected by an increase in circulating levels of certain marker enzymes [31—33]. Since a relatively low dose of AFB was used in the present study, and no increasing trend in plasma βG activity was evident in either the AL or restricted group after dosing (peak lysosomal enzyme levels at 48 h following AFB treatment have been reported) [32], we believe that the significant differences in βG noted between the 2 groups were products of dietary rather than AFB treatment. It is interesting to note that low plasma βG levels not only result from dietary restriction as described here, but were also associated with genetic resistance to AFB in Japanese quail [33]. Furthermore, lower plasma levels of βG may also augment the preservation of hydrophilic glucuronide conjugates, thus promoting more rapid excretion of aflatoxin metabolites in restricted rats [34]. βG is a reliable marker for lysosomal enzyme release and/or induction, so it seems possible that a general effect of dietary restriction is a reduction in the production/release of these hydrolytic catalysts. Mortimore et al. [35] have recently reported that lysosomally-mediated hepatic protein degradation in rats is greatly decreased during short-term starvation. Increased lysosomal permeability and/or greater lysosomal enzyme activities have been associated with hypertensive vascular changes [36], carcinogenesis [37], and the aging process itself [38,39]. One might therefore hypothesize that lysosomal enzymes have a functional role in dietary restriction-related life span extension and carcinogenic protection.
These preliminary results indicate that caloric restriction can beneficially modulate chemical carcinogenesis at the initiation level in rats, and that alterations in both metabolism and DNA synthetic rates may be mechanistically involved. The findings suggest an interesting interrelationship between lysosomal enzyme activities, dietary restriction, carcinogenesis, and aging.

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