BINDING OF CARBON TETRACHLORIDE METABOLITES TO RAT HEPATIC MITOCHONDRIAL DNA

(Carbon tetrachloride metabolism; mitochondrial DNA; mitochondrial metabolism; DNA binding)

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SUMMARY

Radioactivity from $[^{14}C]CCl_4$ was bound to highly purified mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) prepared from livers of rats after a single dose of $[^{14}C]CCl_4$. At a low, non-necrotizing dose as well as at an acutely toxic dose, mtDNA bound 20–50-fold more radioactivity per mg than did nDNA. Extensive enzymatic digestion and purification of mtDNA did not remove radioactivity. Binding of radioactivity to mtDNA could also be demonstrated after anaerobic incubation of isolated mitochondria with $[^{14}C]CCl_4$, NADPH, ADP, and succinate. Our results suggest that CCl_4 can be activated by rat hepatic mitochondrial enzymes to metabolites which bind covalenty to mtDNA.

INTRODUCTION

The covalent binding of chemical carcinogens to nDNA is usually considered to be a crucial step in the malignant transformation of cells. Recently, several reports have shown that mtDNA can also be a site of carcinogen binding. Chemical carcinogens that show significant binding to mtDNA include BPDE [1], several polycyclic aromatic hydrocarbons [2], aflatoxin B_1 [3], and benzene [4]. Metabolic transformation of the xenobiotic within the mitochondrion followed by binding to mtDNA has been suggested for aflatoxin B_1 [5] and benzene [4].

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Abbreviations: BPDE, dihydrodiol-epoxide derivative of benzo(a)pyrene; SDS, sodium dodecyl sulfate.

CCl$_4$ metabolites have been shown to bind to nDNA, proteins, and lipids [6]. Previous studies of rat hepatic mitochondria showed that an increase in the rate of synthesis of mtDNA and a decrease in mtDNA turnover produces an increase in the total amount of mtDNA in the liver after acute CCl$_4$ intoxication [7]. The rate of synthesis and total amount of mtDNA return to normal values following recovery.

These data suggest the possibility of direct interaction between CCl$_4$ and mtDNA. This study examined the binding of radioactivity from [^{14}C]CCl$_4$ to hepatic mtDNA following either exposure of rats to [^{14}C]CCl$_4$, or the incubation of isolated rat hepatic mitochondria with [^{14}C]CCl$_4$.

METHODS

Chemicals

[^{14}C]CCl$_4$ (spec. act. 26.9 mCi/mmol and >99% radiochemical purity) was purchased from Amersham. CsCl, ethidium bromide, RNase (type 1-A) and protease (*Streptomyces griseus*) were purchased from Sigma Chemical Co., St. Louis, MO. Other chemicals were highest grade commercially available.

Animals and treatment

Male Sprague–Dawley rats (Charles River, Portage, MI) weighing 220–280 g were used. Animals were fasted 16 h before use, with drinking water available ad lib.

A single dose of CCl$_4$ at either non-necrotizing (2 μl/kg = 2.1 $\cdot$ 10^{-2} μmol/kg) or acutely toxic (1.1 ml/kg = 11.4 mmol/kg) level was administered. The low dose was diluted in corn oil to a volume of 200 μl. The labelled CCl$_4$ was diluted with unlabelled CCl$_4$ to give a specific activity of 0.048 mCi/mmol. At 5 h and 24 h after the low, and at 24 h after the high dose, pairs of rats were killed by decapitation and mitochondrial and nuclear fractions of the liver prepared following the procedure described by Brabec et al. [7].

Hepatic nDNA was prepared by dissociation of an aliquot of the nuclear fraction in 2% SDS, repeated extraction with chloroform–isoamyl alcohol (20:1) and sequential digestion with RNase and protease. Enzyme treatment was followed by further extractions and 2 cycles of ethanol precipitation. The purified nDNA was dissolved in 0.15 M NaCl and 0.01 M sodium phosphate, pH 7.2.

Hepatic mtDNA was prepared from the isolated washed mitochondria after lysis in 10 mM Tris (pH 7.5), 1 mM EDTA, 1% SDS at 37°C, and extracted 5 times with chloroform–isoamyl alcohol (20:1). The aqueous phase was brought to a density of 1.56 g/cm^3 with CsCl and ethidium bromide added to 400 μg/ml for centrifugation in a Spinco SW41 rotor at 20° for 40 h at 36 000 rev./min. The gradient was fractionated by collecting drops. Aliquots of every other fraction were counted for radioactivity.

The alternate radioactive fractions were pooled and extracted with isoamyl alcohol until free of ethidium bromide, then dialyzed against 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.2.

Incubation of isolated mitochondria

Mitochondria were prepared from livers of untreated rats as described [8]. Additionally, a post-mitochondrial supernatant fraction was prepared by homogenizing 2 g of liver in 3 ml of incubation media (0.25 M sucrose, 1 mM EDTA, 10 mM Tris, 10 mM KCl, pH 7.2), and centrifuging at 10000 × *g* for 20 min. The post-mitochondrial supernatant containing the endoplasmic reticulum was included as a source of activating enzymes in the incubation.

Pooled mitochondria from 2 to 4 rats were suspended in incubation media using 1 ml for each 2 g of original liver, wet weight. When present, the concentration of calf thymus DNA was 2 mg/ml.

Mitochondrial suspension (2.5 ml) or nuclear DNA solution (2.5 ml) plus either 0.5 ml post-mitochondrial supernatant, or 0.5 ml incubation media, were incubated under nitrogen with 1 mM NADPH and 3 mM glutathione [9]. To assure anaerobic conditions, the mitochondrial incubations were made 10 mM in succinate, 3 mM in ADP, and kept at 37°C for 15 min before addition of CCl_4 (final volume 3.0 ml, 30 mM CCl_4, containing 25 μCi [^{14}C]CCl_4). Under the conditions of incubation (succinate, ADP and nitrogen atmosphere), the affinity of mitochondrial cytochrome oxidase is sufficiently high to reduce the dissolved oxygen concentration below $5 \cdot 10^{-8}$ [10]. Following 30 min of anaerobic incubation, mitochondria were sedimented and washed, and mtDNA was prepared. Incubations containing calf thymus nDNA were extracted with chloroform–isoamyl alcohol (20:1) and purified as described.

Radioactivity measurements

Radioactivity was determined using ACS scintillation cocktail and a Packard liquid scintillation spectrometer. Determinations were corrected for quench and converted to mole values based on the specific activity of [^{14}C]CCl_4. DNA was measured by absorbance at 260 nm using 1 *A* unit = 50 μg/ml DNA.

RESULTS

Binding of CCl_4 metabolites to rat liver DNA

Table I illustrates that at the low dose of CCl_4, hepatic mtDNA bound about 20

TABLE I

BINDING OF CCl_4 METABOLITES TO DNA IN VIVO

Dose	Time (h)	pmol CCl_4 metabolite/ mg mtDNA	pmol CCl_4 metabolite/ mg nDNA	Binding ratio: mtDNA binding/ nDNA binding
2 μl/kg	5	$1.9 \cdot 10^1$	$7.1 \cdot 10^{-1}$	27
2 μl/kg	24	$2.5 \cdot 10^1$	$5.0 \cdot 10^{-1}$	50
1.1 ml/kg	24	$5.1 \cdot 10^3$	$2.4 \cdot 10^2$	21

TABLE II

BINDING OF CCl₄ METABOLITES TO DNA IN VITRO

Sample	nmol CCl₄ metabolite mg DNA	mol CCl₄ metabolite/ mol DNA bases[a]
mtDNA[b]	6.2 ± 1.5	$1.9 \cdot 10^{-3}$
mtDNA + PMS[c,d]	6.0 ± 1.8	$1.8 \cdot 10^{-3}$
nDNA[c]	0.8 ± 0.09	$0.2 \cdot 10^{-3}$
nDNA + PMS[c,d]	3.3 ± 0.7	$1.0 \cdot 10^{-3}$

[a]Calculation based on 3.237 μmol nucleotides/mg DNA [7].
[b]Mean of 4 experiments ± standard deviation.
[c]Mean of 3 experiments ± standard deviation.
[d]PMS, post-mitochondrial fraction prepared as described.

pmol/mg DNA. Hepatic nDNA binding was only 2–4% of mtDNA binding on a nucleotide of DNA basis. At the acutely toxic CCl₄ dose, $5 \cdot 10^3$ pmol/mg mtDNA was bound, which was 20-fold greater than the binding to nDNA. Thus, while binding to both nDNA and mtDNA increased with increased dose of CCl₄, mtDNA bound 20–50-fold more radioactivity than nDNA at both low and high dose levels.

Binding of CCl₄ metabolites to rat liver mtDNA following incubation of isolated mitochondria

After incubation of isolated rat hepatic mitochondria and [¹⁴C]CCl₄, radioactivity was bound to mtDNA (Table II). Addition of the post-mitochondrial supernatant fraction did not increase the binding. This suggests that the metabolic pathway for activation of CCl₄ to the binding metabolite may be present in the mitochondrial fraction. Binding of radioactivity to calf thymus DNA in the presence of [¹⁴C]CCl₄ was greatly stimulated by the inclusion of the post-mitochondrial supernatant fraction in the incubation.

DISCUSSION

We have shown that measurable binding of radioactivity from [¹⁴C]CCl₄ to mtDNA occurred in vivo after a single exposure to CCl₄. The degree of binding increased with the dose of CCl₄ and the binding was 20–50-fold greater to mtDNA

TABLE III

AVERAGE NUMBER OF DNA BASES MODIFIED BY CCl₄ METABOLITES

Dose	Time (h)	mtDNA	nDNA
2 μl/kg	5	1 in 170000	1 in 4600000
2 μl/kg	24	1 in 130000	1 in 6500000
1.1 ml/kg	24	1 in 640	1 in 13000

than to nDNA. The binding of the CCl_4 metabolite to mtDNA appeared to be covalent, as the radioactivity remained after the mtDNA had been purified by treatment with detergent, digestion with RNase and protease, extraction with organic solvents, dialysis, and density gradient centrifugation in high-molarity CsCl. These treatments would be expected to remove non-covalently bound radioactivity, and radioactivity associated with protein, RNA, and lipids. The stoichiometry of binding to DNA is shown in Table III. By expressing the results reported in Table I in terms of mol nucleotides/mol CCl_4 metabolite bound, it is seen that at the low level of CCl_4 exposure, only 1 base out of $1.3 \cdot 10^5$ would be expected to be modified in mtDNA. Since each molecule of mammalian mtDNA contains approx. $3.3 \cdot 10^4$ bases [11], only 1 out of every 4 mtDNA molecules would, on the average, contain a modified nucleotide. However, at the higher dosage, 1 out of every 640 nucleotides may be modified, affecting 4% of all mtDNA nucleotide base pairs.

The results of incubation of isolated hepatic mitochondria with CCl_4 would indicate that mitochondria may have the ability to metabolize CCl_4 to an active metabolite which is capable of binding to mtDNA. Mitochondria isolated as described, routinely contain less than 3% contamination by microsomes [12]. Thus, it is not likely that the activation of CCl_4 seen is due to microsomal contamination. Further evidence against microsomal involvement is the failure to increase mtDNA binding upon addition of active post-mitochondrial supernatant fraction.

Hepatic mitochondria contain a cytochrome P-450 system capable of hydroxylating steroids [16]. This system may be capable of metabolizing CCl_4, analogous to microsomal cytochrome P-450 [17]. Also, dehalogenation of allyl chloride [18] and halothane [19] can be catalyzed by reduced hemin. Whatever the route, these results suggest that CCl_4, like benzene [4] and aflatoxin B_1 [3] may be metabolically activated by mitochondrial enzymes.

At the present, we do not know the significance of the high degree of disruption of mtDNA chemistry. The economy of organization of information in mtDNA [13] makes it extremely likely that modification of even a fraction of a percent of the mtDNA sequence will have a functional consequence on mtDNA replication, transcription, or translation. However, the recovery of mitochondrial function after acute CCl_4 intoxication [8] suggests that the binding to mtDNA does not play a role in CCl_4-induced hepatotoxicity. The effects may be more subtle and delayed in expression. For many years the question of the involvement of mitochondria in the process of transformation and malignancy has been discussed (for review, see [14]). Birky [20] discussed mechanisms by which clones of cells with modified mtDNA can arise. Neubert et al. [15] has proposed a mechanism by which modification of a small portion of the mitochondrial population can lead to a malignant cell. However, an understanding of the role of mitochondrial xenobiotic activation and of mtDNA in chemical carcinogenesis awaits futher investigation.

234

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