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Low-level Binding of Halothane Metabolites to Rat Liver Histones In Vivo

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Binding of halothane metabolites to rat liver histones was investigated after *in vivo* administration of ^{14}C-halothane. Animals were injected with either a mixture of triiodothyronine, glucagon and heparin (TGH) to stimulate liver growth or with saline as a control. Twenty-four hours later, animals were administered ^{14}C-halothane and maintained at 8–10 per cent O_2 for 6 hours. Detergent washed nuclei from liver homogenates were subfractionated to allow quantitative measurements of ^{14}C-halothane binding to histones. Although our studies suggest that much of the previously reported binding of halothane metabolites to major cell fractions was a result of redistribution of endoplasmic reticulum components during isolation procedures, carefully controlled experiments demonstrated that the radioactivity associated with histones could not be due to residual microsomal lipid. Of the initial 132 μmol of ^{14}C-halothane administered, 1.1 μmol remained as nonvolatile metabolites in the liver homogenate and 25 pmol were associated with purified histones. This corresponds to approximately one halothane moiety per 15,000 histone molecules. No significant binding to liver cell RNA or DNA was observed. With this low level of histone modification and lack of convincing evidence of halothane metabolite binding to hepatic DNA or RNA, it is unlikely that significant alteration of the genome occurs after exposure to halothane. (Key words: Anesthetics, volatile: halothane. Biotransformation, drug; fluorometabolites; microsomes. Cancer. Cells: chromatin; chromosomal alterations; histones. Complications: hepatic. Metabolism: DNA; metabolites; microsomes; RNA. Toxicity: carcinogenicity; hepatic; metabolites.)

INTEREST in halothane metabolite binding to nuclear components has increased over the last several years due to persistent reports suggesting that long-term exposure to low concentrations of anesthetic gases may be associated with genetic alterations in operating room personnel.[1,2] This interest has been renewed by a recent report which indicated that exposure of rats for one year to trace concentrations of nitrous oxide and halothane resulted in significant dose-dependent increases of chromosomal aberrations in proliferating spermatogonial and bone-marrow cells.[3] In addition, evidence for increased frequencies of mutation as a consequence of exposure to halothane alone has been observed in two separate eucaryotic cell test systems employing either yeast cells[4] or *Drosophila melanogaster*.[5] Animal models for halothane induced hepatotoxicity have shown that post-halothane liver necrosis is a consequence of both increased drug metabolism and hypoxia.[6,7] Low oxygen tension favors the reductive or oxygen independent pathway of halothane metabolism,[8,9] and increases the extent of halothane metabolite binding to liver cell constituents.[10,11] The known reductive metabolites of halothane are CF_3CH_2Cl and CF_2CHCl. These compounds have been detected in the expired breath of both patients and laboratory animals to whom halothane had been administered.[12,13] A third reductive metabolite, CF_2CBrCl, has been inferred from the presence of the corresponding cysteine conjugate in the urine of humans exposed to halothane.[14] The strong electrophilic character of the carbons of these metabolites suggest they may alkylate chemically basic cell constituents. In fact, CF_2CHCl and CF_2CBrCl are weak mutagens in the Ames bacterial test.[15] Although the mutagenic effect of CF_2CHCl and CF_2CBrCl upon bacterial DNA is low, it is nonetheless evidence for the reactivity of these halothane metabolites with DNA, and suggests that other cell constituents could also be modified. Appearance of the mercapturic acid conjugate of CF_2CBrCl in the urine of halothane-exposed patients illustrates the ease of alkylation of cellular sulfhydryl groups,[14] and further suggests that halothane metabolites may alkylate other accessible chemically basic cell constituents. Furthermore, oxidative metabolism of halothane may result in a reactive trifluoroacetyl intermediate which may also alkylate tissue constituents such as ethanolamine, as inferred from the presence of N-trifluoroacetylethanolamide in the urine.[14]

The cell constituents whose modification would most directly alter either genetic structure or the accuracy of protein synthesis are the DNA and chromosomal proteins of the nucleus, nuclear or cytoplasmic RNA, and the ribosomes of the rough endoplasmic reticulum. Since halothane metabolism occurs in the smooth endoplasmic reticulum, and this membrane network is continuous with the rough endoplasmic reticulum which is joined to the membranes of the nuclear envelope, it is possible that lipophilic halothane metabolites could diffuse from the primary site of metabolism through the endoplasmic

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reticulum as far as the nucleus. Furthermore, chromosomal protein histones are synthesized on membrane bound ribosomes in association with specific histone messenger RNA molecules in the rough endoplasmic reticulum.[16,17] It is significant that both halothane metabolism and histone synthesis occur in close proximity in the microsomal or endoplasmic reticulum fraction of the cell. Halothane metabolites may alkylate histones before their transport to the nucleus and resultant interrelation with DNA. There is substantial evidence that normal specific enzymatic histone methylation, acetylation, or phosphorylation at unique times during the cell cycles, are important for regulation of cell growth and development.[18–21] Any modification of basic amino groups in a histone molecule would alter its electrostatic properties and consequently alter the stability of DNA-histone associations. Because chromosomal structure is modulated by histones, nonspecific modification of either histone structure or the normal pattern of enzymatic histone acetylation, deacetylation, methylation or phosphorylation[19,21] by random alkylation by reactive halothane metabolites could adversely effect the natural state of the genome.

New research on the mechanisms of enzymatic histone modification has shown the presence of a histone deacetylase[22,23] and a histone specific protease[24] in chromatin, which are inhibited either by sodium butyrate or by removal of Mg and Ca ions, respectively. Thus, previous studies may not have detected metabolite binding to chromatin due to proteolysis during isolation procedures. Since the rate of liver cell replication and histone synthesis is very slow in adult animals,[25] in this study, a mixture of triiodothyronine, glucagon, and heparin was administered to stimulate liver growth and simulate conditions of long-term exposure of liver cells to halothane metabolites.[26] The importance of histone structure, the possibility of aklylation of chemically basic histone amino groups by electrophilic halothane metabolites, plus our knowledge of the protective influence of the newly-reported histone deacetylase and protease inhibitors, jointly suggested the present investigation of possible halothane metabolite modification of hepatic histones.

Methods

Adult male, 250-g, Fischer 344 rats were maintained with water *ad libitum* and Purina® rat chow for five days prior to experimentation. The animals were pretreated 24 h prior to ^{14}C-halothane exposure with subcutaneous administration of either 0.5 ml of a 5 per cent ethanol in water suspension of 50 μg triiodothyronine, 500 μg glucagon and 50 units heparin,[27] or with 0.5 ml saline as a control. Rats were lightly anesthetized with Ketamine® and a delivery capsule, constructed of number 90 polyethylene tubing and containing 14 μl (26 mg, 132 μmol, 160 μCi, 1.21 μCi/μmol) of ^{14}C-halothane, was inserted intraperitoneally as previously described.[27] One hour later, each animal was placed in a seven-liter glass chamber and maintained for an additional five hours in an atmosphere of 8–10 per cent oxygen in nitrogen. At the end of a six-hour exposure period, approximately 90 per cent of the halothane had been released from the capsule. The livers were perfused with an iced solution of *p*H 7.4 0.25 M sucrose, 10 mM Tris-HCl, 10 mM sodium EDTA, 10 mM sodium butyrate and 10 units/ml heparin, then homogenized in buffer containing 0.34 M sucrose, 10 mM EDTA, 0.5 mM EGTA, 10 mM sodium butyrate, 0.2 mM·phenylmethylsulfonyl fluoride (PMSF), 0.1 per cent butylhydroxytoluene (BHT), and 5 units/ml heparin in buffer A. Buffer A contained 60 mM KCl, 15 mM *p*H 7.4 Tris-acetate, 15 mM mercaptoethanol, 0.50 mM spermidine, and 0.15 mM spermine.[28]

Liver nuclei were isolated as previously described.[29–30] The crude homogenate was layered over 1.22 M sucrose in the homogenization buffer and centrifuged at 10,000 × *g* for 20 min at 4° C to obtain a crude nuclear fraction. The precipitate was gently resuspended in 2.1 M sucrose in homogenization buffer and layered over 2.1 M sucrose in homogenization buffer and centrifuged at 105,000 × *g* for four hours at 4° C. The nuclear pellet was resuspended in 0.25 M sucrose, 10 mM *p*H 7.4 Tris-acetate, 6 mM KCl, 5 mM $Mg(OAc)_2$, 0.1 mM EGTA, 5 mM sodium butyrate, 5 mM sodium trifluoroacetate and saturating concentrations of PMSF and BHT. The resulting solution was adjusted to 0.5 per cent in Triton X-100, and washed nuclei were collected by centrifugation at 5,000 × *g* for 15 min.[31,32] This washing procedure was repeated once, followed by two rinses without detergent, and the white fluffy nuclei were collected at 10,000 × *g* for 10 min.

Chromatin was extracted from purified nuclei by homogenization in an omnimixer in 2.5 mM *p*H 7.4 Tris-HCl, 5 mM EDTA, and 5 mM sodium butyrate.[33] The crude chromatin was separated from residual membrane fragments by centrifugation through 1.32 M sucrose,[31] then washed with 1 per cent Triton X-100 in 65 mM NaCl, 5 mM sodium butyrate, 5 mM sodium trifluoroacetate and 25 mM EDTA.[32] This was followed by two washes without detergent before shearing chromatin in an omnimixer in preparation for extraction of histones.

Histones were extracted from chromatin with

TABLE 1. Isolation of Rat Liver Histones from Saline or TGH-pretreated Animals Exposed to ^{14}C-halothane

Procedure	Saline Pre-Treated Animal* (dpm)	TGH Pre-Treated Animal* (dpm)
Crude liver homogenate	2.31×10^6	2.68×10^6
Nuclear and cell debris fraction	1.36×10^6	1.70×10^6
2.1 M sucrose pellet	7,040	10,930
Washed nuclear pellet	2,480	4,300
Crude chromatin	972	1,508
Washed chromatin	662	949
Acid soluble nuclear proteins	292	307
Histone fraction	225	257
Washed histone fraction	188	223
Dialyzed after storage	151	156
Applied to gels	151	156
Total ^{14}C after PAGE	143	147
^{14}C in histone bands	73	71

* The amount of nonvolatile metabolites is shown during isolation of histones from livers of two rats, one treated with saline and the second with 50 μg triiodothyronine, 500 μg glucagon and 50 units heparin (TGH). Animals were maintained at 8–10 per cent oxygen in nitrogen for 6 hours prior to removal of the livers. Each rat received 160 μCi, 1.21 mCi/mmol, of ^{14}C-halothane.

0.4 N H_2SO_4 and precipitated with ethanol.[33–35] The histones were suspended by sonication in 4 ml of absolute ethanol, 1.3 ml chloroform added, mixed vigorously, and histones recovered by centrifugation at $1,000 \times g$. The histones were additionally washed with 5–6 ml of methanol/chloroform 2/1, ethanol/ether 1/1 and then with absolute ethanol to remove lipids. The histone powders were suspended in 0.1 per cent sodium dodecyl sulfate (SDS) 20 mM 6.8 *p*H Tris HCl, and 5 mM sodium butyrate, and stored at 4° C. Just prior to preparative SDS-polyacrylamide gel electrophoresis, each histone preparation was dialyzed overnight at 4° C in 3500 MW cutoff tubing against 10 mM *p*H 7.4 Tris-HCl, 5 mM sodium butyrate, 5 mM EDTA and 0.02 per cent BHT.

Histones were finally isolated by preparative electrophoresis on 0.1 per cent SDS 12.75 per cent polyacrylamide gels.[36,37] Gel sections which contained histone H1 or histones H2, H3 and H4 were cut out, homogenized in water in an omnimixer, eluted electrophoretically through a medium scintered glass Buchner funnel into 0.01 per cent SDS and the radioactivity determined. Blank portions of the gel were used as controls.

Total DNA and RNA were isolated from livers of two ^{14}C-halothane dosed animals by direct extraction with phenolic solvents.[38] DNA was also isolated from chromatin prepared by saline-EDTA extraction[33–35] of liver from three ^{14}C-halothane treated rats. Polysomes were isolated from livers of four ^{14}C-halothane exposed animals by sodium deoxycholate treatment.[39,40] Ribosomal and messenger RNA were subsequently extracted from ribosomes with phenol or acid.[38,40] Extracted nuclei acids were washed with ethanol/ether to remove any residual phenolic compounds.

Results

In several preliminary experiments, rats were treated with phenobarbital to enhance halothane metabolism, or were partially hepatectomized to stimulate liver growth. Purification of nuclei or chromatin from these animals proved more difficult than from the untreated control animals presumably due to rapid synthesis of glycogen or endoplasmic reticulum.[41,42] Because of both the low yield and purity of nuclei and chromatin isolated from livers of phenobarbital or partially hepatectomized animals, these pretreatments were discontinued in subsequent experiments. Even though direct isolation of chromatin from livers with saline-EDTA solution[32,34–36] was a facile procedure, it was necessary to discontinue this method of chromatin isolation since contamination by cytoplasmic protein and lipid could not be excluded.[31–35] Further quantitative studies of the extent of halothane metabolite binding to nuclear proteins in saline control or TGH-hormone induced animals were completed only with chromatin obtained from detergent purified nuclei.[28,30–33] The TGH-hormone treatment increased DNA and histone synthesis maximally 18–24 h after injection, the stimulatory effect being observable for at least 30 h after treatment.[26]

In four separate experiments with a total of four rats pretreated with TGH-hormone solution, an average of 1.1 ± 0.3 μmol of nonvolatile metabolites were found in the initial liver homogenates. Only 50 per cent of the radioactivity remained after dialysis. This implies that the dialyzable metabolites are bound ionically and only the remaining portion is bound covalently to tissue macromolecules.

Results of complete histone purification from a single saline control and a single TGH-hormone induced animal are shown in table 1. Of the radioactivity recovered from the PAGE gels, 51 per cent was found in the non-histone chromosomal proteins, 11 per cent in histone fraction H1, and 38 per cent in histone fraction H2ab, H3 and H4. Although pure histones were isolated completely only once, in partial purifications, the histone fractions from three saline and three TGH treated animals contained 62, 105, 132 dpm or 59, 85 or 99 dpm of halothane metabolite, respectively. Histones obtained in the partial purifications were believed free of lipid contaminants and shown to be of similar electrophoretic purity as illustrated in figure 1, and

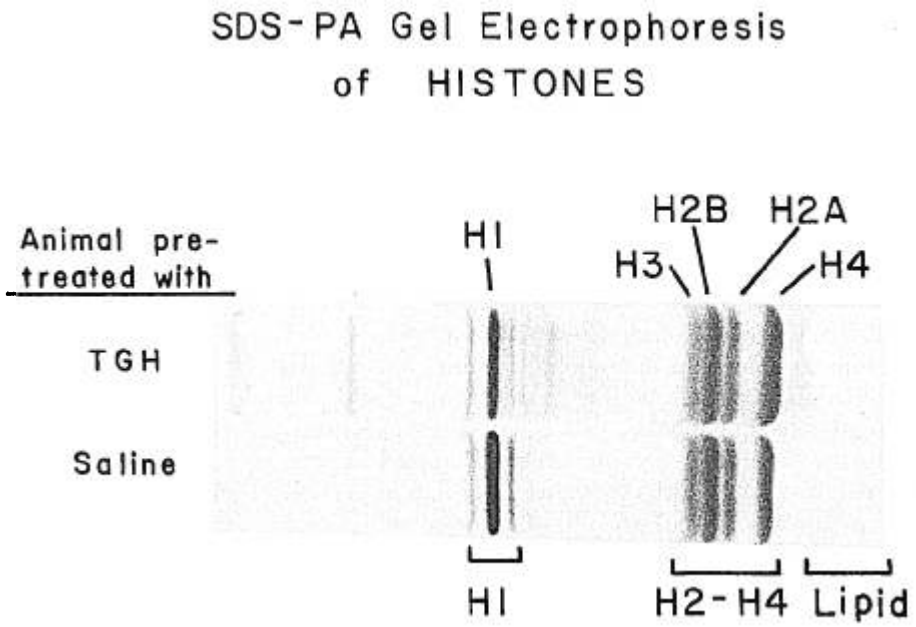

FIG. 1. Isolation of histones by 0.1 per cent sodium dodecyl sulfate–12.75 per cent polyacrylamide gel electrophoresis. Approximately 50 μg of protein were applied to analytical scale slab gels 1.5 mm thick and run at 120 V for 6–7 h. Analytical gels were stained with Coomassie brilliant blue and destained in methanol/acetic acid/water. The indicated histone and lipid region bands were cut out from the corresponding preparative scale gels, eluted and radioactivity measured by liquid scintillation counting. Intervening bands of non-histone protein were also cut out, eluted and counted.

thus substantiate the results of the two lengthy complete purifications.

To directly assess contamination of nuclear histones and major cell fractions by endoplasmic reticulum fragments, hepatic microsomes isolated from a ^{14}C-halothane exposed animal were added to an unexposed liver before subfractionation. Subsequent distribution of the ^{14}C-labeled microsomal components in all cell fractions closely resembled the initial distribution. Of the 200,000 dpm of ^{14}C-labeled microsomes added, 20 per cent were found in the crude nuclear cell debris fraction, 21 per cent in the mitochondrial fraction, 40 per cent in the microsomal fraction, and 19 per cent in the cytosol. Isolation of Triton X-100 washed nuclei from this preparation revealed that only 0.69 per cent or 280 dpm of the initial 40,000 dpm of ^{14}C-microsomal radioactivity remained in the washed nuclear fraction. An additional 48,500 dpm of microsomal lipid was then added to the detergent-washed nuclei before isolation of chromatin and histones. Only 0.033 per cent remained. Therefore, contamination of the histone fraction represented only 0.00023 per cent of the initial non-dialyzed radioactivity or 3 dpm. The washed histone fraction contained 190 to 220 dpm (table 1), indicating that the radioactivity is not attributable to microsomal lipid contamination. Furthermore, all ^{14}C-lipids were clearly separated from histones on the SDS-page gels.

Treatment of purified chromatin with 0.4 N H_2SO_4 to extract histones and other acid soluble nuclear proteins resulted in precipitation of one-half to two-thirds of the radioactivity. This precipitate contained the acid insoluble or non-histone chromosomal proteins (NHCP) as well as residual contaminating cytoplasmic proteins. Absence of adequate criteria of purity plus the minute quantity of each of the minimum of 500 highly diverse NHCP species precluded investigation of metabolite binding to these proteins. The addition of four volumes of ethanol to the supernatant to precipitate histones, and additional washing of the histone precipitate with organic solvents, resulted in some loss of protein and radioactivity but removed any last traces of lipid contamination from the histones. Loss of radioactivity at this step was proportional to the loss of protein. Figure 1 illustrates the separation of histones from cytoplasmic proteins or lipids by preparative scale SDS-polyacrylamide gel electrophoresis. The final recovery of purified histones was approximately 6 mg, which corresponds to a final specific activity of hepatic histones of 4.5 pmol/mg, or one halothane moiety per 15,000 histone molecules. Nucleic acids were isolated from livers of ^{14}C-halothane exposed rats as described in the methods. Even though each liver contained up to 3×10^6 dpm or 1.4 μmol of nonvolatile metabolites, no radioactivity was found associated with either DNA, total RNA or ribosomal RNA.

Discussion

Chromosomal modifications may result in toxic, teratogenic, or carcinogenic effects. Most of the present focus in establishing a biochemical link between epidemiologic or cytologic data has depended

upon DNA modification. Many compounds or their microsomal metabolites, including trichloroethylene[43] and vinyl chloride,[44] have been shown to covalently bind to DNA. These compounds are structurally similar to the reductive halothane metabolites. A prior report from this laboratory has shown that two reductive halothane metabolites, CF_2CBrCl and CF_2CHCl, are mutagenic in the Ames test in the absence of an active metabolic system, which indicates that these compounds interact directly with DNA without further hepatic enzyme-dependent metabolism.[15]

Hepatic halothane metabolite binding was shown here to occur *in vivo* largely to microsomes and to a lesser extent to all other cell fractions as previously reported.[10,11,45] However, when radiolabeled hepatic microsomes were added to a liver homogenate from a non-exposed animal prior to isolation of subcellular components from this mixture, only 40 per cent of the initial purified radiolabeled microsomes was recovered in the new microsomal pellet, and the remainder was recovered in the other three major cell fractions. This demonstrates that metabolites bound to the endoplasmic reticulum are distributed into all subcellular fractions during routine isolation procedures. Consequently, several previous reports[10,11,45] of the subcellular distribution of halothane metabolites may have been due to contamination by endoplasmic reticulum fragments alone. This result emphasizes the importance of excluding microsomal lipid during isolation of hepatic chromatin and histones in our study.

It is known that administration of alkylating agents to the rat results in alterations of liver nuclear protein synthesis,[46] changes in DNA-nuclear protein interactions[47] as well as direct binding of reactive alkylating species such as alkylnitroso compounds and arylepoxides to both histone and non-histone chromosomal proteins.[48–50] Microsomal incubations of benzpyrene with DNA, RNA, or isolated nuclei have been shown to result in significant binding to both exogenous nucleic acids,[51] and the DNA, RNA, histones and non-histone chromosomal proteins of intact nuclei.[52] Since benzpyrene and alkylnitroso compounds are proven carcinogens, this implies that chromosomal protein modifications by chemically reactive species may have deleterious genetic consequence.

That no halothane metabolite binding to cell DNA or RNA was found in these studies is reassuring, and suggests that halothane metabolites do not diffuse appreciably from the endoplasmic reticulum. Rather, they react preferentially with more readily accessible microsomal or cytoplasmic constituents before reaching the nucleus in sufficient quantity to be detected in DNA. There are also active DNA repair enzymes which could either replace an altered nucleotide or remove the reactive moiety which would preclude detection of low frequency DNA binding. It has also been reported that no binding of reactive metabolites to exogenous RNA or albumin was observed during *in vitro* microsomal halothane metabolism, although significant binding occurred concomitantly to microsomal protein and lipid.[53]

The small amount of halothane metabolite binding to histones reported in this study may have occurred in the apparent absence of binding to DNA because the reactive amino groups of DNA bases are too tightly complexed in double stranded form to be alkylated, while the amino groups of histones are more readily accessible.

If binding of halothane metabolites occurred preferentially to histones newly synthesized on endoplasmic reticulum ribosomes rather than to existing nuclear histones, then stimulation of histone synthesis during ^{14}C-halothane metabolism would have increased histone labeling in TGH treated animals. However, no difference in binding occurred, suggesting that there is little binding to newly synthesized histones during the course of halothane exposure. In slowly dividing cells, most of the chromatin is condensed into heterochromatin at the periphery of the nucleus at the nuclear envelope. The remaining small amount of active chromatin is dispersed throughout the nucleus. It might be expected that reactive anesthetic metabolites diffusing from the endoplasmic reticulum toward the nucleus would bind primarily to the dense peripheral heterochromatin, which is relatively inactive, implying a margin of safety concerning metabolite binding to chromatin. It is also possible that histones aklylated during halothane exposure are repaired by chromosomal proteases and deacetylases, which would further decrease the risk of genetic consequence of metabolite binding to chromatin.

The specific activity of metabolite binding to histones is very low and corresponds to approximately one metabolite per 15,000 histone molecules. Since biological histone acetylations or phosphorylations during the normal cell cycle results in 1–3 modifications per histone, interference of normal cell processes by the low level of histone modification observed here is considered minimal. The absence of significant halothane metabolite binding to hepatic DNA or RNA *in vivo*, or to exogenous nucleic acids or protein *in vitro*, makes it further unlikely that significant modification of the genome occurs after exposure to halothane. Thus, whatever the basis

for the cytologic and epidemiologic data[1–5] indicating deleterious effects after prolonged exposure to halothane, the present investigation suggests relatively little covalent modification of chromosomal components by halothane metabolites.

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