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1,2-Dimethylhydrazine-induced premalignant alterations in the *S*-adenosylmethionine / *S*-adenosylhomocysteine ratio and membrane lipid lateral diffusion of the rat distal colon

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Prior studies by our laboratory, utilizing the 1,2-dimethylhydrazine experimental model of colonic cancer, had shown that administration of this procarcinogen for 5 weeks was found to increase phospholipid methyltransferase activity and the fluidity of rat distal colonic brush-border membranes. The present studies were conducted to further explore these 'premalignant' colonic phenomena. Male albino rats of the Sherman strain were subcutaneously injected with dimethylhydrazine (20 mg/kg body weight per week) or diluent for 5 weeks. Animals from each group were killed, distal colonic tissue harvested and the levels of *S*-adenosylmethionine, *S*-adenosylhomocysteine and decarboxylated *S*-adenosylmethionine measured by high performance liquid chromatography. The activity of methionine adenosyltransferase was also examined in these tissues. Additionally, brush-border membranes were isolated from the distal colonocytes of control and treated-animals and examined and compared with respect to their phospholipid methylation activities as well as their lipid fluidity as assessed by the rotational mobilities of the probes 1,6-diphenyl-1,3,5-hexatriene and DL-12-(9-anthroyl)stearic acid and translational mobility of the fluorophore pyrenedecanoic acid. The results of these studies demonstrated: (1) phospholipid methyltransferase activity in rat colonic plasma membranes was increased concomitantly with increases in the cellular levels of *S*-adenosylmethionine and the *S*-adenosylmethionine / *S*-adenosylhomocysteine ratio in the distal colonic segment of treated-animals; and (2) the lateral diffusion of rat distal colonic brush-border membrane lipids, as assessed by the ratio of excimer / monomer fluorescence intensities of the fluorophore pyrenedecanoate, was also increased after dimethylhydrazine administration to these animals for 5 weeks.

Earlier studies by our laboratory [1], using the 1,2-dimethylhydrazine experimental model for colon cancer, have demonstrated that an increase in the specific activity of a phospholipid methyltransferase could be detected in the distal colonic brush-border membranes of Sherman rats administered this carcinogen for only 5 weeks, i.e., well before the development of colonic tumors. Moreover, these alterations were not found to be present in the proximal colonic or small intestinal brush-border membranes of animals administered

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this carcinogen and were not detected in the distal colonic brush-border membranes of dimethylhydrazine-treated Lobund-Wistar rats, a strain which is totally resistant to the induction of colonic tumors by this agent [2]. Taken together, these prior observations strongly suggested that this increased phospholipid methyltransferase activity of rat distal colonic brush-border membranes was an early event in the dimethylhydrazine-induced malignant transformation process in this organ. The mechanism(s) underlying the alteration in this colonic membrane enzymatic activity, however, were unclear.

In this regard, *S*-adenosylmethionine is the methyl donor for most known biological transmethylation reactions [3,4], including those involved in the methylation of phospholipids, nucleic acids and proteins. *S*-Adenosylhomocysteine, one of the products of all transmethylation reactions involving *S*-adenosylmethionine, acts as a potent competitive inhibitor of most methyl-transferring enzymes including those that catalyze the methylation of histones, DNA, tRNA's and phosphatidylethanolamine [5]. Since these latter enzymatic activities have a higher affinity for *S*-adenosylhomocysteine than for *S*-adenosylmethionine [6], a number of investigators have previously proposed that the ratio of *S*-adenosylmethionine/*S*-adenosylhomocysteine may be of critical importance in the regulation of biological methylation reactions [6–8]. It was, therefore, of interest to examine the effects of dimethylhydrazine on the levels of *S*-adenosylmethionine, *S*-adenosylhomocysteine and decarboxylated *S*-adenosylmethionine (*S*-adenosyl-(5′)-deoxy-(5′)-3-methylthiopropylamine) in the distal rat colon.

In our earlier studies [1], the fluidity * of rat distal colonic brush-border membranes was also examined after treatment with this procarcinogen, using various lipid soluble fluorophores and steady-state fluorescence polarization techniques. These experiments demonstrated that the 'dynamic' component of fluidity but not the 'static' component of fluidity, as assessed by r values of DL-12-(9-anthroyl)stearic acid (12-AS), and r_∞ values of 1,6-diphenyl-1,3,5-hexatriene (DPH), respectively, was higher in treated membranes than their control counterparts. Moreover, the increase in phospholipid methyltransferase activity [1] as well as changes in the glycosphingolipid composition of these colonic membranes [9] appeared, at least in part, to explain this alteration in the 'dynamic' component of fluidity in the treated animals.

Recent studies in a number of cell lines derived from tumors [10,11], including human colon cancer cells [11], have suggested that alterations in the lipid lateral diffusion of the plasma membranes of these cells may be involved in their malignant transformation. Since our earlier studies [1] assessed fluidity in terms of the rotational diffusion of lipid soluble fluorophores, in the present experiments it was of interest to further examine the fluidity of control and treated-distal colonic brush-border membranes in terms of translational mobility of the probe pyrenedecanoic acid. The results of the experiments described below demonstrate that alterations in the *S*-adenosylmethionine/*S*-adenosylhomocysteine ratio and membrane lipid lateral diffusion can be detected in the rat distal colon after 5 weeks of dimethylhydrazine treatment and serve as the basis for this report.

Male albino rats of the Sherman strain weighing 75–100 g were given weekly subcutaneous injections of diluent or 1,2-dimethylhydrazine dihydrochloride (Sigma Chemical Co., St. Louis, MO) at a dose of 20 mg/kg body wt. for 5 weeks [1]. The animals were maintained on a pelleted diet (Purina diet, Scientific Small Animals, Arlington Heights, IL) with food and water ad libitum. One week after the last injection, animals were fasted for 18 h with water ad libitum. For each control or treated-preparation, eight animals were killed rapidly by cervical dislocation and their colons excised. The cecum from each animal was discarded and the remaining large intestine divided into proximal and distal parts [1,9]. Epithelial cells, relatively devoid of goblet cells, were then obtained from the distal segment using a technique that combined chelation of divalent cations with mild mechanical dissociation [12].

* The term 'lipid fluidity' as applied to natural membranes is used in this paper to denote the relative motional freedom of the lipid molecules or substituents thereof. A more detailed description has been previously published [28].

The cells from this segment of each group were then pooled separately to isolate brush-border membranes as previously described [12]. The purity of each membrane preparation was assessed by the marker enzyme cysteine-sensitive alkaline phosphatase [12]; specific activity ratios [(purified membrane)/(original homogenate)] ranged from 12 to 20 for this enzyme and were not significantly different between control and treated preparations. The corresponding values of NADPH–cytochrome *c* reductase, succinate dehydrogenase and sodium-potassium-dependent adenosine triphosphatase, marker enzymes for microsomal, mitochondrial and basolateral membranes, respectively, ranged from 0.40 to 1.60 in each of these preparations [12]. Protein was estimated by the method of Lowry et al. [13]. All enzymatic activities were linear with respect to time and protein.

The methylation of phospholipids was measured by incorporation of the [^{3}H]methyl group from *S*-adenosyl-L-[*methyl*-^{3}H]methionine (5.0–15.0 Ci/mmol) into phospholipids as previously described [14,15]. The chemical identity of the methylated products was further established by two-dimensional chromatography [16] and by hydrolysis of the phospholipids and identification of their free bases as described by Schneider and Vance [17]. As shown in Table I, in agreement with prior results from our laboratory [1], the specific activity of phospholipid methyltransferase was significantly increased in distal colonic brush-border membranes of rats administered dimethylhydrazine for 5 weeks compared to controls.

The cellular contents of *S*-adenosyl-L-methionine, *S*-adenosyl-L-homocysteine and decarboxylated *S*-adenosyl-L-methionine of control and treated distal colonic mucosa were determined by reversed-phase, high-performance liquid chromatography (HPLC) according to the method of Wagner et al. [18]. The sample preparation of colonic mucosa was accomplished by rapid tissue removal, rinsing with iced saline and scraping into 0.2 M $HClO_4$. Components were separated using a gradient elution technique and Waters HPLC Systems (Milford, MA). Solvent A consisted of a mixture of 980 ml of 0.1 M NaH_2PO_4 and 20 ml of acetonitrile adjusted to pH 2.65 with HPLC grade phosphoric acid. Solvent B contained 740 ml of 0.15 M NaH_2PO_4 and 260 ml of acetonitrile at pH 3.25. Both solvents contained $8 \cdot 10^{-3}$ M octane sulfonic acid used as the ion-pairing reagent. Optimal separation of compounds was obtained using a linear gradient from 90% A to 100% B over 50 min followed by 10 min of isocratic elution at a flow rate of 1.5 ml/min. An Ultrasphere ion-pair column (5 μm particle size, 250 × 4.6 mm i.d.) from Beckman (Berkeley, CA) was used, fitted with a Guard-Pak pre-column module and C-18 cartridge maintained at 25 °C. The compounds were identified by their relative retention times and quantified by comparison of peak areas to that of known amounts of standard. Peak area responses were linear over the range tested. No internal standard was used as recovery has previously been shown to be quantitative [18].

As shown in Table II, distal colonic tissues of treated-animals were found to possess significantly higher levels of *S*-adenosylmethionine than control tissues. Since the levels of *S*-adenosylhomocysteine were similar in the tissues of both groups, the ratios of *S*-adenosylmethionine/*S*-adenosylhomocysteine were also significantly greater in treated-mucosa compared to their control counterparts. The level of decarboxylated *S*-

TABLE I

SPECIFIC ACTIVITIES OF PHOSPHOLIPID METHYLTRANSFERASE AND METHIONINE ADENOSYLTRANSFERASE IN THE DISTAL COLON OF CONTROL AND 1,2-DIMETHYLHYDRAZINE-TREATED RATS

Values represent means ± S.E. of four separate preparations of each group examined. Phospholipid methyltransferase activity was measured in rat distal colonic brush-border membranes, while methionine adenosyltransferase was measured in rat colonic cell cytoplasm.

Preparation	Specific activity	
	Phospholipid methyltransferase (pmol/mg protein per h)	Methionine adenosyltransferase (pmol/mg protein per min)
Control	315 ± 28	31.3 ± 0.5
Dimethylhydrazine-treated	452 ± 35 *	49.9 ± 0.9 *

* $P < 0.05$ compared to control values.

TABLE II

EFFECTS OF 1,2-DIMETHYLHYDRAZINE ON DISTAL RAT COLONIC EPITHELIAL CELL CONCENTRATIONS OF *S*-ADENOSYLMETHIONINE AND ITS METABOLITES

Values represent means ± S.E. of four separate preparations of each group and are expressed as nmol/mg protein.

	Concentration	
	Control	Dimethylhydrazine-treated
S-Adenosylmethionine	17.8 ± 2.5	25.3 ± 0.4 *
S-Adenosylhomocysteine	1.0 ± 0.1	0.9 ± 0.1
Decarboxylated *S*-adenosylmethionine	0.4 ± 0.1	0.5 ± 0.1
Ratio *S*-adenosylmethionine/*S*-adenosylhomocysteine	17.5 ± 2.0	28.1 ± 0.7 *

* $P < 0.05$ compared to control values.

adenosylmethionine in the distal colonic segments was found to be unaffected by dimethylhydrazine administration for 5 weeks (Table II).

In view of the increased levels of *S*-adenosylmethionine seen in the distal colon of treated-animals, it was of interest to measure and compare the specific activity of the enzyme responsible for the cellular synthesis of *S*-adenosylmethionine, i.e., methionine adenosyltransferase. This enzyme was assayed according to the method of Okada et al. [19] and was found to be increased in the distal colonic segment of treated-animals (Table I).

The foregoing results have therefore documented for the first time an increase in *S*-adenosylmethionine levels in the distal colons of dimethylhydrazine-treated animals. Concomitant with these latter alterations, the phospholipid methyltransferase activity of distal colonic brush-border membranes of treated-animals was also increased. In basic agreement with these observations, previous studies by Hoffman et al. [6] have shown that microsomal phospholipid methyltransferase activity and the ratio of *S*-adenosylmethionine/*S*-adenosylhomocysteine in the liver were directly correlated following bromobenzene treatment or starvation in the rat. Similarly, perfusion of rat livers with homocysteine and adenosine was found to lower the ratio of *S*-adenosylmethionine/*S*-adenosylhomocysteine from 5.6 in control to 0.3 in treated livers and under these conditions, the incorporation of labeled methionine into phosphatidylcholine was reduced almost to zero [8]. Chiang and Cantoni [7] used still another approach to demonstrate that this ratio of methionine metabolites can influence phospholipid methylation in rat liver. These investigators injected animals with 3-deazaadenosine, a potent inhibitor of *S*-adenosylhomocysteine hydrolase, which markedly lowered the hepatic ratio of *S*-adenosylmethionine/*S*-adenosylhomocysteine and concurrently reduced the labeling of phosphatidylcholine with [*methyl*-^{3}H]methionine by 90% [7].

The ratios of *S*-adenosylmethionine to *S*-adenosylhomocysteine in control and treated colonic mucosa are among the highest values reported, to date, in any rat tissue [6-8,20]. In the present studies, to avoid any significant degradation of *S*-adenosylmethionine, colonic tissue was homogenized directly in acid within 5–10 min after the death of the animals as suggested by Eloranta [20], which may at least partially explain these high values. In this regard, however, it is important to note that while the concentration of *S*-adenosylmethionine is high in control tissue and even higher in dimethylhydrazine-treated tissue, these values are well below the K_m for *S*-adenosylmethionine previously determined for the phospholipid methyltransferase present in control (111.3 ± 6.8 μM, $N = 6$) and treated (105.1 ± 9.7 μM, $N = 6$) rat distal colonic brush-border membranes [1]. It would, therefore, appear that the dimethylhydrazine-induced increases in the level of *S*-adenosylmethionine and in the ratio of *S*-adenosylmethionine/*S*-adenosylhomocysteine may have physiological relevance in terms of the regulation of phospholipid transmethylation reactions in distal colonocytes of this organ.

Prior studies have shown that the chronic administration of the hepatic carcinogens 2-acetylaminofluorene [21], diethylnitrosamine [22] and ethionine [23] as well as of the tumor promoters phenobarbital and 1,1-bis(*p*-chlorophenyl)-2,2,2-trichloroethane [24], decreased the levels of *S*-adenosylmethionine in rat livers. In direct contrast to these results, the present studies demonstrate that *S*-adenosylmethionine levels were increased in the distal colon of rats administered dimethylhydrazine for 5 weeks. Taken together, these results would suggest that: (1) alterations in

S-adenosylmethionine may be involved in the malignant transformation process induced by various organ-specific carcinogens in both the liver and colon; and (2) these alterations may vary dramatically depending on the organ.

The mechanism(s) responsible for the increased *S*-adenosylmethionine levels seen in the distal colon of treated-rats, however, remain unclear at this time. While the activity of methionine adenosyltransferase was also found to be increased in treated distal rat colonocytes, based on prior observations in a number of different rat tissues [20,25], this alteration is unlikely to account for the increase in *S*-adenosylmethionine levels seen in these cells. Why this enzymatic activity is elevated in treated colonic tissue is also not apparent from the present studies. In this regard, however, it is now clear that different isoenzymes of methionine adenosyltransferase appear to exist in the rat liver and other tissues [19]. The different forms have been referred to as α, β and γ [19]. Prior studies [19,26,27] using various hepatocarcinogens, have in fact, demonstrated a decrease in the α- and β-forms, with a concomitant rise in the γ-form in the livers of animals during development of hepatomas. While the existence of such isoenzymes of methionine adenosyltransferase has not as yet been documented in the rat colon, it will be of interest in the future to examine this possibility.

Steady-state fluorescence polarization studies were also performed with a Perkin-Elmer 650-40 spectrofluorometer adapted for fluorescence polarization using the lipid soluble fluorophores 1,6-diphenyl-1,3,5-hexatriene (DPH) (Aldrich Chemical Co., Milwaukee, WI) and DL-12-(9-anthroyl)stearic acid (12-AS) (Molecular Probes, Inc., Eugene, OR). The methods used to load the membranes with each of these probes and quantification of fluorescence have been discussed extensively by our laboratory [12,28]. Values of r_∞ for DPH were calculated from r values as previously described by Van Blitterswijk et al. [29]. The lifetimes of DPH and 12-AS in each preparation were estimated by phase fluorometry at 30 mHz in an SLM 4800 polarization spectrophotometer as previously described by our laboratory [30]. No significant differences in the excited-state lifetimes of each of the probes in the various preparations were noted in the present experiments (not shown).

As shown in Table III, in agreement with earlier observations by our laboratory [1], the distal brush-border membranes of treated-animals showed an increase in the 'dynamic' component of fluidity but a similar 'static' component of fluidity, as assessed by lower r values of 12-AS and similar r_∞ values of DPH, respectively, compared to their control counterparts. In order to detect additional differences in the physical state of the lipids of treated and control membranes, studies were conducted using the lipid soluble fluorophore pyrenedecanoate. Pyrene probes, such as pyrenedecanoate which exhibit intermolecular excimer formation, have been widely used to assess and compare the lateral diffusion of biological membranes and model bilayers [31,32].

Excitation and emission spectra and estimation of total fluorescence intensity were obtained with a Perkin-Elmer 650-40 spectrofluorometer equipped with a multitemperature cuvette holder, using the lipid-soluble fluorophore pyrenedecanoic acid (Molecular Probes, Inc., Eugene, OR) at 25°C. The samples were loaded with pyrenedecanoate as previously described [3]. Ex-

TABLE III

FLUORESCENCE STUDIES OF DISTAL COLONIC BRUSH-BORDER MEMBRANES OF CONTROL AND 1,2-DIMETHYLHYDRAZINE-TREATED RATS

Values represent means ± S.E. of four separate preparations of each membrane obtained at 25°C.

Preparation	Excimer/monomer ratios of pyrenedecanoate	Fluorescence anisotropy, r, of 12-AS	Fluorescence anisotropy, r, of DPH	Limiting hindered anisotropy, r_∞, of DPH
Control membranes	0.438 ± 0.052	0.143 ± 0.004	0.187 ± 0.006	0.149 ± 0.007
Treated membranes	0.715 ± 0.033 *	0.132 ± 0.002 *	0.190 ± 0.002	0.153 ± 0.004

* $P < 0.05$ compared to control values.

cimer and monomer fluorescence intensities were determined at an excitation wavelength of 345 nm and emission wavelengths of 397 nm (monomer) and 465 nm (excimer). Both excitation and emission slits were set at 4 nm. Measured intensities were corrected as necessary for background fluorescence of the buffer and/or unlabeled samples [34]. Excimer and monomer lifetimes of the excited-state for pyrenedecanoate in each preparation, using phase and modulation values determined at 6 mHz modulation frequency relative to a POPOP reference solution [35], were determined at 25°C as described by Hresko et al. [34] with an SLM 4800 spectrofluorometer (SLM – Aminco, Urbana, IL). No significant differences in the excited-state lifetimes of the probe in the various preparations were noted in the present studies (not shown).

In the present experiments, the ratio of excimer to monomer intensities of pyrenedecanoate was found to be significantly higher in distal colonic brush-border membranes prepared from dimethylhydrazine-treated rats compared to their control counterparts (Table III), indicating that treated-membranes were more fluid as assessed by this technique [36]. While the significance of these findings is unclear at this time, based on prior observations by our laboratory [12,28,37], it would appear that these dimethylhydrazine-induced changes in the physical state of distal colonic brush-border membranes should lead to important alterations in protein-mediated activities present in these membranes. It is also of interest to note that Dibner et al. [11] have recently shown that several polar organic solvents which induced differentiation in a human colon cancer cell line, concomitantly decreased the lipid lateral diffusion of the plasma membranes of these cells. Searls and Edidin [10] also reported that retinoic acid-induced differentiation of embryonal carcinoma cells was associated with a decrease in the lipid lateral diffusion of their plasma membranes as assessed by a fluorescence photobleaching recovery technique. These latter observations, taken together with the present results, do, therefore, suggest that alterations in the lateral diffusion of the lipids of rat distal colonic membranes may also play a role in the early malignant transformation process induced by dimethylhydrazine in the rat distal colon. Further studies along these lines, using this experimental model of colonic cancer, should therefore be of interest.

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