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Methylation of arsenic trioxide in hamsters with liver damage induced by long-term administration of carbon tetrachloride

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One-time oral administration of arsenic trioxide to hamsters with toxic liver cirrhosis induced by long-term exposure to carbon tetrachloride resulted in significant elevations of the concentrations of dimethylarsenic species in the liver and in blood, and in high urinary excretions of dimethylarsenic species. These concentration changes in the liver, blood and urine indicated that the methylation of inorganic arsenic was not inhibited but promoted in hamsters suffering from experimentally induced toxic liver cirrhosis. Cirrhotic hamsters also had increased urinary excretion of inorganic arsenic. Results of the determinations of *S*-adenosylmethionine in the livers suggested that this compound may accelerate the methylation of inorganic arsenic in the cirrhotic liver.

Keywords: Arsenic trioxide, methylated arsenic species, *S*-adenosylmethionine, methylation, *S*-adenosylhomocysteine, toxic cirrhosis, hamsters

INTRODUCTION

A number of experimental studies with humans and animals have revealed the *in-vivo* conversion of inorganic trivalent arsenic to methylated arsenic compounds.[1–5] The mechanism of this methylation has not yet been elucidated. Several reports[6,7] deny that the methylation takes place in the blood, the urine or the intestinal tract; other reports[8,9] claim that the *in-vivo* methylation of inorganic arsenic occurs chiefly in the cytosol of the liver with *S*-adenosylmethionine (SAM) as the most likely methyl donor. Buchet *et al.*[10] injected arsenic trioxide intravenously into patients with liver cirrhosis and observed that the methylation of inorganic arsenic was not inhibited in such patients.

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The study described in this paper was undertaken to investigate the methylation of inorganic arsenic in hamsters suffering from toxic liver cirrhosis induced by carbon tetrachloride. The behavior of SAM and its metabolite, *S*-adenosylhomocysteine (SAH), was also studied.

EXPERIMENTAL

Administration of carbon tetrachloride

Seven groups (five animals per group) of male Syrian golden hamsters weighing 103.0 ± 5.5 g had free access to a pellet feed manufactured by Japan CLEA Inc., Tokyo, and to distilled water. Three groups were subcutaneously administered twice weekly in the dorsal region a mixture (0.04 cm^3 kg^{-1} body weight) consisting of olive oil containing 5% (v/v) carbon tetrachloride. The first group received this treatment for 15 weeks, the second for 20 weeks and the third for 30 weeks. Olive oil only (0.04 cm^3 kg^{-1}) was similarly administered to the fourth group for 15 weeks, to the fifth group for 20 weeks, and to the sixth group for 30 weeks. The seventh group served as control.

Administration of arsenic trioxide

The hamsters treated with olive oil or the olive oil/carbon tetrachloride mixture were given orally one dose of 0.38 cm^3 (1.12 mg As, 1.5 mg As_2O_3) of an aqueous solution of arsenic trioxide (3 mg As cm^{-3}; Merck AG, West Germany) per kg of body weight 72 h after the last injection of olive oil or of the olive oil/carbon tetrachloride mixture. The hamsters in the control group received the same dose of arsenic trioxide at the same time as the treated hamsters. Each hamster was kept separated in a metabolic cage for 24 h

after the administration of arsenic trioxide. During this time urine and feces were collected. All hamsters were sacrificed 24 h after receiving the arsenic trioxide. All assay materials were kept at −20°C until analyzed.

Determination of arsenic

Each liver, feces (0.5−1.0 g) blood, and urine sample (0.5−1.0 cm^3) was transferred into a 10 cm^3 polymethylpentene test tube. After addition of 2 mol dm^{-3} NaOH (5 cm^3) to each test tube, the test tubes were heated at 95°C for 3 h in a heating block (Yamato Model HF-41). Preliminary experiments proved that monomethylarsenic (MA), dimethylarsenic (DMA), and trimethylarsine oxide (TMAO) species were not degraded to other arsenic species when heated at 95°C in 2 mol dm^{-3} NaOH. Total inorganic arsenic, MA, DMA and TMA (trimethylarsenic) species were determined by hydride generation−atomic absorption spectrometry[11] with detection limits of 0.5 ng As cm^{-3} for each of the four arsenic species. The coefficient of variation was less than 5%. Hydride generation with the reduction performed at pH ≤ 3.5 does not distinguish between trivalent and pentavalent inorganic and methylarsenic compounds, e.g. between $CH_3As(OH)_2$ and $CH_3AsO(OH)_2$. Therefore, arsenic compounds with the same degree of methylation but different valence will be aggregated into one arsenic value. The background excretion of arsenic prior to the administration of arsenic trioxide was 0.89 ± 0.26 μg As day^{-1} (9% inorganic As, 1% MA, 23% DMA and 66% TMA) in urine and 0.50 ± 0.22 μg As day^{-1} (55% inorganic As, 10% MA, 3% DMA and 32% TMA) in feces.

Histopathological examination

Specimens of liver tissue for examination by light microscopy were fixed in a 10% solution of formaldehyde, embedded in paraffin, sliced, and double-stained with hematoxylin and eosin. For electron microscopy, the livers were double-fixed in a 1% solution of glutaraldehyde and a 1% solution of osmium tetroxide, dehydrated with a gradient series of ethanol, embedded in Epon 812, sliced into ultrathin sections, and double-stained with solutions of uranium acetate and lead acetate.

Biochemical examination

Serum glutamic oxaloacetic transaminase (S-GOT) and serum glutamic pyruvic transaminase (S-GPT) were determined with the S.TA-Test Wako. Albumin, globulin and the albumin/globulin ratios (A/G) were determined with the A/G.B-Test Wako (Wako Pure Chemical Industries Ltd, Osaka, Japan).

SAM and SAH concentrations in the liver

SAM and SAH concentrations in the liver were determined by the method of Wagner *et al.*[12] *S*-adenosyl-5-*L*-methionine (Tokyo Kasei Kogyo Co. Ltd, Tokyo, Japan) and *S*-adenosyl-*L*-homocysteine (Sigma Chemical Co. Inc., St Louis, MO, USA) served as standards. An aliquot (0.5−1.0 g) of each liver sample was homogenized with 4 cm^3 of 0.4 mol dm^{-3} perchloric acid. The homogenate was deproteinized by centrifugation at 3000 g for 15 min. The supernatant was filtered through a Millipore membrane filter (0.22 μm, HV). A Shimadzu Model LC-6A chromatograph (Shimadzu Seisakusho Ltd, Kyoto, Japan) was used for high-performance liquid chromatography. An ultrasphere ion-pair column (250 mm × 4.6 mm, particle size 5 μm; Beckman, Berkeley, CA, USA) was fitted with a guard column packed with pellicular octadecylsilane (ODS) (Shimadzu Seisakusho Ltd, Kyoto, Japan). Gradient elution consisting of two mobile phases was used. Mobile Phase A was prepared by mixing 20 cm^3 acetonitrile with 980 cm^3 aqueous 0.1 mol dm^{-3} NaH_2PO_4 solution. The pH of the mixture was adjusted to 2.65 with 3 mol dm^{-3} H_3PO_4 after addition of 1.55 g octanesulfonic acid (OSA). Mobile Phase B was prepared from 740 cm^3 0.15 mol dm^{-3} NaH_2PO_4, 260 cm^3 acetonitrile and 1.55 g OSA. The pH was adjusted to 3.25 with 3 mol dm^{-3} H_3PO_4. Both mobile phases were 0.008 mol dm^{-3} with respect to OSA. A small amount of EDTA (0.01%, w/v) was added to Mobile Phase A to stabilize the baseline of the UV detector. A linear gradient changing during 30 min from 85% A/15% B to 100% B at a flow rate of 1.5 cm^3 min^{-1} and a column temperature of 40°C was used. For SAM and SAH determination (UV detection at 254 nm) 20−50 μl of each sample was injected. The results were treated statistically by means of Student's *t*-test and analysis of variance.

RESULTS AND DISCUSSION

Liver damage induced with carbon tetrachloride

The groups of hamsters on carbon tetrachloride treatment weighed less (96.2 ± 10.5 g at 15 weeks, 102.0 ± 2.3 g at 20 weeks, 117.3 ± 10.3 g at 30 weeks)

than groups on olive oil treatment (143.0 ± 13.2 g at 15 weeks, 149.0 ± 19.8 g at 20 weeks, 148.0 ± 25.1 g at 30 weeks). S-GOT, S-GPT, serum albumin, serum globulin and the albumin/globulin ratios showed no significant difference between the carbon tetrachloride-treated groups, the olive oil-treated groups, and the control group. Therefore, such measurements cannot be used to identify liver cirrhosis in hamsters. The histopathological examination under the light microscope of the liver of hamsters exposed to carbon tetrachloride revealed tissue changes such as extensive centrolobular necrosis, fatty degeneration and sporadic fibrosis suggestive of toxic liver cirrhosis. These changes were also observed under the electron microscope. The administration of carbon tetrachloride to rats or mice is known to induce toxic liver cirrhosis, but there is no report on the induction of liver cirrhosis in hamsters with this agent.

Methylation of inorganic arsenic

The arsenic concentrations in the livers of hamsters 24 h after oral administration of arsenic trioxide are shown in Table 1. Inorganic arsenic, monomethyl-, dimethyl-, and trimethyl-arsenic species were present in the livers of all groups. The concentration of trimethylated arsenic was the same in all the groups. The concentrations of dimethylated arsenic were approximately 1.5-fold higher in the olive oil/carbon tetrachloride group (hamsters with liver cirrhosis) and olive oil groups than in the livers of the controls (hamsters given arsenic trioxide only). A similar, but statistically less significant, difference was found for monomethylated arsenic. Inorganic arsenic concentrations in the livers of hamsters on oil/carbon tetrachloride treatment were lower than in the controls; the concentrations in the oil-treated hamsters were higher than in the controls. However, only the increased concentrations of dimethylated arsenic in the oil and oil/carbon tetrachloride groups are statistically significant at $P < 0.05$. These elevated concentrations of dimethylarsenic species may have been caused by the activation of drug-metabolizing enzymes in the liver in response to the adminstration of olive oil. The total arsenic concentration in the liver was approximately the same in all the groups. Activation of drug-metabolizing enzymes in the liver by polychlorinated biphenyls did not accelerate the methylation of inorganic arsenic.[13]

Inorganic arsenic, monomethylated arsenic and dimethylated arsenic, but no trimethylated arsenic species were detected in the blood. The concentrations of dimethylated arsenic in the blood of the oil/carbon tetrachloride-treated hamsters were approximately twice as high as those in the blood of the controls. The concentrations of arsenic species in all other groups were equal to those of the controls (Table 2).

The urinary excretion of arsenic for each group during the first 24 h after the oral, once-only administration of arsenic trioxide is shown in Fig. 1. All oil/carbon tetrachloride-treated hamsters excreted 1.7 times as much inorganic arsenic and 1.6 to 1.9 times as much dimethylated arsenic as the control animals. Monomethylated arsenic excretion by the animals treated for 15 or 20 weeks with oil/carbon tetrachloride was similar to that for the control group but about 1.7 times higher than the control group after treatment for 30 weeks.

Buchet *et al.*[10] observed a similar increased urinary excretion of methylated arsenic in human subjects with liver damage. After intravenous injection of one dose of sodium arsenite (7.14 $\mu g\ kg^{-1}$ as arsenic) into

Table 1 Concentrations (mean ± SD) of arsenic species in the livers of hamsters after a single oral administration of arsenic trioxide (1.12 mg As kg^{-1})

	Concentration of arsenic (ng As g^{-1} wet wt)				
Group	Inorg. As	MA	DMA	TMA	Total
Controls	160 ± 28	44 ± 7	34 ± 4	23 ± 8	266 ± 21
Oil/CCl_4					
15 weeks	117 ± 29[a]	53 ± 8	46 ± 8[b]	26 ± 3	226 ± 49
20 weeks	148 ± 18	62 ± 19	50 ± 8[a]	30 ± 1	290 ± 34
30 weeks	146 ± 20	63 ± 5	54 ± 12[b]	21 ± 9	294 ± 31
Oil					
15 weeks	195 ± 20	64 ± 14	44 ± 6	13 ± 5	329 ± 28
20 weeks	187 ± 03	66 ± 21	51 ± 10	20 ± 11	331 ± 56
30 weeks	172 ± 29	68 ± 23	54 ± 16	19 ± 11	324 ± 66

[a] $P < 0.01$. [b] $P < 0.05$.

Table 2 Concentrations (mean ± SD) of arsenic species in the blood of hamsters after a single oral administration of arsenic trioxide (1.12 mg As kg^{-1})

Group	Concentration of arsenic (ng As cm^{-3})			
	Inorg. As	MA	DMA	TMA[b]
Controls	44 ± 12	44 ± 13	18 ± 6	—
Oil/CCl_4				
15 weeks	42 ± 24	34 ± 8	41 ± 8	—
20 weeks	43 ± 10	39 ± 13	43 ± 9	—
30 weeks	47 ± 8	49 ± 11	43 ± 8[a]	—
Oil				
15 weeks	40 ± 11	42 ± 7	22 ± 7	—
20 weeks	49 ± 15	47 ± 12	24 ± 3	—
30 weeks	37 ± 7	43 ± 8	24 ± 7	—

[a] $P<0.01$. [b] —, Not detected.

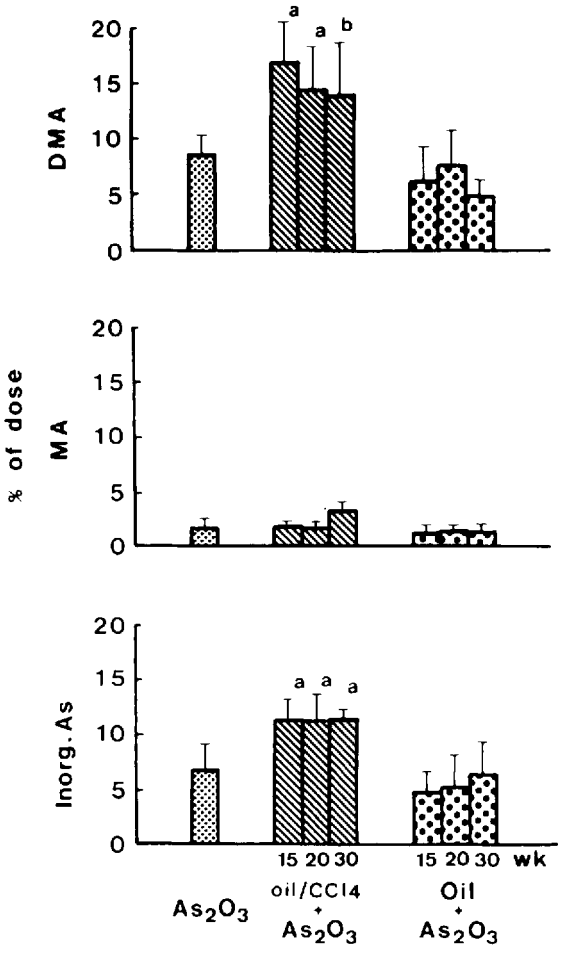

Figure 1 Percentage (mean ± SD) of arsenic species excreted in urine 24 h after a single dose of arsenic trioxide (1.12 mg As kg^{-1}): a, $P<0.01$; b, $P<0.05$.

patients with liver diseases (alcoholic cirrhosis, chronic hepatitis, hemochromatosis, post-necrotic cirrhosis, steatosis and biliary cirrhosis) the urinary excretion of mono- and di-methylated arsenic compounds during the first 24 h was 26% higher than the excretion of normal subjects. The excretion of total arsenic was the same in both groups. The results on humans and hamsters show that the conversion of inorganic trivalent arsenic to mono- and di-methylated arsenic species is accelerated in subjects with liver damage. Hamsters with liver damage excreted more total arsenic in the urine than hamsters with healthy livers.

The difference of fecal excretion of arsenic species among the groups during the 24 h after oral administration of arsenic trioxide are not statistically significant. No TMA was detected in the feces (Table 3).

SAM and SAH concentrations in the liver

No significant differences in the liver SAM and SAH concentrations were found between hamsters with carbon tetrachloride-induced toxic liver cirrhosis and the untreated controls that had not received arsenic trioxide (Fig. 2). Liver cirrhosis does not affect the SAM and SAH concentrations in the liver. Prolonged treatment with olive oil followed by the administration of arsenic trioxide reduced SAM levels. The administration of arsenic trioxide alone resulted in no significant changes in the SAM and SAH concentrations in the livers. Concentrations of SAM and SAH in the liver of normal hamsters or hamsters with liver damage are not available in the literature. The SAM (91 nmol g^{-1}) and SAH (35 nmol g^{-1}) concentrations in the liver of healthy, untreated hamsters used in the present study did not differ greatly from the published background concentrations in the liver of mice[14] and rats.[15]

SAM is a methyl group donor in *in-vivo* methylation

Table 3 Percentage (mean ± SD) of arsenic species excreted in the feces during the 24 h period after administration of a single oral dose of arsenic trioxide (1.12 mg As kg^{-1}).

Group	Percentage of dose			
	Inorg. As	MA	DMA	TMA[a]
Controls	1.3±0.9	0.6±0.4	0.2±0.2	—
Oil/CCl_4				
15 weeks	2.6±1.5	1.8±1.0	0.7±0.5	—
20 weeks	1.6±0.5	0.7±0.4	0.6±0.6	—
30 weeks	1.2±0.7	0.4±0.1	0.5±0.5	—
Oil				
15 weeks	1.3±0.8	0.4±0.2	0.1±0.1	—
20 weeks	1.3±1.2	0.5±0.2	0.3±0.3	—
30 weeks	1.6±1.5	0.6±0.5	0.1±0.1	—

[a] —, Not detected.

reactions. It has been suggested, that SAM is a methyl group donor in the methylation of selenium,[16,17] an element belonging to the group of metalloids as does arsenic. When a methyltransferase inhibitor was first administered to animals followed by arsenic trioxide, the conversion of inorganic arsenic to demethylated arsenic was low, suggesting that SAM may also be involved in the methylation of inorganic arsenic.[9]

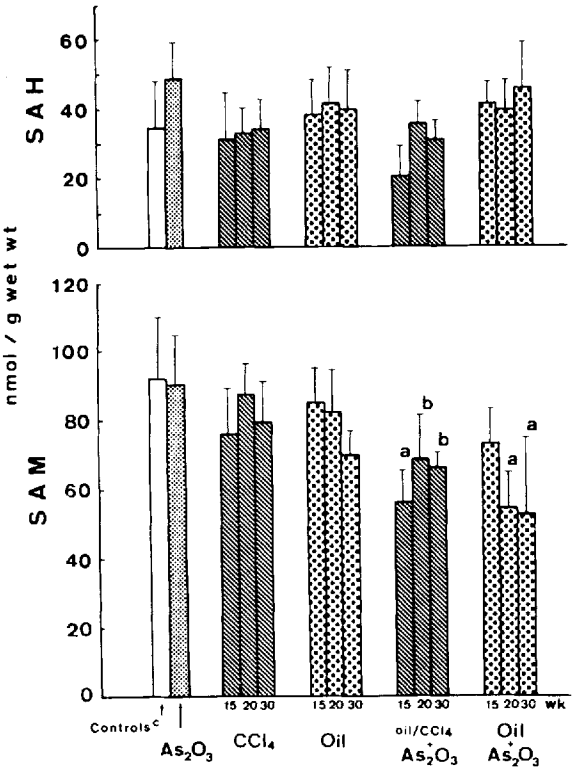

Figure 2 Concentrations (mean ± SD) of *S*-adenosylmethionine (SAM) and *S*-adenosylhomocysteine (SAH) in the livers of hamsters: a, $P<0.01$; b, $P<0.05$; c, untreated hamsters not receiving As_2O_3.

Oil/carbon tetrachloride-treated hamsters that had received arsenic trioxide had SAM concentrations significantly lower than those in the olive oil-treated or untreated hamsters. Because the SAM and SAH concentrations do not decrease with administration of arsenic trioxide alone, SAM may have exerted a specific effect on the methylation of inorganic arsenic in hamsters with cirrhosis. When SAM participates in *in-vivo* methylations, SAH is formed. The decreased SAH concentration in the liver of oil/carbon tetrachloride and then arsenic trioxide-treated hamsters (Fig. 2) suggests a metabolic pathway for SAM which is different from its primary metabolic pathways. No correlation between decreases in SAM concentrations and decreases in SAH concentrations in the liver were observed.

The hamsters treated with olive oil for 20 and 30 weeks (but not those treated for only 15 weeks) and then with arsenic trioxide showed a significant increase in the urinary excretion of methylated arsenic compounds compared with controls (Fig. 1). A similar decrease of SAM concentration in the oil/carbon tetrachloride/arsenic trioxide-treated groups was coupled with an increased excretion of methylated arsenic species. Thus, methylation of inorganic trivalent arsenic is not inhibited in hamsters by drug-induced toxic liver cirrhosis. The effect of other liver disease, such as viral hepatitis, on the methylation of inorganic arsenic is a topic for future studies.

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