Nutritional Sciences 9(1), February, 2006, pp.29-34

Effects of Copper, Zinc and Cadmium on the Recovery Pattern of Aryl Sulfotransferase IV Activity in Rats fed 2-Acetylaminofluorene Diet

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Purified rat liver aryl sulfotransferase IV (AST IV) was found to be inhibited *in vitro* by zinc, copper, cadmium and terbium. Among these four elements, zinc, copper and cadmium were all strongly inhibitory to the AST IV activity at very low concentrations (2.5 μM to 0.025 μM). In rat liver cytosol, zinc, copper and cadmium at 25 μM to 0.025 μM also decreased the AST IV activity to 50% of the controls. In order to assess the possible effects of these metals on the AST IV activity recovery pattern *in vivo*, studies on the relationship between these minerals and dietary 2-acetylaminofluorene were conducted. Total of forty rats were fed one of five diets for 6 weeks: diet 1, Control diet plus 2-acetlyaminofluorene (0.05%); diet 2, zinc-deficient diet plus 2-acetlyaminofluorene; diet 3, zinc-supplement diet plus 2-acetylaminofluorene; diet 4, copper-supplement diet plus 2-acetlyaminofluorene; diet 5, cadmium-supplement diet plus 2-acetylaminofluorene. Half of the rats from each diet were changed to individual diet after 3 weeks of 2-acetylaminofluorene feeding. Placement of rats on the control diet following one cycle of 2-acetylaminofluorene feeding of 3 weeks without 2-acetylaminofluorene resulted in nearly full recovery of AST IV activity within 3 or 4 weeks. However, the rats fed diets that supplemented with zinc, copper or cadmium without 2-acetylaminofluorene showed a new pattern of lowered AST IV activity as early as the first cycle. Also, lowering in cytosolic AST IV contents was appeared in the livers from the rats, following one cycle of 2-acetlyaminofluorene feeding of 3 weeks, fed one of the diets that supplemented with copper, cadmium or zinc without 2-acetylaminofluorene for ensuing 3 weeks.

Key words: Aryl sulfotransferase IV, 2-Acetlyaminofluorene, Copper, Cadmium, Zinc, Recovery pattern

Received December 14, 2005; Revised February 9, 2006; Accepted February 22, 2006

INTRODUCTION

The adminstration of 2-acetylaminofluorene (2-AAF) to rats has been used as an experimental model in the study of hepatocelluar carcinogenesis.[1-4] Liver aryl sulfotransferase (AST IV) activity has been shown to play an important role in the metabolic activation of 2-AAF to highly reactive cytotoxic and genotoxic form.[5-16] In previous reports, liver cytosolic AST IV activity was shown to be rapidly lowered to 10~15% when rats were fed a diet containing 2-AAF.[7-16,17-19] In addition, the analysis of cytosolic AST IV activity gave a pattern of decreased AST IV activity throughout the five-cycle regimen of 2-AAF adminstration. Placement of rats, following one, two, or three cycles of 2-AAF exposure, on control diet without 2-AAF resulted in nearly full recovery of AST IV activity within 3 or 4 weeks. However, the rats that completed four or five-cycle of 2-AAF adminstration showed a new pattern of persistently lowered aryl sulfotransferase activity. In the five-cycle 2-AAF feeding, rats attained a high risk for developing liver cancer.

Although, in previous data, the inhibitory effects of copper and zinc on N-Hydroxy-2-AAF sulfotransferase have been shown *in vitro*, no data are available for *in vivo* or *in vitro* metals effect on AST IV. The work reported here focused on the role of these metals in the inhibition on the recovery pattern of AST IV after feeding rats with 2-AAF diet for three weeks. Data presented here also showed that high level of metals in diets caused inhibitory effects in the recovery pattern of AST IV activity as early as the first cycle.

Furthermore, the studies were designed to estimate AST IV levels in liver cytosol. As shown here, the AST IV level in liver cytosol from rats of each corresponding

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diet showed the same pattern to that of the AST Ⅳ activity. In addition, the study presented here was to compare zinc deficient diet with control diet in the aspect of AST Ⅳ recovery pattern.

MATERIALS AND METHODS

1. Animals and Dietary Protocols

Male Sprague-Dawley rats (8 weeks, SASCO, Inc., Omaha, NE) were fed one of the following diets and free access to water. The basal diet and zinc-deficient diets were purchased from U.S. Biochemical Corp. (Cleveland, OH).

Male Sprague-Dawley rats (8 weeks) were fed one of five diets for 6 weeks:

1. Control diet+2-AAF (0.05%)
2. Zinc-deficient diet+2-AAF
3. Zinc-supplement diet+2-AAF
4. Copper-supplement diet+2-AAF
5. Cadmium-supplement diet+2-AAF

Half of the rats from each diet were changed to individual diet after 3 weeks of 2-AAF feeding. All rats were housed in stainless steel cages and fed 15 g of assigned diet each day throughout the experimental period. Rats were killed after 6 weeks of feeding by cervical dislocation after being inhaled with CO_2. Liver from each rat was removed rapidly and used for enzyme assay and mineral analysis.

2. Chemicals

p-Nitrophenylsulfate, PAP, zinc acetate, cupric sulfate and cadmium chloride were purchased from Sigma Chemical Co. (St. Louis, MO). The N-OH-2-AAF was supplied by Dr. Robert A Froyd (Associate Member, OMRF, Oklahoma City, Oklahoma). 2-Naphthol was purchased from Aldrich Chemical Co. (Milwaukee, WI). All solvents and acids were analytical grade and were obtained from Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). PAPS was purchased from Dr. Sanford Singer, Dept. of Chemistry, University of Dayton, Dayton, OH. Rabbit anti-serum to albumin was purchased from Cappell Co., Westchester, PA.

3. Sulfotransferase Assay

N-OH-AAF sulfotransferase activity was measured as descrby Mulder *et al.*[20] In this assay, *p*-nitrophenylsulfate was used as a sulfate donor in the phenol sulfotransferase catalyzed conversion of PAP to PAPS. The rate of sulfation was monitored spectrometrically by following the accumulation of *p*-nitrophenol (450 nm) that paralleled the formation of the N-OH-AAF sulfate ester. A typical mixture contained in 0.5 mL: 100 mM tris (Ph 8.0), 10 mM *p*-nitrophenylsulfate, 20 μM PAP, 0.5 mM N-OH-AAF, 5% (v/v) ethanol, and 300 μg protein from cytosol at 405 nm was continuously monitored with a Gilford 250 spectrophotometer during a 10 min incubation at 31 ℃.

Sulfotransferase activity was expressed as nmoles of *p*-nitrophenol released per min per mg cytosol protein. Another method, in which 2-napthol was used as a substrate, was also monitored to measure the activity of sulfotranferase.[21]

4. Sample Digestion and Mineral Analysis

Glassware used in the analysis for minerals (copper and zinc) were cleansed in 3.2 N nitric acid (for 24 h) and rinsed at least five times with distilled deionized water. For the analysis of cytosol and tissues, samples were placed in Erlenmeyer flasks and 2~4 mL of 12 N nitric acid was added. Samples were digested (100 ℃) and then evaporated and diluted with water to appropriate volumes. Mineral levels were determined by atomic absorption spectrophotometry (Perkin-Elmer, Model 306, Norwalk, CT).

5. Electroblotting and Immunodetection (Western-blot) of AST Ⅳ

Analysis of liver cytosols for AST Ⅳ and albumin content was performed by electrophoresis, electroblotting, and immunochemical detection with antibodies to AST Ⅳ and albumin as previously described by Ringer *et al.*.[17]

Briefly, 4 μg cytosolic fracions were elctrophoresed using a Phast System single-dimension 10~15% gradient polyacrylamide gel with SDS-buffer strips from Pharmacia LKB Biotechnology, Inc. (Piscataway, NJ). Proteins from the Phast system gels were then electrophoretically transferred from the gels to nitrocellulose paper with the aid of Trans-Blot apparatus (model 250, Bio-Rad, Richmond, CA). The nitrocellulose was then immunochemically developed by the initial 2 h incubation with antiserum to AST Ⅳ (diluted 1:1,000), of antiserum to albumin (diluted 1:10,000), followed by binding of peroxidate-conjugated anti-rabbit IgG as the secondary antibody. Positive signals, localized as purpose bands on the filter paper, were generated by a peroxidase-catalyzed reaction between hydrogen peroxide and 4-chloro-1-naphthol. The stained papers were scanned at 633 nm (Pharmacia LKB Lase Densitometer) and the band intensities integrated as absorbance units/um were used as index of AST Ⅳ and albumin levels in liver cytosols.

RESULTS

Average body weight for control, zinc-supplemented, copper-supplemented, and cadmium-supplemented rats, after 3 weeks feeding of AAF, ranged from 240 to 280 g at the end of experiment (shown in Table 1): in contrast, the average weight of continuous AAF supplemented rats ranged 195 to 239 g. Twenty five to seventy percent of each AAF supplemented rats died by the week 6 except the rats of cadmium-supplement +AAF group; none of the rats in other groups died. It was especially notable that all of the rats of cadmium-supplemented +2-AAF group were alive at the end of the experiment (Table 1). Dietary copper and cadmium had little effect on tissue and cytosol levels of zinc and copper. 2-AAF in the diet also showed no effect on metal accumulation in the liver. As expected, the level of copper in liver and liver cytosol was abnormally high in copper-supplement rats. However, it was unusual that zinc supplementation did not cause a significant elevation in the level of zinc in liver tissue or cytosol ; i. e., there was often an elevation in tissue levels of zinc when rats fed a diet containing 100~1,000 mg Zn/kg.[22]

1. Dietary Effects of Zinc, Copper and Cadmium on AST Ⅳ Activity Recovery Patterns.

As in the previous study[19,36], liver cytosolic AST Ⅳ activity was shown to be rapidly lowered to 10~15% of the normal level when rats were fed a diet containing 2-AAF for more than 3 weeks. This lowering of AST Ⅳ activity appeared to be fully reversible when rats fed 2-AAF for 3 weeks were placed on the control diet without 2-AAF for 3 weeks (Fig. 1 and 3).[36] Rats fed a diet containing 2-AAF and one of these metals for three weeks also showed a rapidly lowered AST Ⅳ activity. However, unlike rats in control diet, rats placed on zinc, copper or cadmium supplement diet for 3 weeks after 3 weeks' of AAF administration did not show a fully recovered AST Ⅳ activity. Furthermore, these lowered recovery pattern appeared as early as the first cycle. Copper, zinc and cadmium showed the strongest inhibitory effects on the recovery pattern.

Table 1. The relationship of dietary zinc, copper and cadmium with AAF on the AST Ⅳ activity and survival.

Diet and supplement	Amount added	Average weight (g) At 6 weeks		Liver		Cytosol		Survival	AST Ⅳ activity[4]
		Rat	Liver	Cu	Zn	Cu	Zn		
	(μg/g diet)			(μg/g fresh tissue)		(μg/mL cytosol)			(% of control)
Control +AAF[1]		279.3± 3.9	11.3±0.3	3.2±0.1	28.8±1.8	1.2±0.0	3.7±0.1	4(4)[3]	100
Zn	50								
Control +AAF[2]		239	7.9	2.9	25.7	0.7	2.4	1(4)	6
Zn	50								
Zinc-deficient +AAF[1]		245.8± 3.6	10.6±0.9	3.2± 0.3	20.9±1.3	0.8±0.1	2.5±0.2	4(4)	27
Zn	<2								
Zinc-deficient +AAF[2]		217.7± 1.8	7.7±0.4	2.8± 0.1	18.6±0.5	0.8±0.1	2.0±0.3	3(4)	9
Zn	<2								
Zinc-supplement +AAF[1]		241.3± 2.8	8.3±0.3	3.0± 0.2	31.1±0.1	0.7±0.1	3.3±0.5	4(4)	47
Zn	2,000								
Zinc-supplement +AAF[2]		195.5± 7.8	7.0±0.5	3.1± 0.1	23.8±3.8	0.6±0.1	2.8±0.1	2(4)	6
Zn	2,000								
Copper-supplement AAF[1]		280.5± 5.3	10.9±0.3	96.2±44.9	28.5±0.6	5.0±0.0	3.4±0.1	4(4)	40
Zn	50								
Cu	1,000								
Copper-supplement +AAF[2]		218.0±12.7	8.7±0.8	84.9±42.9	21.2±0.8	4.2±0.1	2.6±0.7	3(4)	12
Zn	50								
Cu	1,000								
Cadmium-supplement +AAF[1]		263.0± 2.4	10.1±0.3	3.1± 0.3	28.9±5.0	0.5±0.1	4.4±0.2	4(4)	75
Zn	50								
Cd	250								
Cadmium-supplement +AAF[2]		229.0± 2.3	8.0±0.1	3.1± 0.3	30.0±1.6	0.7±0.1	3.1±0.1	4(4)	11
Zn	50								
Cd	250								

1) Fed AAF (0.05%) for 3 weeks and changed to individual diet for 3 weeks
2) Fed AAF (0.05%) for 6 weeks
3) Number of animals
4) p-Nitrophenylsulfate was used as a substrate

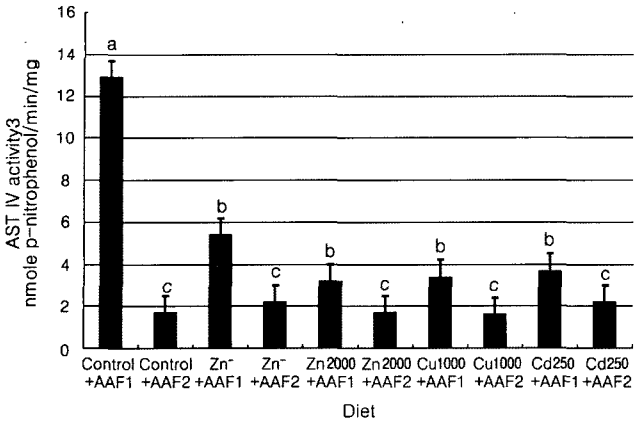

Fig. 1 Effect of dietary metal ions on liver cytosolic sulfotransferase activity of rats during the recovery from 2-AAF administration (bar graph containing 2-napthol assay data).

Recovery pattern following 6 weeks of treatment was conducted as following 1 and 2 patterns:
1. Fed AAF for 3 weeks and changed to individual diet for 3 weeks (recovery)
2. Fed AAF for 6 weeks.

Data were analyzed statistically using the SPSS program.
Values with different small letters on the bars are significantly different at 5% level

2. Effects of Zinc, Copper and Cadmium on Cytosolic AST Ⅳ Levels.

The AST Ⅳ content in liver was high when rats that had been fed 2-AAF for 3 weeks and changed to control diet for 3 weeks (Fig. 2 and 3). However, there was no recovery of AST Ⅳ level in rats fed 2-AAF diet continuously for 6 weeks. These results had similar patterns to those in the previous reports in which the same level of 2-AAF had been used.[19] Lowering in cytosolic AST Ⅳ contents was found in liver from rats fed 2-AAF with individual element for 6 weeks. Particularly, lowered AST Ⅳ levels shown in the liver from rats that were placed on control plus individual element diet for 3 weeks after feeding with 2-AAF for 3 weeks indicated that changes in the amount of AST Ⅳ were closely paralleled with changes in AST Ⅳ activity during the recovery.

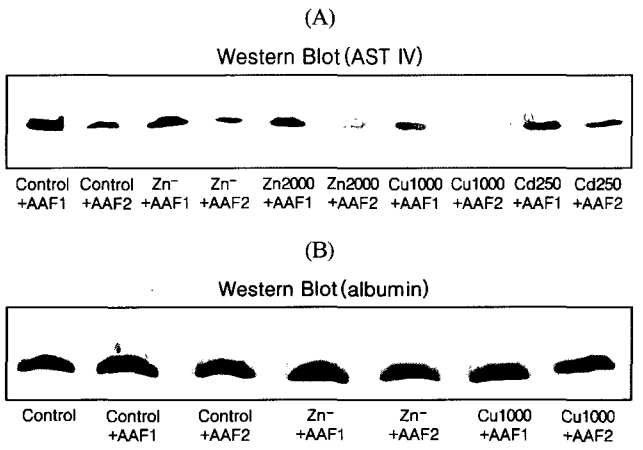

Fig. 2 Immunochemical determination (Western-blot) of relative AST Ⅳ and albumin contents in the cytosols of rats administered Zn, Cu, or Cd metal ions during recovery from 2-AAF administration.

Western-blot immunodetections of AST Ⅳ (A) and albumin (B) levels were performed as described in Material and Methods.
1. Fed 2-AAF for 3 weeks and changed to individual diet for 3 weeks.
2. Fed 2-AAF for 6 weeks.

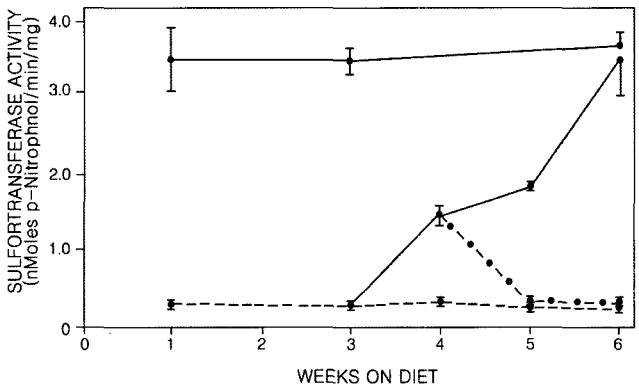

Fig. 3 Recovery of liver AST activity in rats fed AAF diet for 3 weeks then control diet.

Rats were fed AAF diets (---) for 3 weeks then either continued on AAF diet or changed to control diet (—) for another 3 weeks. Some rats were fed 1 cycle of 3 weeks on AAF diet, 1 week on control diet and changed back to AAF diet for 2 weeks (• - •). Rats fed only control diet were also examined. AST activities of the respective rat liver cytosols were determined as described in Materials and Methods.
Each data point represents the mean and S.E.M. for the values of 6 rats/point. From Ringer *et al.*(36)

3. *In vitro* Inhibition of Zinc, Copper, Cadmium and Terbium on AST Ⅳ Activity.

Fig. 4 depicted the effects of these metals on AST Ⅳ activity (A) purified and (B) in cytosol. Zinc, copper

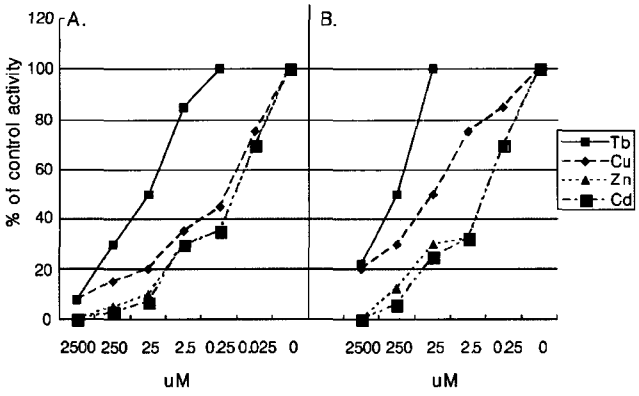

Fig. 4 Inhibition of AST Ⅳ activity by the divalent cations of Tb, Zn, Cu and Cd.

Purified AST Ⅳ (A) and rat liver cytosols (B) were prepared as described in Materials and Methods. PAPS-dependent sulfotmasferase activity was determined by the 2-naphthol assay.
The results are expressed relative to the control without addition of the metal ions.

and cadmium inhibited AST IV activity (purified or in cytosol) at very low concentrations. Terbium was also inhibitory at higher concentrations than these metals. These observations were consistent with previous reports that in which of all the divalent cations (Mg, Mn, Co, Ni, Cu, and Zn) tested, both copper and zinc were strongly inhibitory to N-Hydroxy-2-acetylaminofluorene sulfotransferase at very low concentration.[23]

DISCUSSION

It was demonstrated that among the divalent cations, specially zinc and copper showed strong inhibitory effects at very low concentration in purified N-Hydroxy-2-acetylaminofluorene Sulfotransferase activity.[23] Thus, zinc and copper were purposely chosen to study the inhibitory effects on AST IV activity of purified enzyme or in cytosol. Furthermore, *in vivo* study on the effects of these metals in rats was conducted. Hill and Matrone[24] reported that those elements whose physical and chemical properties are similar will act antagonistically to each other biologically. For example, adversed effects, such as anemia, caused by high levels of zinc in the basal diet could be overcame by the addition of copper to the diet.[1,9,12] Another example of such relationship is the finding that symptoms of cadmium toxicity could be overcome by the addition of zinc to the diet.[25,26] In addition, many other examples of interrelationship have been demonstrated.[27-35]

Accordingly, cadmium was also introduced to study of these metal's inhibitory effects on AST IV activity because cadmium has common chemical parameters with these metals.[24] As expected, in the present work, cadmium also showed the inhibitory effects on AST IV *in vitro* and *in vivo* (Fig. 1 and Fig. 4). Terbium that has the same valence electrons with these metals also had been chosen for the purpose of these studies *in vitro*. *in vivo* studies. Terbium was not involved because its *in vitro* inhibitory effects on AST activity was much less than those of zinc, copper and cadmium.

In recovery patterns, same as a previous study,[19] the lowered AST IV activity in liver cytosol from the rats fed a diet containing 2-AAF for 3 weeks was fully recovered when rats that had been fed 2-AAF were placed on control diet for 3 weeks (Fig 2). Ringer *et al.*[17,18,36] reported that liver cytosolic N-OH-2-AAF sulfotransferase activity was shown to be rapidly lowered to 10~15% of the normal level when rats were fed a diet containing 2-AAF. They also showed that this lowering of AST IV activity was found to be fully reversible when rats that had been fed 2AAF for 1 to 3 weeks were placed on the control diet without 2-AAF for several weeks.[19] Furthermore, placement of rats on the control diet without 2-AAF following one, two, or three cycles of 2-AAF feeding resulted in nearly full recovery of AST IV activity within 3 or 4 weeks. However, when rats completing four or five cycles of 2-AAF administration were placed on the control diet without 2-AAF for 4 weeks, a new pattern of continuously lowered AST IV activity was observed.[36] The data presented here demonstrated that high dietary zinc, copper and cadmium showed inhibitory effects on the recovery pattern of AST IV activity of the rats. As shown in Fig. 1, when rats fed 2-AAF diet for 3 weeks were placed on the control containing zinc, copper or cadmium individually showed lowered AST IV activity compared to that of rats placed on the control diet for 3 weeks after 3 week's of 2-AAF feeding. This new pattern of lowered AST IV appeared as early as the first cycle by the administration of one of these metals.

Cytosolic levels of AST IV detected by Western blot immunochemical detection indicated that lowering of AST IV activity was parallel to the overall lowering of AST IV activity by individually adding one of these metals (Fig. 2). Further study of this process could yield important information concerning the inhibitory mechanisms of these metals on AST IV activity.

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