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Combined Effect of Dietary Cadmium and Benzo(a)pyrene on Metallothionein Induction and Apoptosis in the Liver and Kidneys of Bank Voles

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Abstract Bank voles free living in a contaminated environment have been shown to be more sensitive to cadmium (Cd) toxicity than the rodents exposed to Cd under laboratory conditions. The objective of this study was to find out whether benzo(a)pyrene (BaP), a common environmental co-contaminant, increases Cd toxicity through inhibition of metallothionein (MT) synthesis—a low molecular weight protein that is considered to be primary intracellular component of the protective mechanism. For 6 weeks, the female bank voles were provided with diet containing Cd [less than 0.1 μg/g (control) and 60 μg/g dry wt.] and BaP (0, 5, and 10 μg/g dry wt.) alone or in combination. At the end of exposure period, apoptosis and analyses of MT, Cd, and zinc (Zn) in the liver and kidneys were carried out. Dietary BaP 5 μg/g did not affect but BaP 10 μg/g potentiated rather than inhibited induction of hepatic and renal MT by Cd, and diminished Cd-induced apoptosis in both organs. The hepatic and renal Zn followed a pattern similar to that of MT, attaining the highest level in the Cd+BaP 10-μg/g group. These data indicate that dietary BaP attenuates rather than exacerbates Cd toxicity in bank voles, probably by potentiating MT synthesis and increasing Zn concentration in the liver and kidneys.

Keywords Apoptosis · Benzo(a)pyrene · Cadmium · Metallothionein · Zinc

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Introduction

Cadmium (Cd) is a toxic metal that is present throughout the environment, and in humans and animals accumulates primarily in liver and kidneys [1, 2]. Chronic Cd exposure can result in liver injury including nonspecific inflammation and apoptosis [3, 4], and in kidneys, the metal produces tubular degeneration, apoptosis, interstitial inflammation, and glomerular swelling [5, 6]. Noteworthy, wild animals inhabiting polluted sites are more sensitive to Cd toxicity than animals subjected to Cd exposure under laboratory conditions. For instance, in laboratory rodents, renal or hepatic injury occurs when the Cd concentration exceeds 50 μg/g wet wt. [3–5, 7]; in contrast, in the wildlife such as roe deer, Algerian mice, yellow-necked mice, bank voles, white-toothed shrew, and magpies, the injury occurs at the Cd levels lower than 25 μg/g wet wt. [8–13]. However, the reason for this difference in susceptibility to Cd toxicity is not known and remains to be determined.

It is known that susceptibility to Cd toxicity increases in animals that are unable to synthesize metallothionein (MT), a low molecular weight protein that is induced by and bound to the metal, and is considered to be a primary component of acquired tolerance to toxic effects of Cd [14, 15]. Sensitivity to Cd toxicity is also enhanced by some organic environmental co-contaminants through the reduction of tissue MT levels [16]. Thus, an appropriate amount of MT is required to provide protection against Cd-induced tissue injury. When the content of Cd in the liver and kidneys exceeds the binding capability of MT, the non-MT-bound Cd ions are believed to cause hepato- and nephrotoxicity [7, 17].

Among environmental co-contaminants polycyclic aromatic hydrocarbons (PAH), including benzo(a)pyrene (BaP), are distributed along with metals such as Cd in the

environment [18–20]. BaP exposure has been associated with carcinogenesis as well as with reproductive, hematopoietic, hepatic, and renal abnormalities in both humans and experimental animals [21]. Importantly, BaP has been shown to increase acute Cd toxicity in a fish *Fundulus heteroclitus*, probably through inhibition of MT synthesis in the liver [22]. However, it is unknown whether BaP also inhibits Cd induction of MT in the liver and kidneys of chronically exposed mammals, and whether this effect (if any) is responsible for the enhancement of Cd toxicity.

Therefore, in the present work, we examined the effect of dietary BaP on Cd induction of MT in the liver and kidneys of a small rodent, the bank vole (*Myodes* (=*Clethrionomys*) *glareolus*), which appeared to be vulnerable to Cd toxicity when free living in a contaminated environment [10]. Concurrently, the toxicity was evaluated by measuring liver and kidney histopathology and apoptosis. Because orally administered Cd can affect tissue zinc (Zn) and the metal appears to protect against Cd-induced toxicity [23–25], the concentration of this element was also determined.

Materials and Methods

Animals and Experimental Design

Female bank voles (1 month old, weighing 11–13 g), being the F_1 offspring of the wild-caught population (Knyszyn Old Forest, northeastern Poland), were used throughout the study. The bank voles were randomly assigned into six groups (n=8 each) according to dietary Cd and BaP: (1) control, (2) BaP 5 μg/g, (3) BaP 10 μg/g, (4) Cd 60 μg/g, (5) Cd 60 μg/g+BaP 5 μg/g, and (6) Cd 60 μg/g+BaP 10 μg/g dry wt. The animals were housed for 6 weeks individually in stainless steel cages (lined with peat as absorptive material) at 18–20°C on 12 h light/dark cycle and at 50–70% relative humidity. They received ad libitum distilled water and control or Cd- and BaP-containing whole wheat grains, which appeared to be an adequate quality food for these rodents [4]. In addition, an identical quantity of apple was offered to all voles (3 g/vole/week), who ate it completely. The food intake was monitored throughout the experiment. Prior to the experiment, the grains were contaminated with Cd (soaked in $CdCl_2$ solution) [4] and then 1 kg mixed with 10 mL of corn oil containing 0, 5, or 10 mg BaP (Sigma). Atomic absorption spectrophotometry (AAS) analysis of the grains revealed that actual levels of Cd were between 58 and 63 μg/g (Cd groups) and less than 0.1 μg/g dry wt. (control). The chosen concentration of dietary Cd was twofold higher than that observed in a heavily contaminated environment [26], and it did not induce histopathological changes in voles raised under laboratory conditions [4]. The chosen concentrations of dietary BaP were low and environmentally relevant [21]. All experimental procedures were approved by the local ethical committee (Medical University of Białystok) and were compatible with the standards of the Polish law on experimenting on animals, which implements the European Communities Council Directive (86/609/EEC).

At the end of the 6-week exposure period, the bank voles were weighed and killed by decapitation, and the liver and kidneys were removed, rinsed in cold saline, and blotted dry on absorbent paper. The blood was also taken to determine hemoglobin and hematocrit by using standard methods (spectrophotometrically as cyanmethemoglobin at 540 nm and hematocrit centrifuge, respectively). A portion of the fresh liver (0.25 g) and one kidney were transferred to 1.0 mL chilled 0.25 M sucrose and homogenized with a Teflon pestle in a glass homogenizer. An aliquot (0.5 mL) of the homogenate was taken for determination of metal concentrations. The remaining homogenate was centrifuged at 20,000×g for 20 min at 4°C, and the resulting supernatant was removed for MT assay.

Metallothionein Determination

MT in the liver and kidneys was determined by a Cd saturation method [23]. Briefly, a 0.1-mL sample was incubated in a 1.5-mL vial for 10 min at room temperature with 1.0 mL Tris–HCl buffer (0.03 M, pH 7.8) containing 1.0 μg Cd/mL. To remove non-MT-bound Cd, bovine hemoglobin (Sigma) (0.1 mL of a 5% solution in H_2O) was added, and the sample was heated for 1.5 min at 95°C, cooled, and centrifuged for 5 min at 10,000×g. Addition of hemoglobin, heating, and centrifugation of the sample was repeated three times. Cd bound to MT in the resulting clear supernatant was determined by electrothermal AAS. MT content was expressed in micrograms of the protein per gram of wet tissue, assuming that 1 mol of MT (6600) binds 7 mol of Cd.

Cadmium and Zinc Determination

Metal determinations were performed as described previously [23]. The homogenate (0.5 mL) was placed in a glass tube with 2.0 mL of concentrated nitric acid. After 20 h of sample digestion at room temperature, 72% perchloric acid (0.5 mL) was added and the mixture was heated at 100°C for 3 h. Finally, the temperature was raised to 150–180°C and digestion continued for another 4 h. Deionized water was added to the residue after digestion to a volume of 3.0 mL (first solution). A portion of the first solution (200 μL) was evaporated to dryness in a quartz crucible at 130°C, and the residue was redissolved in an appropriate amount of deionized water (second solution). Cd analyses of these solutions were carried out by electrothermal AAS using a Solaar M6 instrument with a Zeeman correction.

The concentration of Zn in the first solution was determined by AAS in an air–acetylene flame with a deuterium correction. Quality assurance procedures included the analysis of reagent blanks and appropriate standard reference material (NIST bovine liver 1577b). The recovery of Cd was 91–93% and that of Zn was 90–95%. The analytical detection limit for Cd was 0.02 μg/g and that for Zn was 0.5 μg/g.

Histological Examination

A portion of the liver and one kidney from each animal were fixed in 4% formaldehyde, dehydrated in ethanol and xylene, embedded in paraffin, cut into 5-μm sections, and stained with hematoxylin and eosin for microscopic examination. Apoptosis was demonstrated in situ by the TUNEL (TdT-mediated dUTP-fluorescein nick end labeling) assay, using a kit from Roche Diagnostics (Mannheim, Germany) according to their instructions [13]. The numbers of apoptotic cells were determined in 10 random microscopic fields for each vole, using a ×40 objective, and apoptosis was expressed as the mean of the number of apoptotic cells per microscopic field. A photomicrograph showing TUNEL-positive cells is presented in Fig. 1.

Statistical Analysis

Data were expressed as means±SD. The values were analyzed by two-way analysis of variance (ANOVA) followed by the Duncan's multiple range test (SPSS 14.0). Differences at $P<0.05$ were considered statistically significant.

Results

Dietary Cd (60 μg/g) and BaP (5 and 10 μg/g) alone and in combination did not affect significantly the food intake (0.14–0.17 g/g body wt./day), the final body and organ weights, as well as the blood hemoglobin and hematocrit levels in the female bank voles (Table 1). In general, Cd accumulation, MT induction, Zn concentration, and apoptosis in the liver and kidneys were affected significantly by dietary Cd and/or BaP at the concentration of 10 μg/g (BaP-10) but not at 5 μg/g (BaP-5; Tables 2 and 3).

Cd accumulation in the liver was significantly influenced only by dietary Cd (Table 2), while renal Cd was affected by dietary Cd, and interaction between Cd and BaP (Table 3). Notably, renal Cd in the Cd+BaP-10 bank voles was significantly higher than that in the animals exposed only to dietary Cd.

MT induction in the liver and kidneys was significantly affected by dietary Cd, and interaction between Cd and BaP (Tables 2 and 3). The hepatic and renal MT in the Cd+BaP-10 bank voles was significantly higher (by about 50%) than that in rodents exposed only to dietary Cd (Tables 2 and 3). Assuming that 1 mol of MT (6600) binds 7 mol of Cd, the Cd-binding capacity of hepatic and renal MT in the Cd+

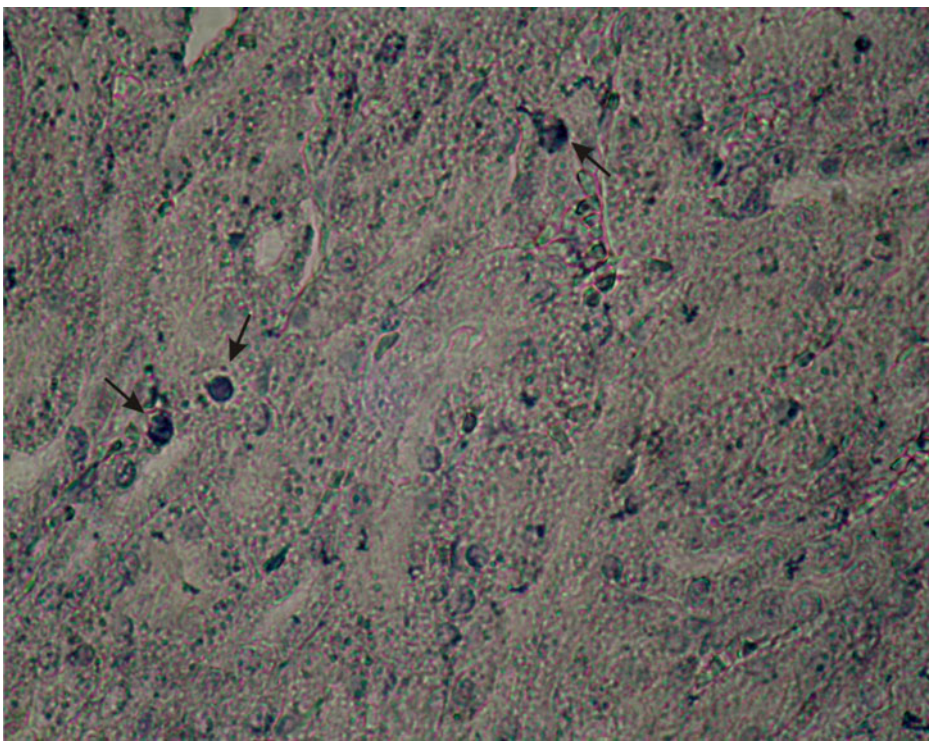

Fig. 1 Immunohistochemical demonstration of apoptotic cells (*arrows*) in the kidney of bank vole exposed to dietary Cd (TUNEL assay). Magnification ×400

Table 1 Body and organ weights, and hematological values in the female bank voles exposed to dietary cadmium (Cd) and/or benzo(a)pyrene (BaP)

Group	Body mass (g)	Liver mass (mg)	Kidneys mass (mg)	Hemoglobin (g/100 mL)	Hematocrit (%)
Control	14.8±0.9	645±81	168±11	15.3±1.1	47.5±2.4
BaP-5	14.6±0.8	642±73	164±10	15.7±1.3	47.9±2.1
BaP-10	14.5±0.9	644±100	162±10	16.9±2.1	49.7±2.0
Cd	14.5±1.0	627±114	166±16	15.1±1.2	49.2±1.9
Cd+BaP-5	14.7±0.9	650±85	164±11	15.3±1.0	48.5±2.2
Cd+BaP-10	14.8±0.6	662±72	162±9	15.0±1.2	51.0±2.5

Values represent the mean±SD for n=8. Bank voles received, for 6 weeks, control diet or diets containing 60 μg Cd/g and/or 5 and 10 μg BaP/g. There were no statistically significant differences between the groups

BaP-10 bank voles exceeded the total concentration of Cd in the liver and kidneys by about 18 and 13 μg Cd/g, while in the Cd alone and Cd+BaP-5 animals only by about 6 and 2 μg Cd/g, respectively.

The hepatic and renal Zn was also significantly influenced by dietary Cd, and interaction between Cd and BaP (Tables 2 and 3). The tissue Zn followed a pattern similar to that of MT concentration, attaining the highest value in the Cd+BaP-10 voles.

Neither dietary Cd and BaP alone nor in combination induced histopathological changes in the liver and kidneys of bank voles (not shown); however, dietary Cd alone and in the presence of BaP-5 increased 9–10 times the numbers of apoptotic cells in both organs, and co-treatment with BaP-10 apparently diminished Cd-induced apoptosis (Tables 2 and 3).

Discussion

It has been previously shown that small mammals, including bank voles free living in a polluted environment, are more sensitive to Cd toxicity than the animals exposed to the metal under laboratory conditions [10]. Based on the literature data [22], we hypothesized that BaP would inhibit Cd induction of MT, thereby enhancing its toxicity in the bank vole. It is well known that hepato- and nephrotoxicity can occur when the tissue Cd exceeds the Cd-binding capacity of intracellular MT [14, 15]. However, the present work demonstrated that Cd induction of hepatic and renal MT increased upon co-exposure to dietary Cd and BaP (Tables 2 and 3), and no increase of Cd toxicity measured by histopathology and apoptosis occurred. Thus, these results suggest that BaP cannot be responsible for the enhancement of Cd toxicity in the bank voles free living in a polluted environment. Recently, we have shown that polychlorinated biphenyls also fail to increase hepato- and nephrotoxicity of Cd in these rodents [27]. It cannot be ruled out, however, that other organic and inorganic (Pb, As) co-contaminants present in natural environmental as well as some ecological factors, e.g., food shortage, bad weather or social stress would make the free-ranging animals more susceptible to Cd intoxication compared to those kept in laboratory conditions [8, 10].

The precise mechanism for potentiation of Cd-induced MT synthesis by BaP in the bank vole is not known. Recently, Roesijadi et al. [28] revealed that dietary BaP also potentiates induction of intestinal MT mRNA by Cd in a fish *F. heteroclitus*. The authors suggest that the potentiation by BaP may lie in interaction at the promoter of MT gene,

Table 2 Cadmium, metallothionein and zinc concentrations as well as apoptosis in the liver of female bank voles exposed to dietary cadmium (Cd) and/or benzo(a)pyrene (BaP)

Group	Cadmium (μg/g wet wt.)	Metallothionein (μg/g wet wt.)	Zinc (μg/g wet wt.)	Apoptosis (apoptotic cells/field)
Control	0.14±0.20a	5.60±1.60a	23.5±1.9a	0.2±0.2a
BaP-5	0.13±0.10a	5.20±1.30a	22.8±2.1a	0.2±0.2a
BaP-10	0.10±0.10a	5.71±1.00a	23.0±2.0a	0.2±0.2a
Cd	19.6±6.1b	218±58b	30.5±5.0b	1.8±0.8b
Cd+BaP-5	20.1±4.0b	235±30b	32.5±4.6b	1.7±0.5b
Cd+BaP-10	20.7±2.9b	324±21c	41.2±5.5c	0.5±0.3a
Source of variation ANOVA: P values				
Cd	0.0000	0.0000	0.0000	0.0001
BaP	NS	NS	NS	NS
Cd×BaP	NS	0.0004	0.0050	0.0062

Values represent the mean±SD for n=8. Bank voles received, for 6 weeks, control diet or diets containing 60 μg Cd/g and/or 5 and 10 μg BaP/g. Means in the same column marked with a different letter are significantly different (P<0.05)

NS not significant

Table 3 Cadmium, metallothionein and zinc concentrations as well as apoptosis in the kidneys of female bank voles exposed to dietary cadmium (Cd) and/or benzo(a)pyrene (BaP)

Group	Cadmium (μg/g wet wt.)	Metallothionein (μg/g wet wt.)	Zinc (μg/g wet wt.)	Apoptosis (apoptotic cells/field)
Control	0.31±0.30a	11.2±3.3a	15.8±2.3a	0.1±0.1a
BaP-5	0.30±0.25a	13.1±4.0a	15.5±3.0a	0.1±0.1a
BaP-10	0.29±0.20a	14.2±2.8a	16.1±2.5a	0.1±0.1a
Cd	25.3±4.0b	228±39b	23.3±4.0b	1.0±0.5b
Cd+BaP-5	26.5±3.5bc	235±40b	24.1±5.0b	1.1±0.4b
Cd+BaP-10	29.5±2.0c	357±38c	32.1±6.0c	0.4±0.2a
Source of variation	ANOVA: *P* values			
Cd	0.0000	0.0000	0.0000	0.0003
BaP	NS	NS	NS	NS
Cd×BaP	0.0476	0.0028	0.0062	0.0173

Values represent the mean±SD for $n=8$. Bank voles received, for 6 weeks, control diet or diets containing 60 μg Cd/g and/or 5 and 10 μg BaP/g. Means in the same column marked with a different letter are significantly different ($P<0.05$)

NS not significant

where the signal transduction pathways for BaP and Cd can converge. Indeed, in the promoter, there are sequences for the metal response elements [29] as well as for the xenobiotic response element that binds aryl hydrocarbon receptor (AhR) activated by PAH [30]; thus, the potentiation might result from a direct interaction of AhR stimulated by BaP. It is worth noting here that fish differ from mammals in having not one but at least two AhRs [31], which may imply some differences in the interaction between BaP and Cd in the two groups of vertebrates. However, irrespective of the molecular mechanism, the higher levels of MT in the presence of BaP could have significant implications for protection against Cd toxicity, notably Cd-induced apoptosis.

It is important to point out that Cd induction of apoptosis in the liver and kidneys of bank voles exposed to dietary Cd alone occurred when the Cd-binding capacity of MT exceeded slightly the total concentration of Cd (Tables 2 and 3). This suggests that there was an appreciable amount of non-MT-bound Cd that could induce apoptosis. Indeed, previous studies demonstrated that approximately 30–50% of the total hepatic and renal Cd is not bound to MT even though the capacity of MT exceeds the Cd concentration, and that the fraction of non-MT-bound Cd decreases as the content of MT increases [4, 7]. Therefore, it may be assumed that the increase in Cd induction of MT by BaP could result in the binding of more Cd ions on the protein, thereby reducing non-MT-bound Cd and thus diminishing Cd-induced apoptosis (Tables 2 and 3). In support, resistance to Cd-induced apoptosis has been documented in liver cells overexpressing MT [32].

In this study, MT could also protect against Cd-induced apoptosis indirectly, through increasing the hepatic and renal Zn concentrations (Tables 2 and 3). It is well known that Zn plays an important role in preventing apoptosis and necrosis [23–25]. Specifically, this element inhibits caspase-3 (a key apoptotic protease) [33] which, in contrast, is activated by Cd [25]. Thus, it is reasonable to conclude that a substantial increase in the tissue Zn upon combined exposure to Cd and BaP could be responsible for the protection, probably through an inhibition of the enzyme. The hepatic and renal Zn increase most likely was related to MT capacity, especially to the binding sites not occupied by Cd (see "Results" section) which could sequester Zn ions. These data confirm an important role of MT in the tissue Zn accumulation in animals intoxicated by Cd [7, 23, 24].

Although MT appears to be an important protein in protecting Cd-induced apoptosis in the bank vole co-exposed to dietary Cd and BaP, it cannot be ruled out that also other factors, e.g., glutathione, which is known to provide protection against Cd toxicity [34], may have been implicated in this process. However, the total glutathione content was not affected by dietary Cd and/or BaP in the bank vole under study (data not shown), which may suggest that this compound could have only a negligible effect.

Another finding of the present study is the rise of Cd accumulation in the kidneys of bank voles by co-treatment with BaP. It has been shown that intestinal MT plays a significant role in the transport of Cd to the kidneys and any increase in the concentration of MT enhances formation of MT–Cd complex and its transport to this organ [35, 36]. Although we did not determine the concentration of intestinal MT in the bank vole, it cannot be excluded that the potentiation of Cd-induced MT synthesis by BaP also occurred in the intestine of these animals, resulting in the higher transport of the MT–Cd complex to the kidneys. Still, the exact role of dietary BaP in Cd disposition remains to be elucidated.

In conclusion, the data indicate that dietary BaP cannot be responsible for an increased susceptibility to Cd toxicity observed in the free-living bank voles. Conversely, dietary BaP attenuates Cd toxicity in these rodents probably by potentiating MT synthesis and increasing Zn concentrations in the liver and kidneys. The present study also indicates that evaluation of apoptotic rate is a more sensitive indicator of intoxication compared to routine histological analysis, and thus it should be included as an end point in toxicological studies.

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