

Cadmium action on aminopeptidase N activity and L-threonine intestinal transport in rabbit

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Summary — Cadmium has been recognized as an environmental contaminant. In oral cadmium intoxication, the immediate target organ is the gastrointestinal tract. The aim of the present work was to study the effect of cadmium on the intestinal absorption of L-threonine and on the aminopeptidase N activity in rabbit jejunum, after *in vitro* addition and/or oral administration of CdCl₂ in drinking water. Results obtained show that cadmium decreases L-threonine absorption in the jejunal tissue. This effect seems to be due to an action mainly on active amino-acid transport at the mucosal border of the intestinal epithelium, because cadmium does not seem to modify the amino-acid diffusion across the intestinal epithelium. Cadmium also inhibits the (Na⁺-K⁺)-ATPase activity of the enterocyte, which might explain the inhibition of the Na⁺-dependent L-threonine transport. Nevertheless, a direct action of the cadmium ion on the carrier of active transport cannot be rejected. Cadmium modifies the aminopeptidase N activity when administered in drinking water for 4 d.

cadmium / L-threonine / small intestine / rabbit

Résumé — Effet du cadmium sur l'absorption de la L-thréonine et l'activité de l'aminopeptidase N chez le lapin. Le cadmium est considéré comme un polluant de l'environnement. Il entre principalement dans l'organisme des animaux par voie orale. Dans l'intoxication par ce cation, le tractus gastro-intestinal est le premier affecté. Le but de ce travail a été de déterminer l'effet du cadmium sur l'absorption de la L-thréonine et sur l'activité de l'aminopeptidase N dans le jéjunum de lapin, après addition *in vitro* et/ou administration orale de CdCl₂ dans l'eau de boisson. Les résultats obtenus ont montré que le cadmium diminue le transport de la L-thréonine dans le jéjunum. Ce cation n'ayant pas modifié la diffusion de l'acide aminé dans l'épithélium intestinal, nous en avons conclu qu'il agit sur le transport actif de la L-thréonine. Par ailleurs, le cadmium a provoqué l'inhibition de l'activité (Na⁺-K⁺) ATPase de l'entérocyte, ce qui pourrait expliquer l'inhibition du transport Na⁺ dépendant de la L-thréonine dans la membrane de la bordure en brosse. Le cadmium a aussi induit une modification de l'activité de l'aminopeptidase N quand il a été administré aux animaux dans l'eau de boisson pendant 4 j.

cadmium / L-thréonine / intestin grêle / lapin

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INTRODUCTION

Cadmium is considered to have no biological function and is highly toxic, hence its release into the environment by anthropogenic activities as well as by natural processes poses a serious threat to humans and animals. Because of its bioaccumulation, it is an environmental contaminant of great concern. Cadmium causes several ailments affecting kidneys, lungs, cardiovascular system and bones (Jamall *et al*, 1989; Nordberg and Nordberg, 1988). Cadmium is predominantly taken up in both the human and animal body through food and water contaminated with the metal. In the digestive system this metal may cause intestinal paralysis, constipation, necrosis of the gastrointestinal epithelia and a decrease in the activity of alkaline phosphatase in the intestinal mucosa (Andersen *et al*, 1988a,b; Bevan and Foulkes, 1989; O'Brien and King, 1989).

It has been reported in many countries that the concentration of cadmium in foodstuffs is distributed over a wide range. Estimations of daily cadmium intake based either on data of cadmium concentration in a single food or on total-diet studies have also been made in several countries. In uncontaminated areas, the average daily intake of cadmium in humans is usually in the range of 10–60 µg/d (Elinder, 1985).

One toxic action of cadmium is located in the small intestine where nutrients are absorbed. Its absorption from the gastrointestinal tract is not controlled by any homeostatic mechanism (Cotzias *et al*, 1961; Schroeder and Balassa, 1961). In experiments with animals and humans, only a small portion of dietary cadmium is taken up by the intestinal mucosa, and less than 1–6% of the metal that enters the mucosa actually passes into the body (Hamilton and Valberg, 1974; Moore *et al*, 1973; Neathery and Miller, 1975). The great capacity of cadmium to bind itself to membranes and intra-

cellular ligands is the reason for its toxicity (Kello *et al*, 1979; Lerner *et al*, 1977).

Inhibition of intestinal absorption of nutrients by Cd²⁺ has been observed in amphibians (Tsuchiya and Okada, 1982), teleosts (Sastri and Subhadra, 1983), pigs, rats and rabbits (Bevan and Foulkes, 1989; Kojima *et al*, 1986; Mesonero *et al*, 1993; Rodríguez-Yoldi *et al*, 1989).

The aim of the present work was to study the action of cadmium on the intestinal transport of L-threonine and on enzymatic digestion (aminopeptidase N activity) in control animals and in animals pre-exposed to a supplementary diet of this metal. L-Threonine is an essential amino acid for rabbits (Lebas, 1987; Moughan *et al*, 1988; Schultze *et al*, 1988).

MATERIALS AND METHODS

Materials

L-Threonine and CdCl₂ were obtained from Sigma (St Louis, MO); L-[U-¹⁴C] threonine and [¹⁴C] polyethylene glycol were obtained from Amersham (Buckinghamshire). Liquid scintillation, formula 989 was obtained from Du Pont (Boston, MA).

Animals, incubation solutions

Handling, equipment used and the sacrifice of animals was in accord with the European Council legislation 86/609/EEC concerning experimental animal protection. Male New-Zealand rabbits weighing 1.5–2.0 kg, were maintained at a constant room temperature (24°C) with free access to water and standard rabbit fodder. Each animal was marked and weighed. Control animals were maintained under these conditions, and experimental groups (test animals) were treated with 20 ppm cadmium in drinking water for 4 d. These animals drank about 500 ml pre-treated water per d. In this experiment, cadmium was given in the form of CdCl₂.

After killing by a blow on the head, the proximal jejunum (5 cm distal to the ligament of Treitz) was removed and rinsed free of intestinal contents with ice-cold Ringer's solution. The tissue was then stripped of its serosal and external muscle layers. The Ringer's solution (KRT) contained in mmol/l: 127 NaCl, 10.18 KCl, 5.44 CaCl₂ and 15 Tris-HCl, and was continuously gassed with O₂/CO₂ (19:1 v/v). Cadmium was added as chloride. After addition of metal or other compounds, the pH was adjusted if necessary to 7.4.

Cell-water determinations

Pieces of tissue (rings of rabbit everted jejunum weighing about 100 mg) were incubated in Ringer's solution at 37°C containing 0.02 µCi/ml [¹⁴C] polyethylene glycol (mol wt 4 000, PEG 4 000, Amersham) for 20 min. The pieces of mucosa were gently blotted on humid filter paper and weighed, then extracted in 0.5 ml 0.1 mol/l HNO₃ overnight at 4°C. Aliquots of the extracts (200 µl) and aliquots of the bathing solutions (200 µl) were then counted in 2 ml liquid scintillation fluid. Following extraction from HNO₃, the tissues were dried at 80°C for 12 h and then reweighed. The test agents (1 mmol/l CdCl₂, 50 mmol/l L-threonine and 0.5 mmol/l L-threonine) were present in the bathing solution from the start of the incubation, and had no significant effect on either the tissue water fraction, calculated as the difference between wet and dry weights (ranging from 0.870 ± 0.003 to 0.872 ± 0.004 ml/g wet weight), or on the extracellular space, calculated by PEG 4 000 (ranging from 0.058 ± 0.005 to 0.068 ± 0.006 ml/g wet weight) or on the cell water fraction, calculated as the difference between tissue and extracellular water fractions (ranging from 0.803 ± 0.004 to 0.812 ± 0.003 ml/g wet weight).

Amino-acid uptake measurements

The experiments were made with rings of rabbit everted jejunum weighing about 100 mg, which were incubated at 37°C in 10 ml Ringer's solution containing 0.01 or 0.04 µCi/ml L-[U-¹⁴C] threonine (according to amino-acid concentration) plus unlabelled L-threonine and cadmium, for 20

min. In this time, the L-threonine absorption arrives at the steady state, which is the highest absorption. At the end of the experiment the tissues were washed with 2 or 3 gentle shakings in ice-cold Ringer's solution and blotted carefully on both sides to remove excess moisture. The tissue was weighed wet and extracted by shaking for 15 h in 0.5 ml 0.1 mol/l HNO₃. Samples were taken from the bathing solutions and from the extracts of the tissues for radioactivity counting. All the modifiers (cadmium, etc) were added to the incubation solution at the beginning of the incubation period. The results are expressed as µmol L-threonine/ml cell water, accumulated in 20 min, after correction by the extracellular space.

A histological study was carried out to prove the tissue viability in the different experimental conditions (control and test animals). Before and after incubation, tissue samples were fixed in 10% buffered formaldehyde and embedded in paraffin. Sections of 5 µm were stained with Hematoxylin-Eosin and PAS (periodic acid Schiff). Another group of samples was embedded in methacrylate and 1 µm sections were stained by methenamine silver. This morphological study showed that cadmium did not modify the epithelium and the basement membrane. In both control (without Cd) and cadmium exposure, the samples showed a slight swelling of the lymphatic vessels and oedema located in the lamina propria. These changes did not seem to be signs of damage.

Na⁺-ATPase and (Na⁺/K⁺)-ATPase activity

Na⁺-ATPase and (Na⁺/K⁺)-ATPase are enzymes located in the basolateral membrane of the enterocytes. The basolateral membrane was purified by the method of Del Castillo and Robinson (1982), and the Na⁺-ATPase and (Na⁺/K⁺)-ATPase activities were measured by the method described by Proverbio and Del Castillo (1981), which determines the Pi liberated from ATP hydrolyses by different ATPases with or without ions and inhibitors. The results are expressed as specific activity (SA) which is defined as nmol Pi liberated from substrate hydrolysed/mg of protein per min at 37°C (nmol Pi/mg protein x minute). Protein was assayed by the method of Bradford (1976), measured with a Bio-Rad assay kit.

Amino-peptidase N activity measurement

Amino-peptidase N is a peptidase located in the brush border of the enterocyte. The brush-border membrane from rabbit jejunum was obtained as described by Brot-Laroche *et al* (1986). Amino-peptidase N was assayed according to the fluorimetric method of Andria *et al* (1980) using L-alanine- β -naftilamide as a substrate, which is hydrolyzed to β -naftilamide. The results are expressed as specific activity (SA) which is defined as nmol substrate hydrolysed/mg of protein per min at 37°C (nmol L-alanine/mg protein x minute).

Statistics

All results are expressed as means \pm SE. The comparison between means was evaluated by a 2-way (animal and treatment) analysis of variance. The Fisher's protected least-significant-difference test (PLSD) was used as a multiple-comparison method to compare data between groups, and considered statistically significant when $p < 0.05$ (Steel and Torrie, 1980).

RESULTS

Effect of cadmium on intestinal L-threonine accumulation

In our laboratory, we have assayed different concentrations of cadmium on D-galactose intestinal absorption. The results showed that 1 mmol/l CdCl₂ inhibited the sugar absorption significantly and did not modify the intestinal epithelium (Mesonero *et al*, 1993).

In order to study if this cation has the same effect on the amino-acid absorption, L-threonine accumulation was measured in jejunum of control and pretreated (test) rabbits with cadmium. It was added to the bath solution to compare the cadmium effect in both conditions to study whether the orga-

nism can adapt to a toxic metal or if this effect is accumulative.

In control conditions (without cadmium added to bath solution), the absorption of 0.5 mmol/l L-threonine in control and test animals were 1.11 and 0.84 μ mol L-threonine/ml cell water in 20 min, respectively. The results show that oral administration of cadmium decreases the L-threonine accumulation by 24%. When 1 mmol/l CdCl₂ was present in the medium, the L-threonine accumulation in rabbit jejunum was inhibited by 25 and 13%, respectively (table I).

The amino-acid absorption across the mucosal border of the jejunum is carried out by diffusion and/or by carrier-mediated transport (Na⁺-dependent system). In order to determine the possible effects of cadmium on L-threonine active transport and diffusion, the active transport was suppressed by competitive inhibition with L-threonine at high concentrations (50 mmol/l). Cadmium did not change the L-threonine diffusion (table I).

Effect of cadmium on (Na⁺/K⁺)-ATPase activity

The above-mentioned results show that Cd²⁺ diminishes the intestinal amino-acid transport due to an effect mainly located on the Na⁺-dependent system of transport at the mucosal border. The Na⁺ gradient in the enterocyte is maintained by the (Na⁺/K⁺)-ATPase located in the basolateral border. The effect of cadmium on (Na⁺/K⁺)-ATPase activity was studied in order to know by which mechanism the cadmium inhibits the active L-threonine transport. The results show that cadmium significantly diminishes the (Na⁺/K⁺)-ATPase activity (fig 1).

Effect of cadmium on Na⁺-ATPase activity

Del Castillo and Whittembury (1987) described a second Na⁺ pump in the basola-

Table I. Effect of cadmium of L-threonine absorption across jejunum of rabbit.

	In vitro additions + 0.5 mmol/l L-threonine			
	0 mmol/l Cadmium		1 mmol/l Cadmium	
	-	50 mmol/l L-thr	-	50 mmol/l L-thr
Control	1.11 ± 0.07 (5)	0.36 ± 0.02 (5)	0.83 ± 0.05 * (6)	0.32 ± 0.01 (6)
Test	0.84 ± 0.07 ** (6)	0.32 ± 0.02 (6)	0.73 ± 0.05 * (6)	0.31 ± 0.03 (6)

Control animals received a normal diet; test animals were pretreated with 20 ppm cadmium in drinking water for 4 d. Results are expressed as μmol L-threonine/ml cell water in 20 min and are presented as means \pm SE. The figures in brackets indicate the number of animals with 5 determinations/rabbit. * $p < 0.05$ compared with 0 mmol/l cadmium. ** $p < 0.05$ compared test with control animals.

teral border of enterocyte (Na^+ -ATPase), which extrudes Na^+ , accompanied by Cl^- and water, and controls the cell volume. The effects of cadmium on this pump were also tested. The results show that cadmium does not significantly alter the Na^+ -ATPase activity (fig 2).

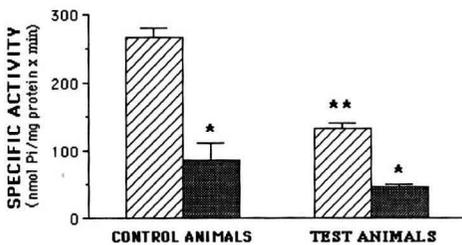


Fig 1. (Na^+ - K^+)-ATPase specific activity measured in jejunum of control rabbits (non-treated) and test rabbits (Cd-pretreated with 20 ppm cadmium in drinking water for 4 d). Each value is the mean of 6 animals. In vitro additions: ▨ 0 mmol/l Cd; ▤ 1 mmol/l Cd. * $p < 0.05$ compared with 0 mmol/l cadmium. ** $p < 0.05$ compared with control animals.

Effect of cadmium on aminopeptidase N activity

In order to improve the knowledge about the effect of cadmium on intestinal amino-acid absorption, a group of experiments was carried out in which the aminopeptidase N activity was measured in the presence of cadmium. The results indicate that cadmium did not significantly modify the aminopeptidase N activity of the control rabbits. However, this activity was increased in the test animals (20 ppm in drinking water) compared with control animals (fig 3).

DISCUSSION

The aim of the present work was to study and compare the effects of cadmium on the intestinal absorption of L-threonine and on the enzymatic digestion in animals, fed on a normal diet and in animals pretreated with CdCl_2 in drinking water for 4 d.

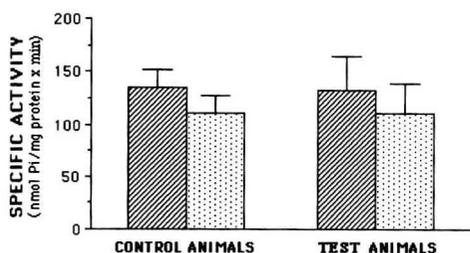


Fig 2. Na⁺-ATPase specific activity measured in jejunum of control rabbits (non-treated) and test rabbits (Cd-pretreated with 20 ppm cadmium in drinking water for 4 d). Each value is the mean of 6 animals. *In vitro* additions: ▨ 0 mmol/l Cd; ▩ 1 mmol/l Cd. There is no significant difference between different groups.

It has been reported that cadmium inhibits the D-galactose, D-glucose, L-phenylalanine and L-histidine absorption across the rat and rabbit small intestine (Bevan and Foulkes, 1989; Kojima *et al*, 1986; Rodriguez-Yoldi *et al*, 1989; Mesonero *et al*, 1993) and the L-alanine, proline and L-lysine transport into renal brush-border membrane vesicles isolated from the winter flounder (Bevan *et al*, 1989) and rat (Kim *et al*, 1990).

The results obtained in our laboratory show that cadmium partially diminishes L-

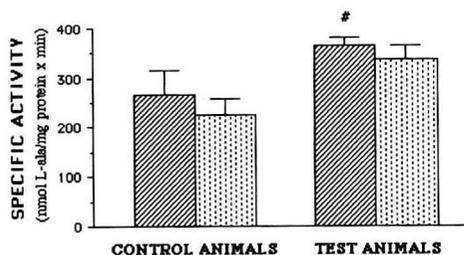


Fig 3. Aminopeptidase N specific activity measured in jejunum of control rabbits (non-treated) and test rabbits (Cd-pretreated with 20 ppm cadmium in drinking water for 4 d). Each value is the mean of 6 animals. *In vitro* additions: ▨ 0 mmol/l Cd; ▩ 1 mmol/l Cd. # $p < 0.05$ compared with control animals.

threonine absorption in rabbit jejunum. The effect of cadmium by *in vitro* addition is smaller in pre-treated than in control animals but the total inhibition is largest in the first group which shows that this action of cadmium is accumulative.

L-Threonine transport across the brush-border is the combined result of 2 mechanisms, called active and passive transport. It was worth determining whether cadmium acts on one transport system or on both. The inhibition of threonine absorption by cadmium appears to be due to exclusive impairment of the active transport component, because when this component was suppressed by competitive inhibition with 50 mmol/l L-threonine, the remaining apparently passive absorption was not affected by the cadmium (table I). This is supported by the histological study which showed that cadmium did not modify the integrity of the intestinal epithelium.

In order to clarify the action of cadmium on either the Na⁺-dependent carrier or the Na⁺ gradient in the enterocyte, the effect of cadmium on (Na⁺/K⁺)-ATPase activity was measured. The results have shown that cadmium significantly diminishes this activity (fig 1).

Since the inhibition of the Na⁺/K⁺ pump leads to inhibition of the active amino-acid transport, this effect might explain the mechanism by which cadmium inhibits the L-threonine absorption. To explain the (Na⁺/K⁺)-ATPase inhibition by cadmium, several authors have suggested the binding of this metal to the sulphhydryl groups of the enzyme (Jacobson and Turner, 1980; Toker and Matte, 1980). Indeed, SH-groups in the Na⁺/K⁺ pump are necessary for activity as suggested by the pump inhibition seen with sulphhydryl reagents like *N*-ethylmaleimide (Fahn *et al*, 1966).

On the other hand, we have found in previous studies that the inhibitory effect of cadmium is partially reversed when the luminal surface of the enterocyte membrane is

washed with dithioerythritol (thiol group protector) (Mesonero *et al*, 1993). Therefore, a possible direct action of the cadmium on the Na⁺-dependent carrier cannot be rejected.

Another enzyme located in basolateral membrane, that related to the control of cell volume is Na⁺-ATPase (Del Castillo and Whitterbury, 1987). The present results (fig 2) show that Na⁺-ATPase activity is not significantly modified by cadmium, which is in agreement with the insignificant effect of cadmium on the cell water.

The amino acids necessary for absorption are provided from dietary proteins by specific enzymes of the brush-border membrane of the small intestine. The aminopeptidases are a source of amino acids and the hydrolytic activity of these enzymes may be a step in the regulation of amino-acid absorption. The polypeptides are hydrolyzed by jejunal mucosal enzymes (aminopeptidase N, *etc*) to their constituent neutral amino acids and small peptides, which are absorbed by active transport and by facilitated diffusion. The disturbance of aminopeptidase N would result in alteration of neutral amino-acid absorption from polypeptides. Consequently, the determination of whether cadmium also alters the aminopeptidase N activity was considered of importance. The results obtained (fig 3) show that cadmium did not modify significantly the aminopeptidase N activity of control animals, whereas the aminopeptidase activity of animals treated with cadmium *in vivo* increased significantly with respect to the control animals.

In summary, the results obtained in the present work and previous studies in our laboratory, show that cadmium diminishes the intestinal absorption of L-threonine across the rabbit jejunum. This effect appears to be due to impairment of the active transport mechanism, which could be altered by modification of carrier proteins and also of (Na⁺/K⁺)-ATPase.

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