REVISTA ESPAÑOLA DE FISIOLOGIA, 47 (2), 75-80, 1991

Cadmium Induction of Metallothioneins in Several Dogfish Organs

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(Received on January 31, 1991)

J. PLANAS, LL. TORT, P. TORRES and R. FLOS. *Cadmium Induction of Metallothioneins in Several Dogfish Organs.* Rev. esp. Fisiol., 47 (2), 75-80, 1991.

Dogfish *Scyliorhinus canicula* were exposed to 50 mg/l Cd for 4 days for inducing metallothionein synthesis. Spleen, pancreas, kidney and gonads were dissected out and metallothionein presence was checked by means of gel filtration chromatography in Sephadex G-75 and sodium dodecyl sulphate polyacrylamide electrophoresis. In pancreas and kidney, a cadmium-binding protein with spectroscopical and electrophoretic properties similar to those of dogfish liver metallothionein was found. In the other organs, the existence of an analogue protein but at very low concentrations is feasible.

Key words: Dogfish *(Scyliorhinus canicula)*, Metallothionein, Heavy metals.

Metallothioneins (MT) are low molecular weight and heat-stable proteins, characterized by an amino acid composition rich in cysteine and lacking aromatic and histidine residues (19), originally found in mammals (12). MT function has not definitively been established, although its involvement in different processes has been suggested: tissue protection against several toxic metals (15), in zinc and copper metabolism (18), in detoxification of hepatotoxins (2) and in free radical scavenging (5).

MT or MT-like proteins have been described in almost all groups of animals; also in elasmobranchs, specifically in the dogfish *Scyliorhinus canicula* (8). Further, dogfish MT was purified and characterized from liver showing its similarity to rat MT (6, 7).

Synthesis of MT is clearly induced by Cd (and several other divalent cations) after exposure or administration. In dogfish, a quicker metal accumulation in pan-

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creas, spleen and kidney than in liver was observed (4). Consequently, it is plausible to consider the possibility of MT production in these organs. Therefore, the aim of this work is to detect the presence of MT after Cd exposure in pancreas, spleen, kidney and, additionally, gonads, of dogfish.

Materials and Methods

Dogfish *Scyliorhinus canicula,* caught off the coast near Barcelona, were kept for a month in big tanks with open circulating seawater and fed sardines *ad libitum* every two days. Then, fish were transferred to smaller experimental tanks, where they remained starved for a week. Twenty-five males and twenty females, all of them weighing between 70 and 170 g, were exposed to 5 mg/l Cd, as $CdCl_2$, for 4 days. Twenty-five males and twenty females were treated as controls. Fish were killed by decapitation and organs were dissected out and stored deep-frozen.

With the exception of testes, the organs from 5-8 animals were pooled in order to achieve an appropriate amount of tissue for the analytic procedures. As a consequence, two treated and two control pools were available for each organ and sex. Pool weights are shown in table I.

Each pool was homogenized (v/w), with a Potter-Elvehjem homogenizer, in ice-cold medium (0.25 M sucrose, 10 mM mercaptoethanol, 10 mM sodium azide and 0.1 mM phenylmethylfluorsulphonate in 10 mM Tris-HCl pH 8.2) and centrifuged for 10 min at 4 °C and 20,000 *g*. The supernatant was heated (70 °C) for 10 min and centrifuged again for 10 min at 4 °C and 28,000 *g*. The supernatant was later loaded to a Sephadex G-75 chromatographic column and eluted with 10 mM Tris-HCl buffer, pH 8.2, at a flow of 20 ml/h. Fractions (2 or 4 ml depending on organs) were collected and monitored for absorbance at 230, 250 and 280 nm and for Cd, Zn and Cu content by means of atomic absorption spectroscopy.

With the low-molecular weight fractions, where MT should be contained, a SDS-PAGE was carried out according to the method described by LAEMMLI (11). Slab gels (1.5 mm thick) presented two regions: an upper stacking gel, with 3 % acrylamide, and a lower running gel, with 15 % acrylamide. Electrophoresis buffer was 25 mM Tris-HCl pH 8.3, with SDS 0.1 % (w/v). For protein detection, gels were finally silver-stained (13).

Results

The different organs showed diverse patterns in Sephadex G-75 chromatographies. The clearest results were found in pancreas. For this organ, 230 nm absorbance (wavelength expressing peptidic bond content) of successive collected frac-

Table I. *Weight of different organ pools and their number for each group.*
TM = treated males, TF = treated females, CM = control males, CF = control females, T = total. Data from testes were referred to individual organs.

Organ	Mean ± SD (g)	Number of pools				
		TM	TF	CM	CF	T
Spleen	4.558 ± 1.195	2	2	2	2	8
Pancreas	0.777 ± 0.182	2	2	2	2	8
Kidney	2.091 ± 0.675	2	2	2	2	8
Ovaries	3.384 ± 0.468		2		2	4
Testes	7.483 ± 1.594	4		4		8

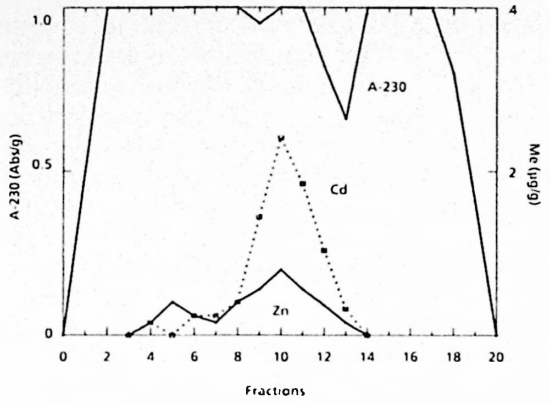

Fig. 1. *Gel filtration chromatography (Sephadex G-75) of dogfish pancreas for Cd-treated animals.*

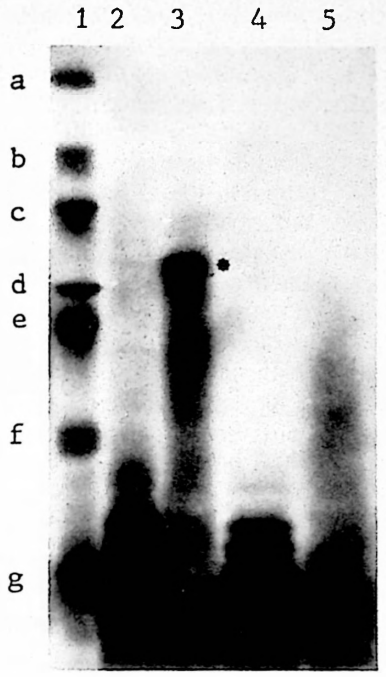

Fig. 2. *Electrophoresis in SDS-polyacrilamide of different protein*[1].
1. Standards (A: bovine serum albumin, 66 kDa. B: ovoalbumin, 45 kDa. C: glyceraldehide-3-phosphate dehydrogenase, 36 kDa. D: carbonic anhydrase, 29 kDa. E: trypsinogen, 24 kDa. F: trypsin inhibitor, 20 kDa. G: lactoalbumin, 14 kDa). 2. Proteins from peak II of Cd-treated kidney. 3. Proteins from peak II of Cd-treated pancreas (the asterisk indicating the band that probably corresponds to MT). 4 and 5. Proteins from peak II of Cd-treated kidney and pancreas, respectively mixed with EDTA.

tions drew three peaks in Cd-treated animals, as occurred in liver (6). Peak II corresponded to low molecular weight proteins, where MT is present. In addition, a peak of cadmium and another of zinc were observed in coincidence with protein peak II. Copper level was inappreciable. An example of this pattern is shown in fig. 1. Both male and female pancreas had a similar figure. Otherwise, in controls neither protein peak II nor cadmium peak were detected.

In order to assess the similarity of protein from peak II with MTs, a SDS-PAGE was performed with eluted fractions comprising this peak. A band with an electrophoretic mobility like that of MT was obtained (fig. 2). When EDTA was added to the sample before SDS-PAGE, the band had a mobility inherent to a lower molecular weight, attributable to metal loss. Furthermore, absorbance spectrum between 220 and 320 nm was scanned (fig. 3). There existed a progressive decrease of absorbance with increasing wavelength, but a shoulder appeared at 250 nm, this wavelength being characteristic of mercaptidecadmium bonds. When HCl was added in order to separate metal atoms from protein by acidification (10), the shoulder faded out giving rise to a spectrum as that from control samples where no notorious absorbance at 250 nm occurred. Besides, the low absorbance at 280 nm indicated the absence of aromatic residues.

Spleen, kidney and testes elution patterns were similar. Both Cd-treated and control animals had only two protein peaks (230 nm absorbance): I and III. Nevertheless, the absorbance level between them is very high, thus indicating the presence of an important amount of low molecular weight protein. Compara-

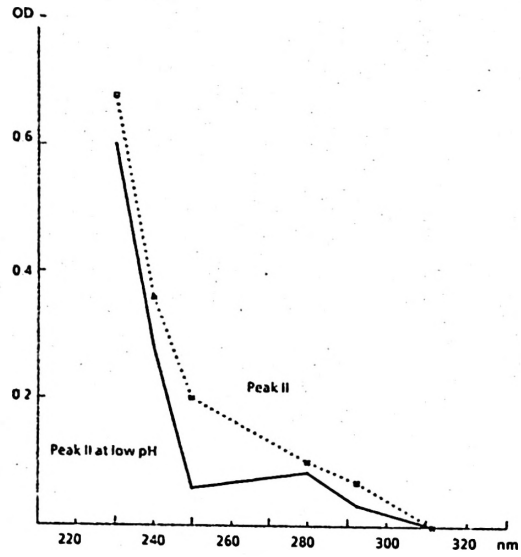

Fig. 3. *Absorption spectra of low-molecular weight protein fractions from Cd-treated dogfish pancreas, before and after HCl addition.*

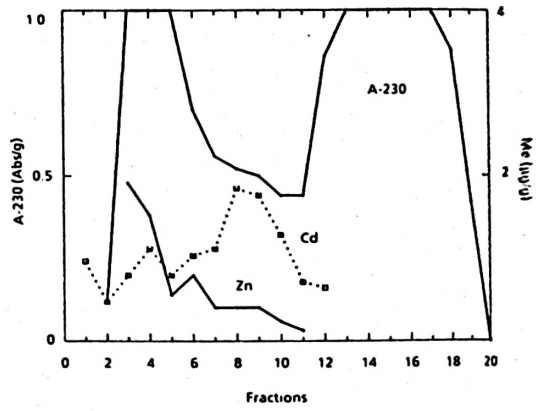

Fig. 4. *Gel filtration chromatography (Sephadex G-75) of dogfish spleen for Cd-treated animals.* Similar curves were obtained for kidney and testes.

ble curves for Zn levels are observed too in both experimental groups. On the other hand, a cadmium peak in the zone of absent protein peak II appeared in Cd-treated animals but not in controls (fig. 4). The levels of metals, summarized in table II, were erratic between samples in testes and differed between sexes in kidney.

In order to deepen in the features of this hidden second peak, an SDS-PAGE of the fractions corresponding to low-molecular weight proteins and high cadmium content from Cd-treated animals was performed. Only kidney developed a band

Table II. *Values of metal concentration (μg/g tissue) determined in fractions corresponding to peak I or II and total metal concentration in peak II (ΣMe)* from a gel filtration chromatography in Sephadex G-75. m: males, f: females, nd: not detected.

	Cd		Zn		Cu		ΣMe
	I	II	I	II	I	II	II
SPLEEN							
Cd-treated m	0.533	1.190	4.175	0.866	nd	0.058	2.114
Control m	nd	nd	3.543	0.511	nd	nd	0.511
Cd-treated f	0.674	1.170	3.304	0.664	nd	0.069	1.903
Control f	nd	nd	5.156	0.521	0.225	nd	0.521
KIDNEY							
Cd-treated m	1.234	3.190	1.599	0.533	nd	nd	3.723
Control m	0.525	0.730	1.465	1.230	nd	nd	1.960
Cd-treated f	0.680	1.760	1.373	0.541	nd	0.092	2.393
Control f	nd	nd	1.424	0.819	0.045	0.328	1.469
TESTES							
Cd-treated	nd	0.259	1.650	0.595	nd	nd	0.854
Control	nd	nd	2.019	0.805	nd	nd	0.805

typical of MT. Effects of EDTA in electrophoretic mobility and HCl in 250 nm absorbance were coincident with those observed in peak II from pancreas. When the peak II fractions from testes were lyophilized before application to SDS-PAGE a band with the same mobility as an MT band appeared and the behaviour on EDTA or HCl addition was the usual. Spleen failed to respond positively to anyone of these tests.

Finally, ovaries showed a third chromatographic pattern: peaks I and II appeared, and the line between them was very low as if there were no low molecular weight protein. Only traces of zinc and cadmium are detected in Cd-treated animals. However, when heat-stable supernatant from ovaries was selectively precipitated with acetone (14), instead of gel filtrated, and applied to SDS-PAGE, a band with MT mobility was detected.

Discussion

Since 1974 (16), various papers have appeared detecting and describing metallothionein in fish, mainly in liver. Metallothionein has also been detected (7) and characterized (5, 6) in dogfish liver.

From our results, it is clear that a protein with the same spectroscopical and electrophoretic properties as dogfish liver MT is present both in pancreas and kidney and it is referred to as cadmium-binding protein as it has been recommended until confirmation of MT identity by amino acid analysis.

The presence of MT in fish kidney has been observed in several species after cadmium treatment (21). Also, pancreas MT has been described in carp (9). Both organs, mainly pancreas, can accumulate important amounts of metals without apparent damage (4). It is feasible that MT could play a protective role. It is known that the exposure to a chronic low dose of a toxic metal inducing MT synthesis enhances the resistance of the animal to subsequent acute intoxications, thus reducing its mortality (1). The same is true for liver catalase inhibition, an index of cadmium poisoning (17).

Although cadmium-binding protein content had not been quantified, it is apparent from the sum of metal concentrations in peak II that a higher amount of this protein is present in Cd-treated animals (table II).

In controls, 230 nm absorbance in the zone of peak II is lower than that measured for Cd-treated animals but not negligible. In addition, small amounts of cadmium had been found in several control samples. It is likely that dogfish used in the experiment had been exposed previously to pollutants in their natural habitat. The shore around Barcelona is a very industrialized and populated zone.

Nevertheless, when animals were subjected to cadmium exposure, cadmium-binding protein had probably been synthesized *de novo*. Besides, zinc and copper could have been displaced by cadmium from preexisting protein, a process that has already been demonstrated as a MT feature (3). It is known that, in absence of cadmium, MT used to be loaded with zinc and copper.

Testes and ovaries showed finally a band with an electrophoretic mobility similar to that of MT. However, it was necessary to concentrate the samples. In spleen, all the trials failed. The presence of a low molecular weight cadmium-binding protein in these organs is only possible at very low concentrations. Further, in rat testes it has been argued that this kind of protein could be a MT (20).

Resumen

Se induce la síntesis de metalotioneína en el pez lija *(Scyliorhinus canicula)* por exposición de los peces a 50 mg/l Cd, durante 4 días. Se comprueba en bazo, páncreas, riñón y góna-

das, tras su extracción, la presencia de metalotioneína, por medio de cromatografía de filtración de gel en Sephadex G-75 y de electroforesis de poliacrilamida de dodecil sulfato sódico. Tanto en páncreas como en riñón se encuentra una proteína ligada al cadmio con propiedades espectroscópicas y electroforéticas semejantes a las de la metalotioneína de hígado del pez lija. Es posible la existencia de una proteína análoga en otros órganos, aunque a muy bajas concentraciones.

Palabras clave: Pez lija, *Scyliorhinus canicula*, Metalotioneínas, Metales pesados.

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