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Effect of an Acute Exposure to Sublethal Concentrations of Cadmium on Liver Carbohydrate Metabolism of Atlantic Salmon (*Salmo salar*)

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Heavy metals have been shown to exert a wide range of effects on fishes, from metabolic and physiological to behavioral and ecological (Forstner and Wittman, 1981). Cadmium is a heavy metal commonly used in ecotoxicological studies because of its concentration arises in the environment due to industrial and domestic sewage waste streams (Pickering et al. 1989). In fish, cadmium has adverse effects growth and reproduction and causes osmoregulatory stress, and it was shown to alter the structure and function of various organs, including liver (Lemaire-Gony and Lemaire, 1992). Reports on the stress response of fish to cadmium are scarce, and the available information is not consistent. Particularly, the specific effects of cadmium on energy-producing metabolic pathways in fishes have received little attention existing only a few studies describing changes in plasma glucose or lactate levels (Thomas and Neff, 1985; Tort and Torres, 1988; Pratap and Wendelaar-Bonga, 1990) but in none of them changes in the pathways of carbohydrate metabolism were assessed. Therefore, the aim of the present study was to characterize some of the effects of cadmium on several pathways of carbohydrate metabolism in livers of Atlantic salmon.

MATERIALS AND METHODS

Immature parr Atlantic salmon (*Salvo salar*) of the strain Landcatch weighing 20.3 ± 0.7 g were obtained on May 1995 from a fish farm. Fish were acclimated, 14 fish per tank, for four weeks under laboratory conditions in 6 aquaria (200 litres) supplied with constantly running and aerated well water at 15 ± 0.2 °C, 6.8 ± 0.02 pH, and under an artificial LD 12:12 regime (lights on at 08.00 h). The fish were fed once daily in the morning (at 11.30 h) before the experiment with commercial dry pellets (ration equivalent to 1.5% body wt. day-‘) and were food-deprived during the experiment. The common water quality criteria were assessed with no major changes being observed. To have enough tissue to analyse all the parameters assessed in liver and in plasma, samples from two fish were pooled.

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After acclimatization, aquaria were randomly allocated to one of three treatment groups: control, cadmium low dose (0.01 mg 1^{-1}), and cadmium high dose (0.1 mg 1^{-1}) in which experimental fish were exposed during 8 h. Cadmium was administered to the water in the aquaria from a stock solution of cadmium chloride. After 8 hours of exposure fish were removed to tanks with clean water and samplings were performed 24 h post-exposure. The control group, exposed to clean water and removed after 8 h, was sampled along with the cadmium-exposed fishes. On each sampling, all fish were removed quickly from the holding aquaria with a dipnet and anesthesized with MS-222 (37.5 mg 1^{-1}) buffered to pH 7.4 with sodium bicarbonate. The liver was quickly removed, weighed, frozen on dry ice and stored at -80°C until further assay. Blood was obtained with ammonium-heparinized syringes from the caudal peduncle. Plasma samples were obtained after centrifugation of blood (10 min at 2000 x g), and were immediately deproteinized (using 6% perchloric acid) and neutralized (using 1 $mol.l^{-1}$ sodium bicarbonate) before freezing them on dry ice and store at -80°C until further assay.

Liver samples were homogenized using a Potter-Elvejhem teflon-in-glass homogenizer held on ice with 10 vols of ice-cold stopping-buffer containing: 50 $mmol.l^{-1}$ imidazole-HCl (pH 7.5) 15 $mmol.l^{-1}$ 2-mercaptoethanol, 100 $mmol.l^{-1}$ KF, 5$mmol.l^{-1}$ EDTA, 5 $mmol.l^{-1}$ EGTA and 0.1 $mmol.l^{-1}$ PMSF (added as dye crystals immediately prior to homogenization). The homogenate was centrifuged (2 min at 9000 x g) and the supernatant was used in enzyme assays. Aliquots of the supernatant were deproteinized and neutralized to assay tissue glycogen using the method of Keppler and Decker (1974). Glucose obtained after glycogen breakdown and plasma glucose levels were determined with a glucose oxidase-peroxidase method (Spinreact, Spain).

Reaction rates of liver enzymes were determined by increase or decrease in absorbance of NADPH or NADH at 340 nm. The reactions were started by the addition of homogenates (0.05 ml), at a preestablished protein concentration, omitting the substrate in control cuvettes (final volume 1.1 ml), and allowing to proceed at 20 °C at preestablished times. Protein was assayed per duplicate in homogenates as detailed by Bradford (1976) using bovine seroalbumin (Sigma, USA) as standard. The specific conditions for enzyme assays were as follows:

Glycogen phosphorylase (EC 2.4.1.1.; GPase) was assayed as described by Moon et al. (1989) and Foster et al. (1993) with the following specific conditions: 50 $mmol.l^{-1}$ phosphate buffer (pH 7.0) 0.5 $mmol.l^{-1}$ NADP, 5 $\mu mol.l^{-1}$ glucose 1,6-biphosphate, 2.5 $mmol.l^{-1}$ AMP, excess phosphoglucomutase, excess glucose 6-phosphate dehydrogenase, and 10 $mg.ml^{-1}$ of glycogen (omitted for control). GPase *a* activities were measured with 10 $mmol.l^{-1}$ caffeine present, and total GPase activities were estimated without caffeine.

Glycogen synthetase (*EC* 2.4.1.11.; GSase) was assayed through PK, as detailed by Passonneau and Rottenberg (1973) and Foster et al. (1993). Specific assay

conditions were: 50 mmol.l^{-1} imidazole-HCl (pH 7.5) 150 mmol.l^{-1} KCl, 15 mmol.l^{-1} $MgCl_2$ 5 mmol.l^{-1} PEP, 0.15 mmol.l^{-1} NADH, 2 mg.ml^{-1} glycogen, excess pyruvate kinase, excess lactate dehydrogenase, and 6 mmol.l^{-1} UDP-glucose (omitted for control). Total GSase activities were measured with 5 mmol.l^{-1} glucose 6-phosphate (G6P) present, and GSase *a* activities were estimated in the absence of G6P.

6-Phosphofructo 1-kinase (*EC* 2.7.1.11.; PFK) was assessed according to Moon et al. (1989), and Su and Storey (1993), using 50 mmo1.l^{-1} imidazole-HCl (PH 7.8), 175 mmol.l^{-1} KCl, 0.25 mmol.l^{-1} NADH, 2 mmol.l^{-1} ATP, 17.5 mmol.l^{-1} $MgCl_2$ and excess aldolase, triose phosphate isomerase and α-glycerol phosphate dehydrogenase. Activities were determined at low (0.05 mmol.l^{-1}) and high (2 mmol.l^{-1}) fructose 6-phosphate (F 6P) concentrations (omitted for control). An activity ratio was calculated in each tissue as the activity at low [F 6P]/high [F 6P]. Similarly, a fructose 2,6-bisphosphate (F 2,6-P_2) activation ratio was determined using low (1 pmol.l^{-1}) and high (5 µmol.l^{-1}) fructose 2,6-bisphosphate concentrations, and 0.05 mmol.l^{-1} [F 6P].

Fructose 1,6-bisphosphatase (EC 3.1.3.11.; FBPase) was assessed mainly following the method of Mommsen et al. (1980). Specific assay conditions were: 50 mmo1.l^{-1} imidazole-HCl (pH 7.2) 0.4 mmol.l^{-1} NADP, 6 mmo1.l^{-1} $MgCl_2$ and excess phosphoglucose isomerase and glucose 6-phosphate dehydrogenase, and 0.03 mmo1.l^{-1} fructose 1,6-bisphosphate (omitted for control).

The normal distribution of variables was tested using the Kolmogorov-Smirnov test, and group variance homogeneity was assessed using the Cochrans' C test. Statistical differences were tested using a one-way analysis of variance, with treatment (control, cadmium low and high doses) being the main factor. In those cases where a significant effect was obtained in the anova, values were compared using a Student-Newman-Keuls multiple range test.

RESULTS AND DISCUSSION

Cadmium was chosen to assess the effects of heavy metals on fish carbohydrate metabolism because of its extensive appearance in the aquatic environment and the availability of literature in its toxicity, chemistry and bioaccumulation during continuous exposure (Handy, 1992). The liver was chosen to be assessed because the concentration of cadmium in such tissue is known to remain high for several days post-exposure (Handy. 1992). Cadmium concentration in unpolluted waters is around 0.036-0.06 µg cadmium.l^{-1} (Gill et al. 1993), then we decided to study the impact of those concentrations of cadmium suitable to induce acute effects in fish metabolism. Thus, we used two experimental cadmium concentrations: low (0.01 mg.l^{-1}) and high (0.1 mg.l^{-1}). Those doses were theoretically sublethal since they were approximately $1/100^{th}$ and $1/10^{th}$ of the reported acute toxicity value (LC_{50}) in salmonids, which is around 1 mg.l^{-1} (Pickering et al. 1989). Accordingly, no mortality was recorded in any group of cadmium-exposed Atlantic salmon in

Table 1. Effects of cadmium exposure on morphological parameters, liver glycogen and protein levels, and plasma glucose and lactate levels in Atlantic salmon

Parameter	Control (7)	Cadmium treatment	
		low dose (7)	high dose (4)
Body weight (g)	20.5 ± 1.19	19.9 ± 1.31	20.8 ± 1.56
Body length (cm)	12.8 ± 0.18	12.9 ± 0.22	12.9 ± 0.30
Liver weight (g)	0.16 ± 0.01	0.15 ± 0.02	0.16 ± 0.01
Hepatosomatic index	0.79 ± 0.05	0.79 ± 0.07	0.83 ± 0.09
Liver protein ($mg.g^{-1}$ wet wt)	86.8 ± 3.8	93.5 ± 4.9	103 ± 2.3
Liver glycogen ($mg.g^{-1}$ wet wt)	44.5 ± 3.5	28.9 ± 4.2*	12.4 ± 2.1* #
Plasma glucose ($mg.100\ ml^{-1}$)	86.6 ± 5.1	95.6 ± 3.5	120 ± 13.7* #
Plasma lactate ($\mu mol.ml^{-1}$)	3.37 ± 0.11	4.09 ± 0.07*	4.32 ± 0.11*

Experimental fish were exposed during 8 h to 0.01 mg cadmium.l^{-1} (low dose) or 0.1 mg cadmium.l^{-1} (high dose).Data are shown as mean ± S.E.M. of (N) pools of two fish and were analyzed by one-way ANOVA. *, significantly different (P<0.05) from the control fish. #, significantly different (P<0.05) from the fish exposed to the low dose of cadmium

agreement with studies using similar doses in other fish species (Tort and Torres, 1988) indicating a clear sublethal effect of the two doses of cadmium used. No significant changes were observed in the morphological parameters of the different fish used in the study (Table 1). Also, no changes were observed in liver protein levels (Table l), which allowed us to express the data of enzyme activity in terms of mg protein.

Stress in fish, including that due to toxicants, imposes a metabolic load that consists in two components: an energy demand required to cope with the disturbance and an energy cost to correct the accompanying hydromineral balance (Barton and Iwama, 1991). Cortisol is known to increase in plasma after cadmium exposure, with the levels remaining high during several days in species like striped mullet (Thomas and Neff, 1985) rainbow trout *Oncorhynchus mykiss* (James and Wigham, 1986) tilapia *Oreochromis mossambicus* (Pratap and Wendelaar-Bonga, 1990) and American eel *Anguilla rostrata* (Gill et al. 1993). It has been suggested that this increased cortisol levels may function to sustain high levels of circulating glucose after the initial catecholamine-induced increase in response to stressors (Barton and Iwama, 1991; Gill and Epple, 1992). Thus, increased plasma glucose and lactate levels were found in cadmium-exposed Atlantic salmon (Table l), which we may hypothesize are due to the effect of cortisol. These increased plasma glucose and lactate levels are also in agreement with other studies performed in dogfish *Scyliorhinus canicula* (Tort and Torres, 1988) while increases in glucose have also been found in striped mullet (Thomas and Neff, 1985) and tilapia (Pratap and Wendelaar-Bonga, 1990). However, in some cases, no increases were found in plasma glucose levels after cadmium exposure such as in American eel (Gill et al. 1993).

Table 2. Effects of cadmium exposure on the activities of potential regulatory enzymes of glycogenolysis, glycogenesis, glycolysis and gluconeogenesis assayed in liver of Atlantic salmon.

		Cadmium treatment	
Parameter	Control (7)	Low dose (7)	High dose (4)
Glycogen phosphorylase			
Total activity (U mg^{-1} protein)	1.15 ± 0.10	0.93 ± 0.09	0.94 ± 0.01
%GPase *a*	55.2 ± 2.3	65.9 ± 2.1 *	75.9 ± 2.5 * #
Glycogen synthetase			
Total activity (U mg^{-1} protein)	0.23 ± 0.01	0.26 ± 0.02	0.16 ± 0.01 * #
%GSase *a*	34.3 ± 0.8	24.5 ± 0.9 *	21.7 ± 0.6 *
GSase *a*/GPase *a* activity ratio	0.13 ± 0.01	0.11 ± 0.01 *	0.09 ± 0.01 * #
6-phosphofructo 1-kinase			
Optimal activity (U mg^{-1} protein)	0.29 ± 0.01	0.40 ± 0.02 *	0.49 ± 0.04 * #
Activity ratio	0.15 ± 0.01	0.34 ± 0.04 *	0.40 ± 0.01 *
F 2,6-P_2 activation ratio	0.36 ± 0.07	0.42 ± 0.03	0.45 ± 0.03
Fructose 1,6-bisphosphatase	0.23 ± 0.01	0.24 ± 0.01	0.28 ± 0.02

Experrmental fish were exposed during 8 h to 0.01 mg cadmium.l^{-1} (low dose) or 0.1 mg cadmium.l^{-1} (high dose). Data are shown as mean ± S.E.M. of (N) pools of two fish and were analyzed by one way ANOVA. *, significantly different (P<0.05) from the control fish. #, significantly different (P<0.05) from the fish exposed to the low dose of cadmium.

One unit of enzyme activity is defined for glycogen phosphorylase as that which produces 1 µmol NADPH min^{-1}, for glycogen synthetase as that which utilizes 1µmol NADH min^{-1}, for 6-phosphofructo l-kinase as that which utilizes 1 µmol fructose 6-phosphate min^{-1}, and for fructose 1,6-bisphosphatase as that which utilizes 1 µmol fructose 1,6-bisphosphate min^{-1}.

% GPase *a*, percentage of total glycogen phosphorylase (a + b) in the active form (a).

% GSase *a*, percentage of total glycogen synthetase (a + b) in the active form (a).

The activity ratio of 6-phosphofructo 1-kinase is defined as activity at low (0.05 mmol.l^{-1})/high (2 mmol.l^{-1}) substrate (fructose 6P) concentration. Similarly, a fructose 2,6-bisphosphate activation ratio was determined using low (1 µmol.l^{-1}) and high (5 µmol.l-1) fructose 2,6-bisphosphate concentrations, and 0.05 mmol.l^{-1} fructose 6-phosphate concentrations.

The source for the increased glucose levels in plasma may be related to an increased potential for glycogenolysis in liver. A clear fall was detected in liver glycogen levels of cadmium-exposed Atlantic salmon (Table 1), which was dose-dependent since the low glycogen levels were detected in those fish exposed to the high cadmium concentration. Changes observed in the activity of the enzymes involved in glycogen metabolism clearly correlated to changes in glycogen levels in cadmium-exposed fish (Table 2). Thus, a dose-dependent increase in the % GPase *a* as well as a dose-dependent decrease in the % GSase *a* were observed suggesting an increased glycogenolytic potential in livers of cadmium-exposed fish. The clear dose-dependent decrease in the GSase *a*/GPase *a* activation ratio found in cadmium-exposed fish (Table 2) also supports this contention since such a decrease favours glycogen depletion. This increased glycogenolysis due to the cadmium effect may be also related to the action of increased plasma cortisol levels since this hormone is known to activate glycogenolysis in salmonid livers (Vijayan and Moon, 1992).

Analyzing the effects of cadmium on liver glycolytic potential there was observed a clear increase in PFK activity not only considering the optimal activity but also the activity ratio of the enzyme (Table 2) which means the existence of a less phosphorylated enzyme and a subsequent more efficient catalysis at physiological concentrations of substrate, i.e. that the flux through glycolysis would be favoured. An increased flux through glycolysis may be related to an increased energy demand in liver. One of the possible uses for this energy would be the activation of those metabolic pathways involved in detoxicant activities and/or the synthesis of metallothioneins in the liver (Olsson et al. 1989). This is the first time, as far as we are aware, in which this increased energy demand has been reported to occur in the liver of a teleost fish due to cadmium exposure, though increases in the activity of the tricarboxilic acid cycle have been reported to occur after mercury exposure in mosquitofish *Gumbusia holbrooki* (Kramer et al. 1992). On the other hand, the absence of changes in the activity of the gluconeogenic enzyme FBPase (Table 2) is not surprising considering the above data indicating a general gearing up of liver carbohydrate metabolism to provide energy.

The results obtained in the present study demonstrate for the first time, as far as we are aware, the existence in a teleost fish of changes in liver carbohydrate metabolism after only an 8 h exposure to sublethal concentrations of cadmium. These changes include an activation of liver glycogenolysis and glycolysis as well as increased levels of plasma glucose and lactate. All these changes may be attributed to a secondary stress response related to a primary increase of plasma cortisol levels in cadmium-exposed fish.

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