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Changes in concentrations of glycolysis and Krebs cycle metabolites in mosquitofish, *Gambusia holbrooki*, induced by mercuric chloride and starvation

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Synopsis

Concentrations of glycolysis and Krebs cycle metabolites in the tail tissues of mosquitofish, *Gambusia holbrooki*, were measured in response to starvation and to exposure to 0.62 mg Hg l^{-1} (as $HgCl_2$) for 48 h. In control fish, starvation caused decreased concentrations of glucose-6-phosphate (G6P, − 58%) and fructose-6-phosphate (F6P, − 79%). Mercury exposure resulted in decreased concentrations of G6P (− 56%) and F6P (− 79%), and to increased concentrations of pyruvate (+ 75%), α-ketoglutarate (+ 41%), succinate (+ 39%), and malate (+ 47%). Krebs cycle activity increased in response to mercury exposure, perhaps in response to greater energy needs associated with maintaining homeostasis under stressful conditions. We conclude that glycolytic activity is reduced in fish exposed to mercury and that this response is similar to that caused by a cessation of feeding.

Introduction

The toxicity of inorganic mercury (Hg^{2+}, hereafter Hg) is well documented. In addition to acute lethality, a wide range of toxicological responses has been observed in a number of fish species (USEPA 1980). Early research noted secretion of a thick mucus layer over the body and gills, resulting in respiratory distress and suffocation within 30 min in the minnow, *Phoxinus phoxinus*, at a concentration of 200 mg Hg l^{-1} (Carpentar 1927). Severe histological changes in gill lamellae, including hyperplasia, fusion, and desquamation occurred in the mosquitofish, *Gambusia affinis*, at 0.02 mg Hg decreased gill tissue respiration after 30 and 60 days exposure but increased respiration after 120 days exposure (Dawson et al. 1977).

Inorganic mercury alters the physiology of the organs in which it accumulates. One mode of physiological damage involves disruption of plasma membrane function. Exposure to 1 mM $HgCl_2$ (200 mg Hg l^{-1}) significantly reduced para-aminohippurate transport activity, Na^+, K^+-ATPase activity and respiration in flounder renal tubule preparations (Miller 1981). Evans (1987) suggested that the membrane toxicity of Hg results from altered ionic permeability of membranes and inhibition of membrane-bound transport systems. Another as-

sulfhydryl groups (Berlin 1979). The activity of a number of enzymes in fishes is diminished by exposure to Hg including succinate and lactate dehydrogenases (Naidu et al. 1984) and pyruvate dehydrogenase (Sastry & Sharma 1980).

As an alternative to the description of morphological and enzymological changes in fishes exposed to Hg, the measurement of the tissue concentrations of glycolysis and Krebs cycle metabolites could lead to a better understanding of the physiological status of Hg-exposed fish. Also, the specific effects of Hg on energy-producing metabolic pathways in fishes have received little attention. In this study, the physiological response of eastern mosquitofish, *Gambusia holbrooki*, to acute Hg exposure was determined by the measurement of glycolysis and Krebs cycle metabolite concentrations. It was observed that Hg-intoxicated fish did not feed. Therefore, the effects of starvation were also compared to Hg intoxication. Three hypotheses were tested in this study. First, mercury-exposure does not alter metabolite concentrations in *Gambusia holbrooki* tail tissues. Second, starvation does not alter metabolite concentrations in mosquitofish tail tissues. Finally, mercury-induced alterations, if any, in metabolite concentrations are similar to those caused by starvation.

We report that Hg exposure caused decreased concentrations of glucose-6-phosphate and fructose-6-phosphate in tail tissues identical to the decrease caused by starvation in the same time period. Pyruvate and three Krebs cycle metabolite concentrations increased with Hg exposure. Within control fish, starvation had no effect on pyruvate or Krebs cycle metabolite concentrations.

Methods

Chemicals

Glutamate dehydrogenase (EC 1.4.1.3, bovine liver), lactate dehydrogenase (EC 1.1.1.27, bovine heart), pyruvate kinase (EC 2.7.1.40, porcine heart), G6P dehydrogenase (EC 1.1.1.49, *Leuconostoc mesenteroides*), malate dehydrogenase (EC 1.1.1.37, porcine heart), and inosine-5′-triphosphate were purchased from U.S. Biochemical, Cleveland, OH 44128. Succinate thiokinase (EC 6.2.1.4, porcine heart), glucosephosphate isomerase (EC 5.3.1.9, rabbit muscle), isocitrate dehydrogenase (EC 1.1.1.42, porcine heart), citrate lyase (EC 4.1.3.6, *Enterobacter aerogenes*), threo-D_s-(+)-isocitric acid, pyruvic acid, oxaloacetic acid, F6P, nicotinamide adenine dinucleotide phosphate (NADP), and coenzyme A (yeast) were purchased from Sigma. Citric acid, L-malic acid, and succinic acid were obtained from Aldrich. G6P, nicotinamide adenine dinucleotide reduced (NADH), nicotinamide adenine dinucleotide (NAD), and NADP were obtained from Calbiochem. A 1000 ppm (± 1% as $HgCl_2$) mercury reference solution was purchased from Fisher. Reagent grade mercuric chloride was obtained from Baker Chemical.

Fish collection and maintenance

In March 1990, eastern mosquitofish, *Gambusia holbrooki*, Girard 1859, were collected by dipnetting from Risher Pond, a 1.1 ha abandoned farm pond, on the U.S. Department of Energy's Savannah River Site, Barnwell County, South Carolina. Forty five male and 60 female fish were transported at ambient water temperatures to the laboratory in aerated water with an added stress treatment (Stresscoat, Aquarium Pharmaceuticals, Chalfont, PA 18914). Fish were held in 30 l glass tanks containing soft stream water (Upper Three Runs Creek, Aiken Co., SC) maintained at 16.0 ± 0.5° C on a 9–15 h light-dark cycle with constant aeration. Fish were fed commercially prepared flake food (Tetramin, Tetrawerke, Germany) twice daily, receiving an average of 8 mg food $fish^{-1}$ d^{-1}. Fish were acclimated to laboratory conditions for 72 h prior to the start of the experiment.

Mercuric chloride exposure

Four experimental groups of 20 fish each included: Group 1, 13 females, 7 males fed normal regimen, no mercuric chloride; Group 2, 12 females, 8 males

not fed, no mercuric cloride; Group 3, 11 females, 9 males fed normal regimen, 1 mg Hg l^{-1} (as $HgCl_2$) static renewal; Group 4, 13 females, 7 males not fed, 1 mg Hg l^{-1} static renewal. Fish exposed to mercuric chloride were kept in the same 30 l tank, separated by a plastic perforated partition through which food particles could not flow. Unexposed fish were held in a similar manner. A solution of 1000 mg Hg l^{-1} was added to the exposure tank at t = 0 and t = 24 h to bring the concentration of Hg to a maximum of 1 mg l^{-1}. The average concentration of Hg in the exposure tank over the experimental period of 48 h was 0.62 mg l^{-1} (range 0.28–0.91 mg Hg l^{-1}). The concentration of Hg in the control tank was less than the detection limit of 0.1 μg Hg l^{-1}. Two males were removed from the control tank (Group 1) during the experiment because they were showing signs of stress. One female in Group 3 died. All other fish survived until the termination of the experiment. In general, mercury-treated fish were listless, exhibited labored gill movements, and did not feed. Control fish were active and fed readily.

Water quality

Total alkalinity (potentiometric titration, APHA 1980) and pH were measured with an Orion Research Ionalyzer Model 901 using a Ross Combination Electrode Model 81-02. Total inorganic mercury was analyzed by cold vapor atomic absorption (APHA 1980) using a Perkin-Elmer 50A atomic absorption spectrophotometer. Other water quality parameters were not measured in this study. However, more detailed analyses of the same stream water are available in other studies (Newman 1986, Diamond et al. 1989). Median pH (N = 7) in the control tank was 7.0 (range 6.5–7.3); in the mercury-exposure tank, 6.8 (5.0–7.2). Total alkalinity (N = 2) ranged from 7.3 to 16.4 mg $CaCO_3$ l^{-1} in the control tank and from 7.1 to 8.0 mg $CaCO_3$ l^{-1} in the mercury-exposure tank.

Extraction of metabolites

Fish tail tissue, 0.089 to 0.675 g wet weight, was cut from live fish (mass range of females = 0.21–2.25 g of males = 0.17–0.51 g wet weight) along a dorsoventral line from the anterior of the dorsal fin to just caudad of the anus. Included in the tail section were the following tissues, by mean percent (standard error of mean, N = 5) of total tissue wet weight: skin and scales, 9.7 (0.9)%; vertebral column, 8.9 (1.3)%; caudal, dorsal, and anal fins, 7.4 (0.7)%; muscle, 67.0 (2.2)%; unaccounted tissues, 6.8 (3.6)%. The tissue was immediately plunged into liquid nitrogen. Freezing time, determined as the time required for gaseous nitrogen to cease its release from the tissue, was 5–15 sec. Frozen tails were stored in liquid nitrogen for no more than 3 wk prior to analysis.

Metabolites were extracted from the fish tissue using a modification of the method described by Burlina (1985). The frozen tail tissues were ground to a homogeneous powder with an agate mortar and pestle cooled in liquid nitrogen. The powder was placed in a tared, 1.5 ml microcentrifuge tube, cooled in liquid nitrogen, and closed with a vented cap. The tube containing the frozen tissue was then chilled in liquid nitrogen while 0.3–0.8 ml (approximately twice the weight : volume) of 3 M perchloric acid ($HClO_4$) in 12 ml conical polystyrene centrifuge tubes was being chilled in a dry ice-propanol bath at −30° C. The frozen tissue was then placed on the chilled $HClO_4$ and promptly mixed into the acid by vigorous agitation. After a 20 min extraction, the mixture was brought to 4° C and diluted with 1 ml double-deionized water. Following centrifugation for 15 min at 4° C (2300 rpm, 1100 g, Dynac II swinging bucket rotor) the decanted supernatant was neutralized by mixing with 0.2–0.6 ml (half the volume of acid added) of 2 M K_2CO_3, 0.5 M triethanolamine base. The neutralized extract (pH 8–9) was chilled at −20° C for 10 min to enhance precipitation of $KClO_4$. After centrifugation, the clear neutralized supernatant was decanted and frozen in liquid nitrogen until enzyme analysis, usually for less than 24 h.

Enzyme analysis

The procedures for the enzyme analysis of the metabolites were modified from Bergmeyer (1985). The reactions were monitored on a Beckman Spectrophotometer Model DU 70 (path length 10 mm, temperature maintained at 27° C by a circulating water-jacketed cuvette holder) as the change in absorbance at 339 nm caused by the oxidation or reduction of the nucleotide cofactor. The following metabolites were measured: G6P, F6P, pyruvate, citrate, isocitrate, α-ketoglutarate, succinate, malate, and oxaloacetate. The median difference between duplicate measurements of the metabolites was 3.2% (N = 97, 25th and 75th percentiles, 1.3% and 6.5%). Recovery of metabolites by the extraction technique was tested by spiking the frozen tissue with a known amount of each metabolite. Median recovery was 93.2% (N = 13, 25th and 75th percentiles, 84.2% and 112%).

Results

Metabolite concentrations (mean ± standard error) in the tail tissues of *Gambusia holbrooki* were calculated for each group (Tables 1, 2). Sample sizes were less than 20 in each group because in cases in which individual tail tissue mass was less than 0.1 g, the tissues of two fish were pooled. Also, some tissue samples from female fish were lost during storage: two each from Groups 1 and 2 and one each from Groups 3 and 4. Analysis of variance (SAS Proc GLM, SAS Institute 1987) was performed on the class variables sex, mercury treatment, and feeding regimen (Table 3). Only citrate variance was affected by sex ($F = 6.23$, $p = 0.016$).

Specifically, Group 3 females had a significantly greater concentration of citrate in their tail tissues than Group 3 males (females: 486 ± 22 nmol g^{-1} wet weight vs. males: 385 ± 23 nmol g^{-1} wet weight, $t = 3.17$, $p = 0.006$). Mercury treatment significantly affected variance in all metabolite concentrations except citrate, isocitrate, and oxaloacetate. Also, starvation was associated with G6P, F6P, and pyruvate variability (Table 3).

There were no differences in metabolite concentrations between fed and unfed groups within the mercury treatment (Groups 3 and 4) except for pyruvate (fed: 103 ± 6 nmol g^{-1} wet weight vs. unfed: 72 ± 6 nmol g^{-1} wet weight, $t = 3.69$, $p = 0.001$). G6P and F6P concentrations were markedly reduced due to starvation within the control group (fed: G6P 254 ± 59 nmol g^{-1} wet weight and F6P 42 ± 11 nmol g^{-1} wet weight vs. unfed: G6P 107 ± 14 nmol g^{-1} wet weight and F6P 9 ± 2 nmol g^{-1} wet weight; G6P $t = 2.43$, $p = 0.029$, F6P $t = 2.86$, $p = 0.013$).

Comparisons of metabolite concentrations in response to mercury treatment exhibited pronounced differences. Pyruvate, malate, succinate, and α-ketoglutarate concentrations all increased

Table 1. Glycolysis metabolite concentrations (nmol g^{-1} wet weight) in tail tissues of mosquitofish in response to mercury treatment and starvation, mean (SE). Student-Newman-Keuls multiple range test applied to each metabolite, $p < 0.05$. Means with same letter are not different.

Sample size metabolite	Mercury		Control	
	Unfed 16	Fed 18	Unfed 16	Fed 14
G6P	108 (11)[a]	118 (13)[a]	107 (14)[a]	254 (59)[b]

Table 2. Krebs cycle metabolite concentrations (nmol g^{-1} wet weight) in tail tissues of mosquitofish in response to mercury treatment, mean (SE). Starvation did not affect Krebs cycle metabolites. Therefore, values were pooled for the analysis of the mercury response, Student's t test, $\alpha = 0.05$.

Sample size metabolite	Mercury 34	Control 30	t	p
Citrate	434 (14)	423 (26)	0.35	0.727
Isocitrate	11 (2)	14 (2)	1.09	0.282
α-Ketoglutarate	41 (4)*	29 (3)	2.15	0.036
Succinate	305 (21)*	220 (21)	2.86	0.006
Malate**	94 (9)*	64 (6)	2.82	0.007
Oxaloacetate	17 (3)	27 (4)	1.92	0.060

*Indicates the mean is significantly different from the control

due to mercury treatment (Tables 1, 2). Mercury treatment also caused significant reductions in G6P and F6P concentrations when compared to the fed control (Group 1) concentrations. In fact, unfed control (Group 2) concentrations of G6P and F6P were the same as the mercury-treated (Groups 3 and 4). (Student-Newman-Keuls test, $p < 0.05$; fed mercury = unfed mercury = unfed control < fed control, Table 1).

Discussion

Although the toxicity of inorganic mercury to fish has been well defined, the effects of mercury poisoning on energy-producing metabolic pathways in fish have been less well documented. Ramalingam (1988) demonstrated in a study of *Oreochromis mossambicus* exposed to 0.09 mg Hg l^{-1} (as mercuric chloride) that muscle glycogen concentration decreased while pyruvate and lactate concentrations increased within the first 24 h of exposure. However, while glycogen concentration continued to fall over the remaining 15 days of the experiment, pyruvate and lactate concentrations returned to control levels during this period (Ramalingam 1988).

Table 3. Analysis of variance of metabolite concentrations in mosquitofish tail tissues with respect to mercury treatment, feeding regime, and sex. Sample sizes can be found in Tables 1 and 2. F statistic derived from Type I sum of squares, $\alpha = 0.05$.

Metabolite	Class		
	Mercury treatment F	Feeding regime F	Sex F
G6P	4.66*	6.62*	0.01
F6P	8.28*	9.22*	0.69
Pyruvate	24.78*	8.19*	3.26
Citrate	0.14	2.32	6.23*
Isocitrate	1.20	0.08	3.16
α-Ketoglutarate	5.70*	0.41	2.70
Succinate	9.41*	1.58	0.00
Malate	7.24*	0.54	0.03
Oxaloacetate	3.88	0.70	3.90

* Indicates $p < 0.05$.

In this study, conclusions about overall glycolytic activity in the tail tissues of *Gambusia holbrooki* are somewhat less decisive because glycogen (a glycolytic starting material) and lactate (a glycolytic end product) were not measured. Pyruvate concentrations increased with the mercury treatment, although perhaps not due to enhanced glycolytic activity. Rather, starvation in the control and mercury-treated groups resulted in greatly reduced concentrations of G6P and F6P, suggesting diminished glycolytic activity. Starvation is known to reduce muscle glycogen in fish (Lim & Ip 1989, Love 1980). Liver slices taken from roach, *Rutilus rutilus*, exposed to 1 mg l^{-1} phenylmercuric hydroxide, exhibited a 20–25% reduction in glycolytic activity, measured as lactate production. Lindahl & Hell (1970) suggested that the phenylmercuric ion inhibited glycolytic enzyme activity in the liver slices.

Krebs cycle metabolite concentrations were not affected by starvation in this experiment. However, mercury treatment caused significant increases in α-ketoglutarate, succinate, and malate, suggesting that Krebs cycle activity increased in response to the mercury stress. Lindahl & Hell (1970) demonstrated that Krebs cycle activity increased 15–19% in liver slices and liver mitochondria (measured as oxygen consumption) taken from *Rutilus rutilus*, exposed to 1 mg l^{-1} phenylmercuric hydroxide. In contrast, Begum (1987) concluded that ammonia toxicity decreased Krebs cycle activity in the liver of *Oreochromis mossambicus*, indicated by decreased activity of succinate, malate, and NAD-isocitrate dehydrogenases. Morales et al. (1990) concluded that handling stress in rainbow trout, *Onchorhynchus mykiss*, caused increases in plasma amino acids that could be made available to the Krebs cycle through deamination and transamination reactions leading to pyruvate and α-ketoglutarate. Also, Dalla Via et al. (1989) showed that the concentrations of pyruvate, α-ketoglutarate, fumarate, and malate all increased after the stress of forced exercise in juvenile *Rutilus rutilus*.

The pattern of responses in intermediary metabolite concentrations in *Gambusia holbrooki* tail tissues suggests a generalized stress response mechanism. The response to mercury poisoning included

reduced glycolytic activity similar to that caused by starvation and enhanced Krebs cycle activity that could have provided the energy required to maintain the homeostatic condition of the fish under stressful conditions.

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