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Inorganic mercury binding to fish oocyte plasma membrane induces steroidogenesis and translatable messenger RNA synthesis

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Both *in vitro* and *in vivo* $HgCl_2$ treatment demonstrated a remarkably high rate of progesterone synthesis accompanied by a low rate of conversion to 17β-estradiol in the oocyte of *Channa punctatus*. On depuration, however, there was a reversal of the steroidogenic scenario with a low progesterone and high estradiol level. The accumulation of progesterone was positively correlated with the significant increase in 3β-hydroxysteroid dehydrogenase activity in the Hg-treated fish. Thus, it was clear that at the early stage of intoxication Hg does play a role in the induction of 3β-hydroxysteroid dehydrogenase in the oocyte of fish at the spawning stage. The induction of this enzyme was found to be mediated by specific binding of Hg to the plasma membrane Na^+–K^+-ATPase (B_{max}: 14 nmoles mg^{-1} protein; K_a 1.14×10^8 moles) and increase in the specific messenger RNA translating 3β-hydroxysteroid dehydrogenase. It is concluded that inorganic mercury is able to initiate translatable messenger RNA synthesis in fish oocyte at a low degree of intoxication.

Keywords: 3β-HSD, fish oocyte, mercury, Na^+–K^+-ATPase, steroidogenesis

Introduction

There are ample reports on the effect of heavy metals on ovarian morphology, maturation, ovulation, spawning, egg number and viability (Victor *et al*. 1986, Hatakeyama & Yasuno 1987, Kirubagaran & Joy 1988, Dey & Bhattacharya 1989, Munkittrick & Dixon 1989, Tulasi *et al*. 1989, Anderson *et al*. 1991, Pereira *et al*. 1993, Kime 1995) but only a few are available on ovarian steroidogenesis in fish under the stress of heavy metals (Singh 1989, Thomas 1989, 1990). Little is also known about the effect of other xenobiotics on steroidogenic enzymes in the ovary, except for the pesticidal action on ovarian 3β-hydroxysteroid dehydrogenase (3β-HSD) activity (Kapur *et al*. 1978, Haider & Upadhyaya 1985).

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It is generally thought that heavy metals demonstrate their toxicity through avid binding to sulphydryl or nitrogen (Durnam & Palmiter 1981). It has also been reported earlier that inorganic mercury is partitioned to the nucleus within a very short span of time of intramuscular administration in both fish and rat liver (Bose *et al*. 1994). With the exception of a preliminary study demonstrating specific binding of inorganic mercury to fish oocyte plasma membrane (Ghosh *et al*. 1991), no work has been done to suggest a plausible signal transduction mechanism of a heavy metal in fish oocytes. The present study therefore addresses the impact of a heavy metal, mercuric chloride, during the spawning phase of a freshwater teleost, *Channa punctatus*. An attempt was also made to elucidate the mechanism of action of mercuric chloride on ovarian steroidogenesis by following the binding of the heavy metal to the plasma membrane together with *in vitro* translation of ovarian mRNA and to search for a probable link with the ovarian 3β-HSD activity.

Materials and methods

Adult, healthy, female *Channa punctatus* (average weight 35 ± 5 g and average length 17 ± 2 cm) at the spawning stage, were collected locally. The fish were acclimatized under laboratory conditions for 10 days and kept in batches of 10 in glass aquaria (60 × 30 × 30 cm) containing 30 l of tap water.

The 48 h LC 50 was determined following the method of Doudoroff *et al.* (1951), before deciding the experimental doses. The 2 day LC 50 for $HgCl_2$ was found to be 1.15 mg l^{-1}. The exposure doses were selected on the basis of no mortality and absence of any sign of physiological distress in the fish over the experimental period. Accordingly, in the short-term 2 day exposure, 115 μg l^{-1} (1/10 LC 50) was selected, and in the chronic 35 day exposure 23 μg l^{-1} (1/50 LC 50) was selected. The short-term exposure was followed by a 7 day depuration period. Concurrent controls were maintained throughout the experiments.

In vitro toxicant treatments were initially done with 10, 100 and 1000 μg of $HgCl_2$. Interestingly, no significant difference from the control could be recorded at 10 and 1000 μg of $HgCl_2$, while significant changes were observed at 100 μg of the treatment. Therefore, the *in vitro* steroidogenesis was followed only at the dose of 100 μg (0.37 mM) of $HgCl_2$.

Both control and treated fish were anaesthetized with tricane methane sulphonate (MS 222) at 125 mg l^{-1} and the entire ovary was carefully dissected out and kept under ice. The tissue was divided into three parts to determine: (i) the concentration of steroids – progesterone (P_4) and estradiol (E_2); (ii) 3β-HSD activity; and (iii) the rate of cell-free translation of mRNA. Soluble released polypeptides were further assayed for 3β-HSD activity spectrophotometrically.

In vitro ovarian steroidogenesis was followed in fish treated *in vivo* with $HgCl_2$, and in the ovary collected from untreated fish subsequently treated *in vitro* with $HgCl_2$ (in the absence or presence of exogenously added 20 ng of P_4) according to the protocol described below.

100 mg pieces of finely chopped ovary were incubated in a continuous shaking water bath for 2 h at 30°C in 1 ml of Krebs Ringer Bicarbonate saline solution (KRB; 0.103 M NaCl, 0.154 M KCl, 0.11 M $CaCl_2$, 0.154 M KH_2PO_4, 0.154 M $MgSO_4 \cdot 7H_2O$ and 0.15 M $NaHCO_3$). All incubations were stopped by rapid freezing and centrifuged at 5000 rpm for 10 min at 4°C. The supernatant containing the incubation medium was collected and stored at –24°C until further use. General extraction and radioimmunoassay procedures used were basically as described by Kime & Dolben (1985). The antisera to progesterone and 17β-estradiol were procured from Steranti Research Limited (S. Klinger), St Albans, UK. ^{3}H–progesterone and ^{3}H-estradiol were procured from Amersham Life Sciences, Amersham, UK. The antibodies were used at 1:1000 dilution and the tritiated antigens in the range of 10 000–12 000 dpm. Radioactivity was counted in an automatic Liquid Scintillation Counter (Beckman, LS 6000 SC) using Ready Safe (Beckman) scintillant. A set of standards were run under identical conditions and the results were calculated from a Beckman Software Package of RIA and expressed at pg steroid per mg tissue.

3β-HSD was prepared from the ovarian tissue (250 mg) collected from control and $HgCl_2$-treated fish (2 day, 23 μg l^{-1}). It was homogenized (20% w/v) under ice with Na-phosphate buffer–sucrose solution (pH 7.4) containing 50 mM sucrose, 1.0 mM EDTA and 5% glycerol, and centrifuged at 10 000 *g* for 20 min at 4°C (Beckman GS-15R centrifuge). The supernatant 3β-HSD activity was determined according to the method of Weibe (1976) with minor modification to suit the fish system. The rate of formation of reduced NAD was measured spectrophotometrically (Beckman DU 640) at 340 nm. The activity of the enzyme was expressed at μmole NAD reduced per min per mg protein. Protein was assayed (Lowry *et al.* 1951) using bovine serum albumin as standard.

Polysomes were isolated from 2.5 g of ovary collected from control and Hg-treated (2 day, 23 μg l^{-1}) fish by magnesium precipitation followed by extraction with a 1:1 phenol–chloroform mixture (Palmiter 1974). The Poly(A)$^+$-RNA was finally isolated from the polysomal RNA using Poly"U"-Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) affinity chromatography. All the fractions after elution from the column were monitored at 260 nm and 280 nm. Only the fractions having A_{260}/A_{280} ratios of 1.8 to 2.0 were taken for the *in vitro* translation experiments in a cell-free protein-synthesizing system prepared from raw wheat germ (Sigma, St Louis, USA) following the procedure of Comstock *et al.* (1987).

Isolated Poly(A)$^+$-RNAs were translated *in vitro* in an assay mixture containing wheat germ extract and other ingredients of protein synthesis (Comstock *et al.* 1987). The reaction mixture in a final volume of 50 μl, contained 20 mM Hepes buffer (pH 7.6), 2 mM DTT, 2.5 mM $Mg(OAc)_2$, 75 mM KCl, 1 mM ATP, 20 μM GTP, 8 mM creatine phosphate, creatine phosphokinase (0.01 mg ml^{-1}), 20 μM each of the 19 amino acids except leucine, ^{14}C-leucine – 36 000 dpm per assay (specific activity 300 mCi $mmol^{-1}$; Bhabha Atomic Research Centre, Trombay, India) and 4 μg of fish oocyte mRNA. After incubation at 25°C for 2 h, the reaction was terminated by transferring the tubes to 0°C. The translation products were analysed for total protein synthesis and soluble released polypeptide after treatment with 10% TCA (ice cold) followed by three washes with 5% TCA containing non-radioactive leucine. Counts were taken in a Liquid Scintillation Counter (Beckman LS 6000 SC) using Beckman Ready Safe liquid scintillant.

Fish oocyte plasma membrane was prepared according to Jamaluddin & Bhattacharya (1986). In brief, fish ovaries were placed in ice cold sterile culture medium (191 mM NaCl, 5 mM KCl, 6 mM glucose, 2.26 mM $MgCl_2$, 8 mM Tris, 4.99 mM $NaHCO_3$ and 0.44 mM NaH_2PO_4, pH 7.4). The homogenate was prepared from freed oocytes in 0.01 M sodium phosphate buffer (pH 7.4), filtered through cheese-cloth; (mesh size 120 μm) and centrifuged at 3000 rpm, at 4°C for 15 min. The pellet was collected and

recentrifuged at 20 000 rpm, at 4°C for 20 min. The purity of the isolated membrane preparation was checked by assaying Na^+–K^+-ATPase, 5-nucleotidase and glucose-6-phosphatase activity (Plummer 1988). Inorganic phosphate was measured according to Fiske & Subbarow (1925). The fish oocyte plasma membranes had only a 10% level of contamination by endoplasmic reticular membranes. All preparations were checked under the phase contrast microscope and were found to be comprised of 5–10 μm fragments. The binding of radiolabelled mercury (^{203}Hg, specific activity 94.4 mCi g^{-1}, BARC) to the plasma membrane was followed as per the protocol reported earlier by Bose *et al.* (1994). Plasma membrane protein (2 mg) from control fish oocyte was incubated with varying concentrations of hot mercury in the absence (total binding) or presence of 1000-fold excess of cold mercury to measure the non-specific binding. The tubes were incubated at 30°C for 2 h in a shaking water bath and the reaction was terminated by adding 1 ml ice cold washing medium (0.1% bovine gamma globulin and 0.1 M NaCl dissolved in 0.01 M phosphate buffer, pH 8.0). The bound fraction was obtained by adding 20% PEG (w/v) followed by centrifugation. The final pellet was solubilized by Beckman tissue solubilizer (BTS 450) and an aliquot was added to Ready Safe Liquid Scintillation Cocktail (Beckman) and counted in a Beckman Liquid Scintillation Counter (LS 6000 SC) having 90% efficiency for ^{203}Hg. The ^{203}Hg-bound membrane fragments were separated from the unbound fractions by using two chromatography steps, Sephadex G-75 and Sephacryl S-300 as reported by Bhattacharya *et al.* (1997).

Values are expressed as mean ± SE of three individual experiments. Data were analysed for statistical significance using Student's *t* test (Snedecor & Cochran 1967).

Results and discussion

The *in vitro* Hg-treated fish ovarian steroidogenesis from endogenous precursors demonstrated an increased rate of P_4 synthesis accompanied by a reduced rate of E_2 production. Interestingly, in presence of exogenously added P_4 the rate of conversion to E_2 was much higher in the Hg-treated ovary compared with that of the control (Figure 1).

In vivo steroidogenesis in Hg-treated (115 μg l^{-1}) fish demonstrated a higher rate of P_4 synthesis, with the rate of conversion to E_2 almost halved in comparison with the control. On depuration, P_4 production lowered significantly ($P < 0.01$), with a concomitant rise in E_2 synthesis (Figure 2).

During the chronic exposure the spawning phase of the fish gradually passed over to the post-spawning stage which is reflected in the reduced conversions to both P_4 and E_2. Initially, on 2 day exposure to $HgCl_2$ (23 μg l^{-1}) P_4 production was

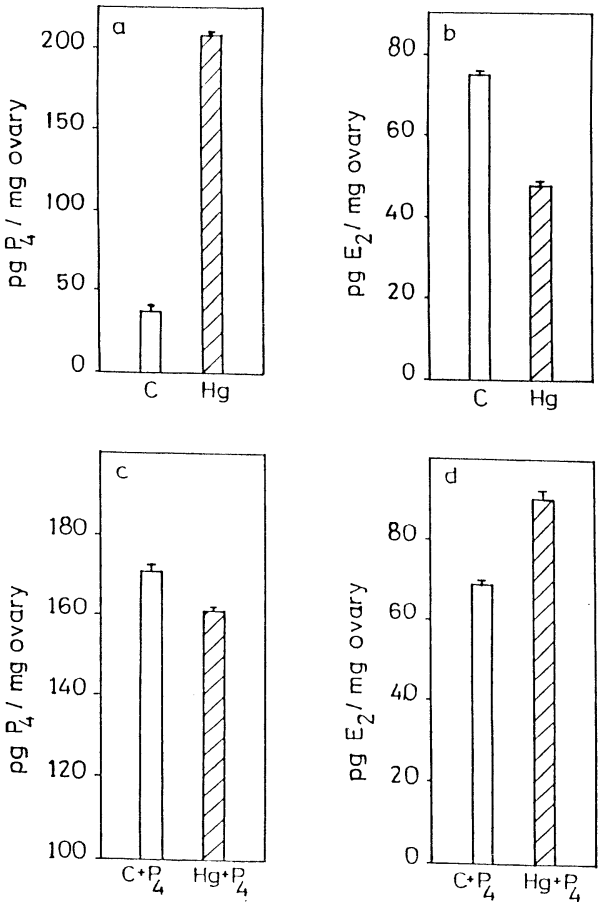

Figure 1. *In vitro* effect of 100 μg inorganic mercury on progesterone (P_4) and 17β-estradiol (E_2) synthesis in ovarian tissue of *Channa punctatus* in the absence **(a,b)** or presence **(c,d)** of 20 ng P_4. (C, control; Hg, treated). Data are shown as mean ± SE of three different experiments with tissues pooled from five fish.

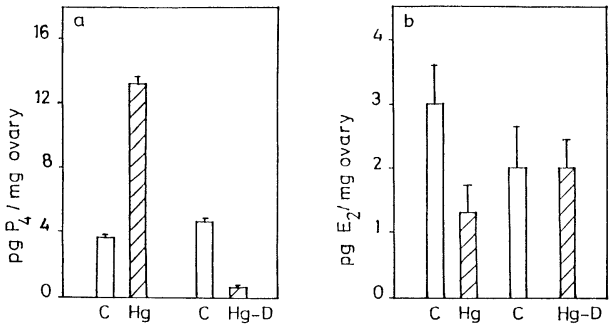

Figure 2. Synthesis of: **(a)** progesterone (P_4); and **(b)** 17β-estradiol (E_2) in the ovary of *Channa punctatus* is affected by *in vivo* exposure to $HgCl_2$ (115 μg l^{-1}) and depuration (Hg–D). (C, control; Hg, treated). Data are shown as mean ± SE of three different experiments with tissues pooled from five fish.

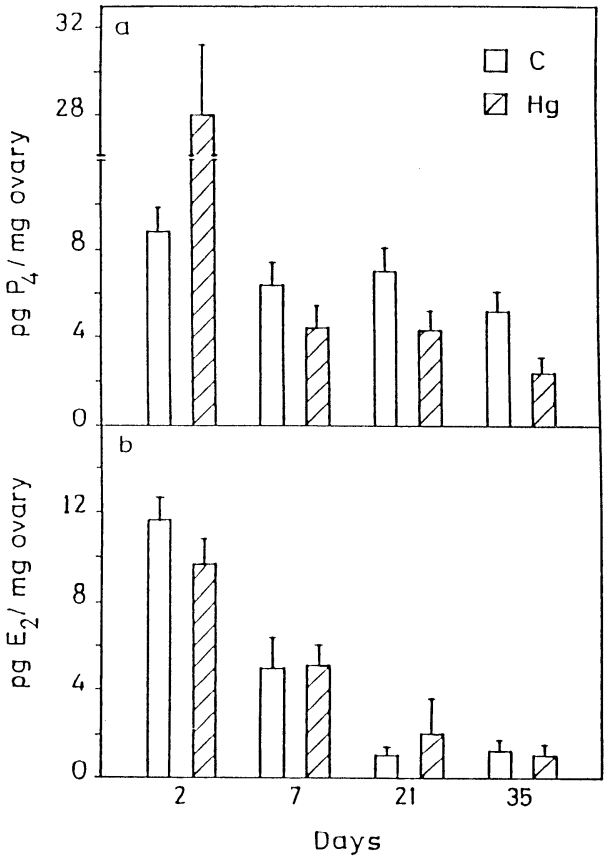

Figure 3. Levels of: (a) progesterone (P_4); and (b) 17β-estradiol (E_2) in the ovary of *Channa punctatus* exposed chronically to $HgCl_2$ (23 μg l^{-1}). Data are shown as mean ± SE of three different experiments with tissues pooled from five fish.

significantly ($P < 0.0025$) higher than the control but during the prolonged duration of exposure the rate of P_4 synthesis gradually lowered. In the case of E_2, however, no significant change in the rate of synthesis was noted during the toxicant exposure (Figure 3).

Thus, from both *in vitro* and *in vivo* experiments with Hg at different treatment regimens it was abundantly clear that there is a remarkable build up of P_4 in the ovary, which can be related to increased 3β-HSD activity. However, the rise in P_4 is reversed during depuration indicating the induction of steroidogenic enzymes other than 3β-HSD. With exogenously added P_4 the conversion to E_2 is enhanced, suggesting that excess P_4 has a stimulatory effect on steroidogenesis in the presence of Hg.

The 3β-HSD activity assayed in the homogenate and in the protein synthesized under *in vitro* cell-free translation system by the mRNA purified from the ovaries of control and Hg-treated fish demonstrated a remarkable increase in enzyme activity caused by Hg treatment (Table 1). This could be positively correlated with an increased rate of protein synthesis, as exemplified by ^{14}C-leucine incorporation into protein synthesized by mRNA in a cell-free condition (Table 2). The rate of ^{14}C-leucine incorporation was found to be much higher in the Hg-treated system compared with the control as revealed by both direct protein synthesis and released polypeptide. The data clearly indicate the role of inorganic mercury in the *de novo* protein synthesis in fish oocytes.

The Hg-bound membrane fragments appeared in the void volumes of the two columns used and were also found to contain Na^+–K^+-ATPase activity (data not shown) as seen in the liver plasma membrane of rat treated with Hg (Bhattacharya *et al.* 1997). The Scatchard analysis reveals the maximum binding capacity to be 14 nmoles mg^{-1} protein, having a K_a of 1.14×10^8 moles (Figure 4). The specific binding

Table 1. $HgCl_2$ treatment of fish stimulates 3β-hydroxysteroid dehydrogenase (3β-HSD) activity in the ovarian homogenate and in the cell-free translation product

System	μmole NAD reduced per min per mg protein	% Stimulation
Homogenate		
Control	0.011 ± 0.006	–
Hg-treated	0.131 ± 0.016*	190
Cell-free translation product		
Control	0.73 ± 0.2	–
Hg-treated	11.78 ± 0.49**	614

Data are shown as mean ± SE of three different experiments with tissues pooled from five fish; * and **, significantly different ($P < 0.0125$ and $P < 0.0025$, respectively) from the controls.

Table 2. Stimulation of ^{14}C-leucine incorporation in the proteins synthesized by fish ovarian mRNA under cell-free condition by Hg-treatment *in vivo*

System	^{14}C-leucine incorporation	
	dpm μg^{-1} mRNA	% Stimulation
Direct protein synthesized		
Control	143 ± 8	–
Hg-treated	175 ± 6*	22
Released polypeptides		
Control	30 ± 2	–
Hg-treated	41 ± 3*	36

Data are shown as mean ± SE of three different experiments with tissues pooled from five fish; * significantly different ($P < 0.05$) from the controls.

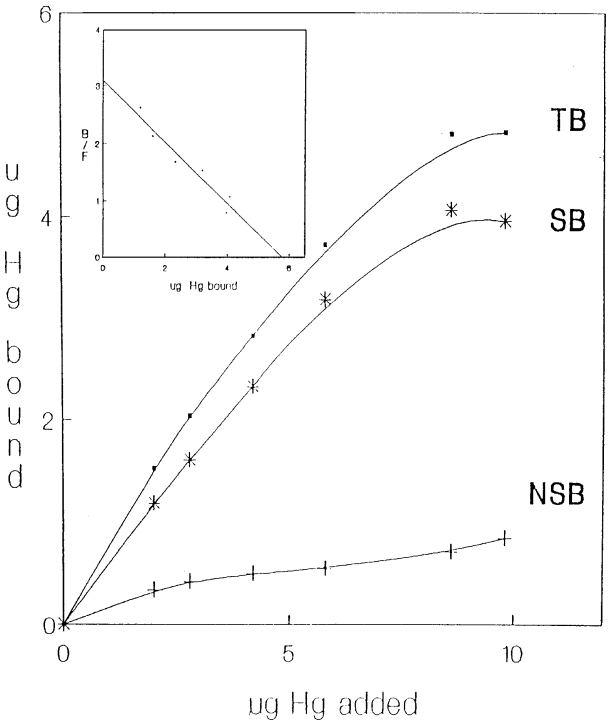

Figure 4. ^{203}Hg binding to 2 mg fish oocyte plasma membrane occurred in an incubation medium containing 5 mg $MgCl_2$, 0.1 M sucrose and 0.1% BSA in 0.01 M Na–phosphate buffer, pH 7.4, at 30°C. TB = total binding; SB = specific binding; NSB = non-specific binding. Scatchard analysis of the data as bound/free (B/F) versus bound Hg is shown in the inset.

pattern of inorganic mercury to the oocyte plasma membrane and inhibition of Na^+–K^+-ATPase activity suggest that the inorganic mercury has a common signal transduction mechanism in two different types of cells from two animal species. In the rat liver inorganic mercury binds to the plasma membrane Na^+–K^+-ATPase at the inner surface of the membrane to the SH– group which allows Na^+ to accumulate in the cytosol. In the presence of cytosolar glutathione-S-transferase Na^+ accelerates the dissociation of Hg from the plasma membrane to subsequently bind to the cytosolar nucleophile, glutathione (Bhattacharya *et al.* 1997). Since the cytosol of the oocyte also contains a high level of GSH (data not shown) the transport of inorganic mercury to the nucleus and successive binding to the response elements of the specific gene to express the specific mRNA for 3β-HSD may follow the same pathway as proposed for metallothionein gene expression in rat liver.

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