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Effects of the industrial pollution on glutathione s-tranferase in the liver of rainbow trout

R. Farzad[1], D. N. Inanlou[1], R. A. Cohan[2], M. Ghorbani[2]*

[1]Department of Research and Development, Research and Production Complex, Pasteur Institute of Iran, Tehran, Iran

[2]Department of Virology, Pasteur Institute of Iran, Tehran, Iran

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Glutathione S-transferase (GST) isozymes are widely distributed in nature and found in many organisms including microbes, insects, plants, fish, birds and mammals (Sherratt and Hayes). These enzymes show various activities toward different types of reactions, mostly by dissociation of the reduced glutathione (GSH) from the binding compound (Mannervik 1985). Fish are some of the species in the industrial world that are exposed to increasing level of polluted water. To investigate the effects of pollution on glutathione S-transferase activity we exposed rainbow trout to industrial pollution for a period of 4-6 weeks. Exposure to pollutants such as phenol, ammonia, mercuric chloride, cadmium chloride and mixture of agricultural anti parasitic agents significantly increased glutathione S-transferase activity with a sharp decrease of reduced glutathione (GSH) profile. By increasing the pH to 8.0 and the water temperature to 18°C, the GST activity also increased. We concluded that GST acts as a strong defense mechanism against environmental stresses by detoxificating industrial pollutants and some natural phenomena that reduce the water quality.

Key words: Glutathione S-transferase, rainbow trout, detoxification, toxicology.

INTRODUCTION

One of the important enzyme families that are involved in transformation of endogenous and xenobiotic compounds is glutathione S-transferase (GSTs) (1-2). These enzymes play a major role in detoxication by conjugating the tri-peptide glutathione (GSH) to electrophilic substrates produced during the oxidative stress and participate in the intracellular binding and transport of lipophilic compounds (3).

It is assumed that one of the major mechanisms of survival in a polluted water source is due to the detoxication of xenobiotics; and both GSH and glutathione-S-transferase are mainly responsible for this task (4-5). It is also suggested that glutathione can bind electrophilic carbon atoms or atoms such as cadmium, zinc, lead and mercury (6), as well as ammonia (7).

GSTs and their activity have been extensively characterized in rats, mice, and humans (8). Based on the protein sequence, substrate specificity and immunological activity of GSTs, they have been classified into different forms such as Alpha, Mu, Pi (9) and Theta (10) classes that are mainly located in the cytosole. Although there is no specific classification for GSTs in fish, they have been studied and characterized in a few species and it is suggested that GSTs activity in fish is similar to that in mammals (11-12).

It is reported that the major GST isoform in the liver of rainbow trout is pi (13) which is the same as the predominant class in human brain (14). Pi function is to detoxify toxic substrates that are carried by the fish blood (13) or cerebrospinal fluid in human brain (15).

The enzyme has only recently been described in some fresh waterfish (16) and there are little reports available on the involvement of this enzyme in detoxication of industrial pollutants in fresh water fish. The present study on the profiles of reduced glutathione and glutathione-S transferase in the liver of a rainbow trout may reveal a probable mechanism of detoxification in fish.

MATERIALS AND METHODS

In this study, 90 rainbow trouts weighing 50-150 g and between 10 and 20 cm in length were obtained from a regional trout farm (Karaj, Iran) and acclimatized in aerated glass tanks (60 × 40 ×100) containing 200 liter de-chlorinated water and maintained at 13°C with pH 7.5 for 72 hours prior to the experiment. All experiments were performed according to the IACUC standard protocols. All chemical compounds were purchased from Sigma Company (Canada).

* To whom all correspondence should be sent:
E-mail: mghorbani@irimc.org

The water quality in the fish farm was evaluated prior to the experiments using a Tytronics Sentinel analyzer (Galvanic Applied Sciences Inc., Canada). The composition of the water was as follows:

Component	PPM
Ammonia	1
Copper	0.12
Chloride	22
Dissolved oxygen	0.05
Mercury	No detectable
Phenol	18
Sulphide	18
Total hardness	105
Total solids	420

Fishes were divided into 6 groups of 15, including a control group, and exposed to non-lethal concentrations of ammonia (60 ppm), $CdCl_2$ (160 ppm), $HgCl_2$ (0.16 ppm) and phenol (22 ppm) for 2 days. A mixture of all toxins including phenol (6 ppm), $CdCl_2$ (60 ppm), ammonia (20 ppm) and $HgCl_2$ (0.08 ppm) was added to the fish tank containing the sixth group. After the exposure, fishes were transferred to toxicant free fresh water (with the same quality as before) and kept for 30 days. Group one was elected as the control group and maintained under identical conditions. Five fish from each tank were sampled immediately after 2 days of exposure and on days 15 and 30 after exposure.

Preparation of hepatocytes

Hepatocytes were collected according to the general method described by MelgarRiol et al. (17). Fish were anaesthetized by exposure to 3-aminobenzoic acid ethyl ester (Sigma, Canada) for a few minutes. Fish were then placed on a cutting board and a ventral incision was made along the medial line. The portal vein was cleaned from the surrounding tissues and cannulated by placing a ligature around the vein 1-2 cm away from the liver. The liver was then perfused using a peristaltic pump at a flow rate of 10 ml per min for 20 min to clear all the blood using a calcium-free solution containing 11.76 mM Hepes, 160.8 mM NaCl, 3.15 mM KCl, 0.5 mM EDTA and 0.33 mM Na_2HPO_4, pH 7.65, while the heart was cut to avoid high pressure in the system. The perfusion buffer was then replaced with a collagenase solution containing 6.67 mM $CaCl_2$ and 0.5 mg collagenase for 20 min, at a flow rate of 5 ml. The liver was then removed and the gall bladder was dissected carefully. The liver was then smashed against a sterile wire mesh strainer (100-µm) and washed with cold PBS. The cells were collected in a sterile falcon tube and centrifuged at 1500×g for 3 min. The supernatant was decanted and the cell pellet washed in 15 ml EMDM medium and centrifuged as before. The supernatant was removed and the pellet was re-suspended in 25 ml EMDM, and cell viability was evaluated by the trypan blue exclusion test (18).

Isolation of GSTs

Hepatocytes were homogenized followed by sonication for 2 min and cytosolic fractions were collected after centrifugation at 100000×g for 20 min (19). The cytosol was then dialyzed against 10 mM potassium phosphate buffer pH 6.5. The dialyzed cytosol containing approximately 105 units of GST activity towards 1-chloro-2, 4-dinitrobenzene (CDNB) (Sigma, Canada) was used in previously packed CM-cellulose column (1.7 × 25 cm) (Sigma, USA), equilibrated in the cold room with 1 liter of equilibration buffer (10 mM potassium phosphate buffer, pH 6.5) at a flow rate of 42 ml/h. The column was washed at a flow rate of 37.5 ml/h with 500 ml of the equilibration buffer until no absorption was detected at 280 nm wavelength. After a few washings, the GSTs were eluted from the CM-cellulose column with a linear KC1 gradient of 200 ml potassium phosphate buffer (10 mM, pH 6.5) and 200 ml of the same buffer containing 50 mM KC1. The fractions of about 2.5 ml were collected during the gradient elution with a flow rate of 35 ml/h. The protein profile of chromatography was determined by measuring the absorbance of fractions at 344 nm. The GSTs activities were then determined using CDNB as substrate and expressed as pg GSH/mg liver protein.

The result was expressed in terms of aa/min/mg protein. The liver protein was determined by the method of Lowry et al. (20) using bovine serum albumin (Sigma, Canada) as the standard.

Determination of liver GST activity

CDNB was used as a substrate to determine the GSTs activities through formation of thioether and detection at 344 nm. The CDNB reaction mixture included 100mM potassium phosphate, 1 mM CDNB and 1 mM GSH. The cytosolic proteins were diluted 1/50 in 20mM potassium phosphate buffer at pH 7.4 and stored at 25°C prior to addition to the reaction mixture. The result was expressed in terms of nmoles/min/mg protein. The optical densities were determined at every 5 seconds for 100 seconds.

Table 1. Reduced glutathione level in the liver of rainbow trout during and after exposure to non-lethal concentrations of ammonia, cadmium chloride, mercuric chloride, phenol and a mixture of toxicants. The significance is shown as $p<0.05$.

Toxicants	Glutathione level (pg GSH/mg liver protein)		
	Days of Toxicant exposure	Days after exposure	
	2 Days	15 Days	30 Days
Control	110.32 ±5.2	122.85 ± 8.8	132.64 ± 5.5
Ammonia	124.99 ± 7.3*	111.81 ± 12.2	126.02 ± 11
Cadmium chloride ($CdCl_2$)	74.71 ± 5.7*	128.99 ± 17	112.99 ± 7.3
Mercuric chloride ($HgCl_2$)	82.92 ± 8.3*	117.99 ± 13	124.99 ± 9.2
Phenol	69.69 ± 7.3*	125.99 ± 6.3	120.99 ± 9.3
Mixture of toxicants	61.99 ± 6.7*	114.99 ± 10.6	128.99 ± 13.2

Statistical analysis

Results are presented as mean ±SEM. Statistical analysis used Instate software for analysis of variance followed by a Student-Newman-Keuls post hoc test. Significant differences were assessed at $P<0.05$.

RESULTS

Glutathione

All toxicants used in this study reduced the GSH content in the liver during the 2 days exposure which returned to the normal level after transferring the fish to fresh water (Table 1).

Glutathion-S-Transferase activity

Fish intoxication with ammonia increased the level of glutathione S-transferase activity which was concomitant with the reduction of GSH level in the liver (Fig 1, Table 1). The enzyme activity declined sharply after transferring fish to fresh water (Fig 1).

Fish exposed to Cadmium Chloride also exhibited a significant enhancement of glutathione S-transferase activity in the liver which was declined on day 15 and returned to normal level on day 30 after exposure (Fig 2).

Treatment of rainbow trout with mercury ($HgCl_2$) induced a similar response as compared to other toxicants. However, it showed only a slight decrease on days 15 and 30 after exposure and was still significantly above the normal level (Fig 3).

Treated fish with phenol, exhibited a significant increase in GST activation on day 2 of exposure up to 2.5 times higher than the control group, whereas, the level of GSH returned to below normal level on day 15 and 30 of exposure (Fig 4).

The mixture of toxicants caused the activation of glutathione S-transferase to the maximum level (Fig 5). Again, like other toxicants, the level of enzyme activity returned to almost basal level in this group of fish.

DISCUSSION

It has been reported that glutathione S-transferase enzyme (GSTs) in the liver of animals is able to detoxify poisons in the blood flow (21). It appears that GSTs can bind to a large number of compounds. GSTs can be resolved into their ionic isozymes by ion-exchange columns depending on their ionic properties (21).

GSTs can appear in an organism in multiple forms. The establishment of such multiplicity has been discovered by chromatography and electrophoresis as well as by measuring the activity of the isozymes using CDNB as the electrophilic substrate (22).

In a number of studies of glutathione S-transferases, glutathione affinity chromatography has been extensively used (23). It has been shown that GST specific activity towards CDNB in freshly isolated hepatocytes was significantly higher as compared to the GST activity in cell culture (24).

The GST ligands are nucleophilic centers and intend to attack electrophilic compounds. Such compounds will be considered as the substrate for GST enzyme. Due to the large reactivity of these compounds to interact with GST enzyme system and the wide catalytic capability of GST enzyme, it appears that GSTs are involved with detoxication of both xenobiotics and normal constituents of food which are converted to less reactive compounds (24).

It has also been suggested that GSH may be involved in the protection of liver against a number of toxic compounds (25).

GSH depletion from liver could activate xenobiotics circulation which in turn increases hepatotoxic action. Meanwhile, hepatic toxicity of certain xenobiotics would be decreased by elevation of the GSH concentration in the liver (26).

In an industrial environment and due to the availability of excessive amount of toxicants such as cobalt and lead, glutathione depletion from the

liver of mice may occur, suggesting that the GST peroxidase is inhibited and resulted in the elevation of liver GSH levels due to the antioxidant properties of cobalt (27).

It seems that physiological and xenobiotic stimulations may activate the liver functions by increasing the levels of hepatic glutathione in both mammals and fish. There are many compounds such as acrolein and acrylonirile that cause elevation of glutathione during the incubation of hepatocytes (28).

Purification of cytosolic GSTs from sheep lung has shown that metal ions such as Ni^{2+},Cd^{2+}, Ba^{2+}, Mn^{2+}, Co^{2+}, Cu^{2+}, Pb^{2+} and Zn^{2+} inhibit the activity of GSTs. Glutathione-S-transferase activity has also been detected in the cytosolic fractions of tissue homogenates, especially in rat brain and liver, as well as avian brain homogenate (29).

It has been suggested that the release of glutathione from fish liver will increase the toxicity of several compounds and glutathione-S-transferase activity in fish that is similar to that in rat (30).

It is assumed that both glutathione and glutathione-S-transferase play an important role in the detoxification mechanism in both fish and mammals. In this study, we demonstrated that the decrease of glutathione is associated with the enhancement of glutathione-S-transferase activity.

We suggest that exposure to xenobiotics and toxins in many organisms causes the induction of glutathione that helps the organism to adapt itself to the changes in the environment and resist against acute pollution, as well as reducing the harmful effects of the toxicants. In the mean time, the isolated rainbow trout hepatocytes appear to be an ideal approach to analyze the effect of toxicants on the activity of glutathione-S-trasferase. Since all GST subunits are not acting the same there are needs for comparative experiments to investigate the effects of different toxicants on various glutathione subunits.

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ЕФЕКТИ НА ПРОМИШЛЕНОТО ЗАМЪРСЯВАНЕ ВЪРХУ ГЛУТАТИОН S-ТРАНСФЕРАЗАТА В ЧЕРНИЯ РОБ НА ДЪГОВАТА ПЪСТЪРВА

Р. Фарзад[1], Д. Н. Инанлу[1], Р. А. Коан[2], М. Горбани[2]*

[1]*Департамент за изследване и развитие, Изследователско-производствен комплекс, Ирански Пастьор-институт, Техеран, Иран*
[2]*Департамент по вирусология, Ирански Пастьор-институт, Техеран, Иран*

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(Резюме)

Глутатион-S-трансферазните (GST) изозими са широко разпространени в природата и са намерени в много организми като микроби, насекоми, растения, риби, птици и бозайници (Sherratt и Hayes). Тези ензими показват разнообразна активност спрямо различни типове реакции, главно чрез дисоциацията на редуцирания глутатион (GSH) от свързващите съединения (Mannervik, 1985). Рибите са сред видовете, най-изложени на замърсените води в промишлено-развитото общество. За изучаването на тези ефекти беше използвана дъговата пъстърва като тестов вид при експозиции от 4 до 6 седмици. Експозицията към замърсители като фенол, амоняк, меркури-хлорид, кадмиев хлорид и смес от земеделски и паразитни агенти значително повишава активността на глутатион-S-трансферазата с рязко понижаване на профила на редуцирания глутатион (GSH). Активността на GST се повишава при pH 8.0 и температура на водата 18°C. Беше установено, че GST действа като силен защитен механизъм срещу екологичните стресове, като обезвредява промишлените замърсители и някои естествени фактори, влошаващи качеството на водите.