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Quantitative RT-PCR Analysis of Polyubiquitin Gene Expression in *Biomphalaria arabica* after Exposure to Heat Stress and Cadmium

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Abstract: This study focused on the analysis of polyubiquitin gene expression in *Biomphalaria orabica* after exposure to heat stress (42°C, 1 h). a treatment known to induce polyubiquitin gene expression from cell stress responsive promoters and exposure to lethal and sublethal levels of cadmium (1, 2 and 3 mg/L, 336 h) . Polyadenylated RNA was extracted and polyubiquitin gene expression was analysed using quantitative reverse transcription-polymerase chain reaction technology (RT-PCR) with primers complement to the 3' and 5' prime regions of ubiquitin repeats (polyubiquitin). Results were expressed in arbitrary units as a ratio of optic al density of polyubiquitn/βactin electrophoretic bands. In heat stressed snails, the expression was 0.242 ± 0. 012 and 0.785 ± 0.039 for control and heat-treated individuals, respectively . After cadmium exposur e, the expression was 0.327 ± 0.013, 0.298 ± 0.012, 0.523 ± 0.021 and 0.612 ± 0.024 at concentrations of 1, 2 and 3 mg/L respectively . This suggests that alterations in polyubiquitin gene expression may be cruc ial to withstand cadmium toxicity and may be considered as a biomonitor for tox icity.

Key words: Biomphalaria arabica, cadmium, polyubiquitin, RT-PCR

Introduction

Cells from various organisms respond rapidly to toxic stress by altering their growth rates, metabolic systems and gene expression controlling normal and basic functions.

In particular, changes in the expression of cell stress or so called heat shock genes may be considered as a biomonitor to assess wether organisms are experiencing cellular stress within their environment.

Heat shock gene family includes a multigene repeates incoding for a highly conserved protein, present universally in all eukaryotic cells, known as ubiquitin (Wilkinson, 1988). Substantially, ubiquitin is involved in non-lysosomal protein catabolic pathway of abnormal and short-lived proteins (Rechsteiner, 1991; Hershko and Ciechanover, 1992; Jentsch, 1992) .

In all species studied, ubiquitin is coded lor by three classes of genes. Class I and II genes encode two proteins, each consists of a single copy of ubiquitin fused to ribosomal proteins (Cabrera *et al*., 1992; Baker and Board, 1991; Bishoff and Schwartz, 1990) . The class lll gene encodes multiple ubiquitin repeats (polyubiquiutin genes) . The number of repeats var ies with the organism (Wiborg *et al*. , 1985; Neves *et al.,* 1991; Wempe and Schiet, 1993; Hayashia *et al.,* 1994). These genes are strongly induced by heat stress and contain a consensus heat shock promoter (Bond and Schlesinger, 1985; Finley *et al..* 1987; Simon *et al*., 1999). It is thought that polyubiquitin transcripts accumulate in response to increased demand for ubiquitin during stress.

The current study was undertaken to determine the expression of polyubiquitin in the freshwater snail, *Biomphalaria arabica,* alter heat exposure and exposure to lethal and sublethal levels of cadmium.

Here, the expression of polyubiquitin was quantified using raverse transcription-polymerase chain reaction technology (RT-PCR) with primers complementary to ubiquitin lequences on the bases of polyubiquitin cDNA homology from various organisms (Wiborg *et al.,* 1985; Neves *et al*., 1991; Wempe and Schiet, 1993; Hayashia *et al.,* 1994). The primers were designed to amplify the 3' and 5' flanking egions of ubiquitin, which resulted in the amplification of cDNA fragments from polyubiquitin transcripts but not from that of ubiquitin fused transcripts. Here, βactin gene expression was used as an internal standard (Lardans *et al*., 1997) .

Determination of polyubiquitin gene expression may be considered as a bio-indicator for water pollutants and specially cadmium.

Materials and Methods

Snail collection and screening: Individuals of *B. arabica* were collected from drainage canals located near Al-Hafuf of Al- Hassa region in eastern province of Saudi Arabia . Animals were screened for cercaria! emergence according to Al-Khedhairy, 1999.

Heat and cadmium exposure: Snails were heat- shocked at 42°C for 1 h period. Also, a different set of animals was exposed to $CdCl_2$ (1, 2 and 3 mg/L, 336 h) according to Al-Khedhairy, 2000.

RNA praparation and mANA selection: Total cellular RNA was prepared from heat-shocked and cadmium- exposed animals using the guanidinium thiocyanate/phenol- chloroform method (Chomczynski and Sacchi, 1987) . Polyadenylated RNA was purified with PolyATract® mRNA isolation system (Promega Co., Madison, USA) .

Primer design: Polyubiquitin eDNA sequences of human, rat, bovine and *Tetrahymena pyriformis*, were compared for sequence homology using Blast2 software from GenBank database.

RT-PCR: Reactions were completed with AMV reverse transcriptase (AMV RT) and Tfl DNA polymerase (Promega Co., Madison, USA). Reaction components were adjusted at the follow ing concentrations, 25 ng poly (A) RNA, 200 μ M of reverse and forward primers, 100 μM dNTPs, 2 mM $MgSO_4$, 1 x Tfi/AMV reaction buffer, 5 units of AMV RT and 5 units of Tfi DNA polymerase. Amplification was done with specific primers for the target sequences of polyubiquit in (CTGACTACAACATCCA and

GGGTCTTCACGAAGA) and βactin (TCGTCGGTATCCATGAAACA and ATTTCCTCTCTGGTGGAGCA, Gulf Biotech, Dallah Healthcare Co, Riyadh, S.A.) on a Biornetra therrnocycler, Germany, First-strand synthesis was accomplished in 45 minutes at 48°C. Subsequently, samples were incubated at 94°C for 2 minutes, 58°C for 1 minute and 68°C for 1 minute over 40 cycles. Final extension was carried out at 68°C for 7 minutes.

DNA fractionation: DNA fragments were fractionated in agarose gels. DNA was loaded into gels in DNA loading buffer [20% ($^{W}/_{v}$) Ficoll 400, 0.25% ($^{W}/_{v}$) bromophenol blue and 0.25% ($^{W}/_{v}$) xylene cyanol FF]. Electrophoresis was performed in 1 x TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA, pH 8.0) supplied with ethidium bromide (Sambrook *et al.,* 1989). DNA was visualised using a 302 nM UV transilluminator supplied with a Photoman-Kodak camera.

Quantitation and statistics: Density of electrophoretic bands was quantitated by image analysis of agarose cDNA bands. All values given are means ± SEM.

Results

Figure 1 shows the RT-PCR strategy employed and all possible polyubiquitin cDNA size fragments that may be generated after RT-PCR amplification.

Figure 2A shows the amplification of cDNA sequences for polyubiquitin and Pectin from control and heat-shocked snails. Primers for both polyubiquitin and pectin assisted the amplification of a 300 by and 190 by uDNA fragments, respectively. Figure 2B shows the quantitation of polyubiquitin mRNA levels. Polyubiquitin gene expression was 0.242 ± 0.012 and 0.785 ± 0.039 for control and heat shocked snails, respectively.

Figure 3 shows the quantitation of polyubiquitin mRNA levels. In control animals, polyubiquitin gene expression was 0.327 ± 0.013. At 1 mg/L of cadmium concentration, the mRNA level was 0.298 ± 0.012. Higher levels of polyubiquitin mRNA were quantitated at 2 and 3 mg/L and found to be 0.523 ± 0.021 and 0.612 ± 0.024 respectively. These values showed that mRNA was increased to an average of 1.62 fold at 2 mg/L and 1.87- fold at 3 mg/L.

Discussion

Recently, it was shown that the expression of HSP70 in the snail *B. arabica* was induced after exposure to various lethal and sublethal concentrations of cadmium (Al-Khedhairy, 2000). However, it was thought that cadmium may also alter the expression of other genes since it has a broad effect on cellullar proteins.

In this respect, cadmium is known to interact with thiol groups of proteins and can substitute zinc in essential proteins, which subsequently causes a formation of abnormal and denatured proteins (Valle and Ulmer, 1972). This seems to be a primary mechanism of toxicity caused by cell exposure to adverse chemical and physical conditions, which is generally thought to result from the weakening of polar bonds and exposure of hydrophobic residues (Brown *et al.,* 1982; Wedler, 1987).

Cadmium was selected among known heavy metal pollutants for its high toxicity, which may affect living systems in various ways. This heavy metal is a substantial industrial and environmental pollutant that injures a variety of organs, including the liver, testes, kidneys and brain (Morselt, 1991) possibly by the oxidative damage of essential cellular macromolecules. For example, cadmium can increase lipid peroxidation in leydig cells (Koizumi and Li, 1992), hepatocytes (Muller, 1986) and brain (Acan and Tezcan, 1995).

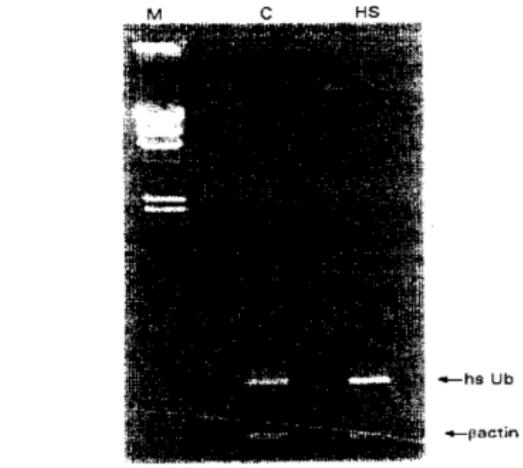
Primarly, it was essential to design primers for amplification of ubiquitin cDNA fragments from polyubiquitin mRNA repeats since it is will documented that the gene is a heat-shock gene (Bond and Schlesinger, 1985; Finley *et al*., 1987; Simon *et al.,* 1999). Highly conserved primers from man to the ciliate, *T. pyriformis,* were found to assist the amplification of a 300 bp (hsUb) fragment (Fig. 2A). Because, both forward and reverse primers are complement to sequences within the 3' and 5' prime regions of ubiquitin, the amplified cDNA fragment is thought to correspond solely for polyubiquitin.

It was found that hsUb was significantly increased in heat-shocked animals. Quantitation of optical density ratio of hsUb/Bactin electrophretic bands showed that the expression was 0.242 and 0.785 in control and heat-shocked animals respectivily (Fig. 2B). This represents a 3.24-fold increase in the expression of polyubiquitin after 1 h treatment.

Fig. 1: Schematic diagram showing RT-PCR strategy and all possible polyubiquitin cDNA size fragments that may be generated after RT-PCR amplifications. Positions of forward (▸) and reserse (◂) primers indicated

Here, it was aslo found that the expresssion of polyubiquitin was increased after exposure to various levels of cadmium. The expression was 0.298, 0.523 and 0.612 at concentations of 1, 2 and 3 mg/L respectively, while in control animals the expression was 0.327. Thus, cadmium increased the expression of polyubiquitin at only high concentrations.

The stimulation of polyubiquitin gene expression may be a consequence of the stress-induced accumulation of damaged proteins in a mechanism similar to the stimulation of HSP70 (Jungmann *et al.,* 1993; Lee and Hahn, 1988; Ananthan *et al.,* 1986). Although this mechanism has not been demonstrated in the snail *B. arabica*, it seems that both polyubiquitin and HSP70 are induced via binding of the heat shock transcription factorfs) (HSFs) to the heal shock element (HSE) present within the promoter region of both genes. This conclusion is supported by recent findings of several heat shock-responsive polyubiquitin genes from human, chicken and *Saccharornyces cerevisiae* (Bond and Schlesinger, 1985; Finley *et al.,* 1987; Nenoi *et al.,* 1996; Simon *et al.,* 1999) Thus, the production of more polyubiquitin transcripts is



(A)

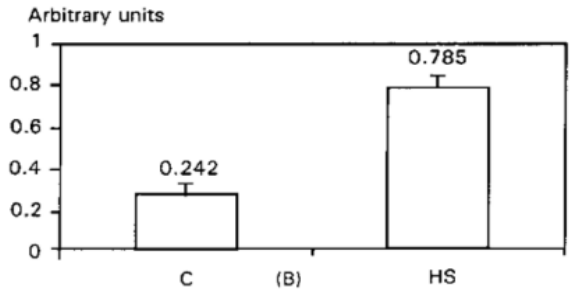


Fig. 2(A-B): RT-PCR analysis of polyubiquitin mRNA levels from *B. arabica* after heat stress. (A) Amplification products were separated in 1.2% agarose gel. Digests of A-DNA with Hind III were used as DNA size marker (M). (B) Quantitation of polyubiquitin mRNA levels. Results are expressed in arbitrary units as the ratio of opitcal density of polyubiquitin/βactin electrophoretic bands

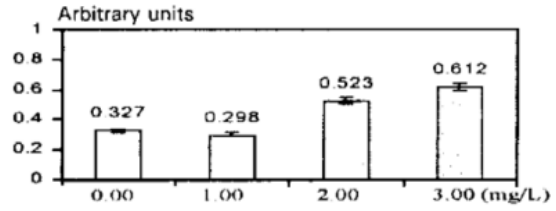


Fig. 3: Quantitation of polyubiquitin mRNA levels from *B. arabica* exposed to different concentrations of cadmium (0, 1, 2 and 3 mg/L, 336 h)

likely to be the primary mechanism for increasing the pool of ubiquitin for protein ubiquitination and subsequently nonlysosomal degradation of stress-damaged proteins. Such mechanism requires, primarily, attachment of ubiquitin to stress-damaged proteins prior to degradation (Rechsteiner, 1991; Hershko and Ciechanover, 1992; Jentsch, 1992). It was concluded that both heat and cadmium stress can markedly increase the expression of polyubiquitin similar to what has been recently recorded for the expression of HSP70, possibly through a common mechanism of gene induction (Al-Khedhairy, 2000, 2001). Further more, alterations in the expression of polyubiquitin may be necessary for the degradation of damaged or missfolded proteins and that this may be used as a biomonitor to assist water pollution with cadmium.

Future work will focus on the effect of other pollutants on the expression of polyubiquitin.

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