EUROPEAN ACADEMIC RESEARCH
Vol. III, Issue 8/ November 2015

Impact Factor: 3.4546 (UIF)
DRJI Value: 5.9 (B+)

ISSN 2286-4822
www.euacademic.org

Screening of chicken RBC's for the induction of heat stable protein (HSP40) in response to anisomysin

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Abstract:

Stress response is a universal mechanism developed by all organisms to deal with adverse changes in the environment, which leads to the synthesis of heat shock proteins (HSPs). Cells exposed to conditions of proteotoxic stress, like heat shock express a family of proteins called heat shock proteins including members of the HSP100, HSP70, HSP60, HSP40 and a group of small heat shock proteins. These proteins have a critical role in recovery of cells from stress and cytoprotection, guarding cells from subsequent insults. They prevent stressed cells by their ability to recognize nascent polypeptides and exposed hydrophobic stretches of amino acids. In doing so, chaperones hold, translocate or refold stress denatured proteins and prevent their irreversible aggregation with other proteins in the cell. In addition to their role in protecting cells from stress nearly all heat shock proteins are constitutively expressed under normal growth condition, where they function to maintain protein homeostasis by regulating protein-folding quality. In the present study chicken RBCs were exposed to anisomysin to show stress protein induction in the molecular weight range of 40kDa in a time dependent manner. It was observed that

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HSP40 level increased with time and reached maximum at 5hrs in response to anisomysin.

Key words: HSP40, Heat shock protein, Chick RBC

INTRODUCTION

Living organisms from simple unicellular bacteria to complex multicellular organisms like humans when confronted with any kind of environmental stress elicit a common and highly conserved response called "Stress Response" [1]. Diverse conditions like treatments with drugs, exposure to higher concentration of amino acids, transition series metals and growth at elevated non-morphological temperatures may give rise to stress response [2]. The continued variations in the living environment of cells and organisms have necessitated the evolution of stress response network to detect, monitor and respond to environmental changes. Prolonged exposure to stress interferes with the efficient operations of the cell with negative consequences on the biochemical properties of proteins that under ideal conditions exist in thermodynamically stable state [3] .

In stressed environment proteins can unfold, misfold or aggregate [1]. The stress response results in the induction of a limited number of proteins, which eventually becomes the major polypeptide products of the stressed cell, both in terms of synthesis and accumulation. Most of the stressed proteins are expressed constitutively in cells under normal growing conditions and represent essential gene products involved in a number of important biological pathways. Increase in the synthesis of these proteins in stressed cells appear to result from the near exclusive synthesis of mRNA coding for these proteins as well as reduced translation of preexisting mRNA coding for other cellular polypeptides [4].

Expression of stress proteins also called as heat shock proteins is regulated at the level of transcription in both eukaryotes and prokaryotes. In prokaryotic cells this expression is under the control of a specific sigma factor called σ32, which directs the bacterial cell RNA polymerase to heat shock promoter [5-7]. In eukaryotes, heat shock genes are under the control of conserved regulators referred to as heat shock transcription factors (HSF). Heat shock proteins have been studied extensively in lower organisms and in mammalian tissues. Some of these proteins have been cloned and sequenced. Avian tissues respond to stress in a manner analogous to other organisms. The patterns of heat shock protein synthesis in response to different stimuli have been studied in some chicken tissues. Heat shock proteins have been classified into six major families – HSP100, HSP90, HSP70, HSP60, HSP40 and small heat shock proteins [8-12]. Keeping in view the importance of these heat shock proteins present study was under taken as a preliminary study to observe the induction of HSP40 when chicken RBCs were exposed to anisomysin.

MATERIALS AND METHODS

Freshly collected chicken blood was used for the present study. 0.45mM EDTA was used as an anticoagulant. RBCs were isolated by centrifugation at 5000 g for 5 minutes. Cells were lysed by adding lysis buffer (Table 1, A). Hemolysate was centrifuged at 10,000g for 30 minutes to pellet down ghosts. Supernatant was removed carefully and was heated up to 90-95^{o}C. Denatured proteins were removed by centrifuging the solution at 10,000g for 10 minutes. Above step was repeated several times. After final step of centrifugation supernatant was removed carefully and the pellet was discarded. Supernatant containing only heat stable proteins was analyzed on 10% SDS PAGE gel. Heat stable proteins were isolated from

heat shock (at 42°C) and anisomysin (1mM) treated cells. Freshly collected blood was subjected to heat shock and anisomysin treatments for different time periods i.e. 1hr, 2hrs, 3hrs and 5hrs. Heat stable extract isolated from the treated cells were analyzed by SDS PAGE and Western Blot for expression of heat stable proteins.

SDS-PAGE (Sodium Dodecyl Polyacrylamide Gel Electrophoresis):

SDS PAGE was performed using the method of Lamelli [13]. Samples were electrophoresed on slab gel (0.75nm thick) that consisted of 5% stacker and 10-12% resolving gel. On an average 1.5cm long staking gel and 4cm long separating gels were used. Electrophoretic run was performed at a constant current of 12mA until the bromophenol tracking dye reached the bottom of separating gel.

Silver Staining:

Gel was first soaked in a prefixing solution A (Table 1, B) for 30 minutes and then in a prefixing solution B (Table 1, C) for another 30 minutes. Finally the gel was fixed with 10% glutraldehyde for 30 minutes to retard protein movement or diffusion. After rinsing the gel with double distilled water several times, gel was soaked in 5µg /ml DTT for 30 minutes. After DTT treatment gel was soaked in 0.1% silver nitrate for 30 minutes and was rinsed once with double distilled water and twice with a developing solution (7% formaldehyde) until the protein bands become visible. Staining process was stopped by adding 5 ml of 2.3 M citric acid.

Western Blot Analysis:

Cytosolic proteins from liver were extracted by homogenizing the tissue in lysis buffer containing 20 mM Tris acetate pH 7.6, 20 mM NaCl, 0.1mM EDTA and 0.1 mM β-mercaptoethanol.

Then, the mixture was centrifuged and the supernatant was collected for use as protein sample. Protein quantification was performed by the Bradfold method.

Procedure:

Proteins solution was electrophoresed on 10% SDS PAGE gel and transferred onto a PVDF membrane. For this PVDF membrane was first soaked in methanol, rinsed several times with water and then soaked in 1X transfer buffer. Blotting papers and porous pads were also soaked in 1X transfer buffer and the transfer apparatus was set as follows – bottom plate anode, porous pads, blotting paper, PVDF membrane, gel, blotting paper, porous pads and finally cathode. PVDF membrane was marked to follow orientation. Air bubbles were removed by using gloved hands and by rolling a clean pipette over the sandwich. Transfer was commenced by connecting the electrodes and was run for 60 minutes at a constant voltage (70V). Apparatus was carefully disassembled and the PVDF membrane was stained with Commassie Brilliant Blue R250 for one minute and was destained using methanol. The transferred blots were blocked with 3% BSA in TBS for 4 hours. The blot was then incubated with the required antibodies in 3% BSA plus 0.02% Tween 20. After incubation blot was washed several times with TBS containing 0.02% Tween 20. Finally the blot was incubated with anti goat ALP secondary antibody (Genei) for 1 hour. After incubation blot was washed with TBS (three times). Following the final wash 10ml of BCIP/NBT solution (Fermentas) was added to the blot and incubated for 5- 10 minutes until the desired color intensity was obtained.

Table: Composition of various buffers and reagents used

A. Lysis Buffer

1.5 M Tris Cl (pH-7.6)	0.67ml
1M KCl	1 ml
1M MgCl2	1 ml
0.5M EDTA	0.4ml

The volume of the solution was made up to 100ml with distilled water and was stored at 4°C.

B. Silver stain Solution A

Methanol	50ml
Acetic acid	10ml
Water	40ml

C. Silver stain Solution B

Methanol	5ml
Acetic acid	88ml
Water	7ml

RESULTS AND DISCUSSION

The enhanced synthesis of few proteins immediately after stress was first reported for Drosophila cells [14] and the universality of the response from bacteria to human was recognized shortly afterwards [15]. The genes encoding HSPs are highly conserved and occur in every species in which they have been sought [16]. Many of the genes and their products can be assigned to families on the basis of sequence homology and molecular weight [17]. In eukaryotes many families comprise multiple members that differ in inducibility, intracellular localization and function. Depending on their geographic locale, organisms in nature risk exposure to temperatures ranging from -100 to more than +100°C and comparable extremes of chemical and gaseous substance, food and water availability, hydrostatic pressures, radiations and other toxic substances. Seemingly HSP expressions should be a common occurrence in nature. In reality, however, movement and or other behaviors may often enable organisms to avoid

HSP inducing stress in nature by exploiting equable microhabitats in otherwise stressful environment. Also biochemical specializations other than HSPs may stabilize many organisms (or particular stages of their life cycle) so that environmental extremes are not particularly stressful. But it has been found that even equable environments can contain HSP inducing microhabitats, and even mild stresses can induce HSPs when multiple stresses act in combination [2].

The RBC's of chicken, like those of other non-mammalian vertebrates, retain their nucleus, mitochondria and other cytoplasmic organelles and are capable of transcription and translation. SDS PAGE analysis shows that circulating chicken RBC's synthesize a range of heat stable proteins including heat shock proteins. Thus chicken RBC's represent a useful model for protein synthesis studies. HSP40, HSP70 and HSC70 are present in all areas of both adults and embryonic chicken lens. Presence of HSP in the deep cortex and nucleus is intriguing as no detectable metabolic activities are reported in these areas. However, it has been proposed that HSP40, HSP70 and HSC70 interact with protein of these areas and protects them from stress induced denaturation [18]. In cultured chick pineal cells heat shock proteins HSP25, HSP70 and HSP90 are synthesized under temperature conditions that also causes changes in melanin production [19,20].

We investigated the expression of heat stable proteins in chicken RBC's in response to heat and anisomysin. Although different heat shock proteins are synthesized in avian cells in response to any kind of stress, we have focused on HSP40 owing to their description as major stress inducible proteins in chicken cells. They have also been shown to be the most consistent in their expression after stress. Their expression in response to heat and anisomysin was studied. In response to anisomysin heat stable protein in the molecular weight range 40KDa was induced. To characterize the protein synthesized in

response to anisomysin heat stable extracts from chicken erythrocytes were analyzed by western blotting.

A time course study showed the expression of HSP40 in response to anisomysin (Fig1). Nature of the proteins induced in response to anisomysin was confirmed by western blotting analysis using αHSP40 antibodies (Fig 2).

In response to heat, protein in the molecular weight range of 20-40 was induced as revealed by SDS PAGE analysis (Fig1). Time course study indicated that the concentration of this protein increases with time and reached maximum at 5 hrs.

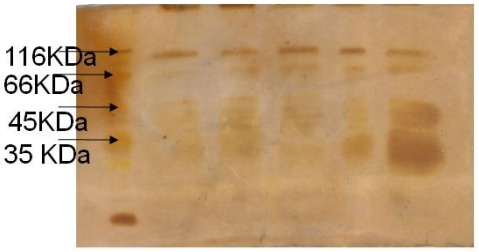

Fig 1: Analysis of protein induction by heat shock at different times. After incubation at 42°C for 1hr (lane 3), 2hrs (lane 4), 3hrs (lane 5) and 5hrs (lane 6) cellular extracts were prepared and analyzed by SDS PAGE. Equal volumes were loaded in each lane. Control sample was treated similarly.

Fig 2: Western blot analysis of HSP40 induction in response to anisomysin. RBCs were treated with 0.1mM anisomysin for different time periods. After incubation for 1hr (lane 1), 2hrs (lane 2), 3hrs (lane 3) and 5hrs (lane 4) heat stable proteins were isolated and subjected to one-dimensional electrophoresis followed by western blotting. Equal volumes were loaded in each lane. Protein transfer was performed at 70 V for 1hr. 3% BSA was used as a blocking agent. Primary antibodies were used at a concentration of 1μg /ml and the

concentration of secondary antibodies used was 1µg /5ml. ALP conjugated anti goat secondary antibody was used. Blot was developed with NBT/BCIP mix

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