EXPRESSION PROFILING OF HEAT SHOCK, SUPEROXIDE DISMUTASE AND CATALASE GENES IN BLACK TEA EXTRACT (BTE) TREATED *Drosophila melanogaster*

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Abstract

Black Tea extract (BTE) is a mixture of the aflavins and epicatechins. They act as effective antioxidant because of their free radical scavenging and metal chelating properties. We have investigated the effects of BTE on heat stressed Drosophila melanogaster. Our results revealed that acute heat stress (37°C for 30 min - 4hrs) caused induction of heat shock puffs in salivary gland chromosomes of the third instar larvae and increased level of reactive oxygen species in mitochondria. Addition of 400µg/ml BTE in Ringer, caused changes in transcription profiling in heat shock puffs in larval salivary gland chromosomes of D. melanogaster OR strain. Similarly, 10mg/ml BTE in food media enhanced the level of expression pattern of superoxide dismutase (SOD1 and SOD2) and catalase (CAT) enzymes. These results have been interpreted to have suggested that anti-stress activities of tea polyphenols at least in part are mediated through changes in the expression pattern of heat shock, sod and cat genes.

Keywords: Tea polyphenol, heat shock, catalase, superoxide dismutase

INTRODUCTION

All living organisms experience a variety of stressful situation as a part of their normal life. The stresses range from toxin, heavy metals, oxidants, elevated temperature, ionizing and non-ionizing radiations, and emotion or neural stress. Stress cause DNA damage, protein denaturation and mis-folding by weakening of polar bonds and exposure of hydrophilic groups and ultimately lead to death (Biamonti and Caceres, 2009). Organisms encounter these stressors as a homeostatic mechanism to protect the cellular machinery from damages inflicted by a variety of adverse environmental factors to minimize their effect. To encounter stress, cell initiate a dramatic change in pattern of gene expression including elevated synthesis of a family of heat shock (hsp) and/or stress response genes (Craig *et al.,* 1993; Wallin *et al.,* 2002). These co-ordinated changes in transcriptional and translational activities result a massive accumulation of newly synthesized transcriptional and translational products in the stressed cells. Thus, the stress response is not only a good for studying co-ordinated gene function, but also has acquired considerable applied importance from the point of view of health research because of the involvement of HSPs in many human disorders or diseases including immune response.

In both vertebrates and invertebrates, two antioxidant systems are active to scavenge ROS and minimize their associated adverse effect. First, they produce endogenously a group of antioxidant enzymes including superoxide dismutase (SOD), glutathione peroxidase (GPs) and catalase (CAT), which serves as a first line of defense against ROS (Li *et al.,* 2007). Second, exogenous antioxidants such as vitamin C and E build a secondary defense base to terminate the propagation of ROS reactions and show the aging process.

Although, there are some reports on the heath benefit of a group of theaflavins (TF) (Kuroda and Hara, 1999; Pietta, 2000; Gupta *et al.*, 2002; McKay and Blumberg, 2002; Deb and Chatterjee, 2008; Yang *et al.,* 2008) or thearubigins (TR) (Weisburger ., 1996; Nakachi *et al.,* 2003; Sharma and Rao, 2009; Bhattacharyya *et al.*, 2009) of black tea, in different system, the combined effects of the complex bioactive compounds of tea in considering the overall health benefit of food have not been examined adequately. Therefore, biological activities and chemo-preventive properties of black tea and its components are poorly understood since the total antioxidant capacity of tea is not related to a particular kind of polyphenol and the combined activity of diverse anti-oxidants including phenolic acids and polyphenols in black tea extract; we must consider the combined effects of BTE, for understanding the overall health benefit of teas. BTE contains TF and epicatechins, act as an excellent source of dietary antioxidants (McKay and Blumberg, 2002). Since a conserved stress response pathway exist in both invertebrates and vertebrates and since the molecular mechanisms by which tea polyphenols extended life span have not been fully revealed in any organism, it therefore, remains unproven that anti-stress activities of tea polyphenols is accomplished by similar mechanism in different species or not.

Fruit fly, *Drosophila melanogaster* has been employed in stress and aging research because it shares many conserved biological pathways and >70% of known disease causing genes in human are conserved in the species (Minois, 2006). Furthermore, the fly has all usual advantages of model organism for genetic manipulation and molecular physiological analysis. Since Drosophila is sexually dimorphic, it is easy to investigate sex specific differences of effect of the tea polyphenols. Therefore, *D. melanogaster*, has many strength as a model organism for studying of stress response and extension of life span by tea polyphenols. Information on the mechanisms by which stress response by intake of tea polyphenols in these invertebrates is ikely to inform our understanding the process in mammals. The present study was therefore, conducted to examine (i) whether like other stress response, BTE can modulate the expression profile of heat shock puffs in Drosophila melanogaster and (ii) the interaction between dietary tea polyphenols, on gene expression patterns of endogenous antioxidant enzymes, SOD, catalase in D. melanogaster.

MATERIALS AND METHODS

Reagents: All the chemicals used were of analytical grade and procured from Sigma, USA.

i) *Drosophila* stocks:

Following *Drosophila* mutant stocks are used in present investigation:

a) *Drosophila* wild type strain (Oregon R)

b) SOD^{n108}/TM3 (SOD^{n108}) and OE^{-}/SM5; Cat^{n1}/TM3 (Cat^{n1}), obtained from Bloomington *Drosophila* stock center, Department of Biology, Indiana University Bloomington, USA)

ii) Culturing procedure

Standard food medium containing corn-meal-molasses-yeast-agar-agar was prepared. Propionic acid was added as a mold-inhibitor. The food medium was supplemented with live yeast for better nourishment. Adults and all developmental stages were reared in a BOD incubator at 24°±1°C with 80% relative humidity.

iii) Synchronization of age

To obtain third instar larvae and adults of similar group, eggs were collected in bottles at 2 hour interval. Adults as well as larvae were reared in *Drosophila* food media.

iv) ^{3}H-uridine autoradiography

For uridine autoradiography, excised salivary glands were incubated in 10µl of *Drosophila* Ringer containing 5µCi of 3H-uridine for 10 min (specific activity 13,600 mCi/mM, obtained from BARC Trombay). The glands were thereafter prepared as Chatterjee (1990) and slides

were processed for autoradiography following usual methods. The exposure time was 10 days.

v) Measurement SOD activity:

The principle is that a tetrazonlium salt can detect superoxide anion generated by xanthine oxidase and hypoxanthine while SOD is able to remove the superoxide anion. In general, one unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide anions. Based on this to measure the SOD 200 fruit flies were homogenized in 1 ml of cold 20mM HEPES buffer (pH 7.2,with 1mM EDTA 210mM mannitol and 70mM sucrose) and then centrifuge at a speed of 1500g for 5 min at 4°C. The supernatant was transferred to centrifugation at 10,000g for 15 min at 4°C. The supernatant contain the cytosolic copper-zinc containing SOD (CuZn Sod), and the pellet contained mitochondrial manganese containing SOD (Mn SOD). The supernatant was transferred to a new tube and the mitochondrial pellet was resuspended in 0.5 ml cold HEPES buffer. The sample (10µl) in triplicates was used for each test. The diluted radical detector containing tetrazolium salt. (200µl) was added onto 96-well plates together with 10µl sample. The reaction was initiated by adding 20µl of diluted xanthine oxidase and then shaking the plate for 20 min at room temperature. After incubation, the absorbance was recorded at 450nm using a spectrophotometer.

vi) Measurement of Catalase activity:

To measure the Catalase (CAT), we measured the hydrogen peroxide substrate remaining after the action of CAT present in the sample. 100 flies were homogenized in 1 ml enzyme dilution buffer and then centrifuged at a speed of 1500g for 5 min at 4°C. The supernatant was moved into a new tube and diluted 15 times by 1x assay buffer (5mM potassium phosphate buffer, pH 7.0) in triplicates. The resultant sample (10µl) was diluted again with 65µl of 1x assay buffer. Then, 25µl of 200 mM hydrogen peroxide solution was added to initiate the reaction. At exactly 1 min, 900µl of stop solution (15mM Sodium azide) was added. The reaction mixture(10µl) was mixed with 1 ml color reagent containing 0.25mM 4-aminoantipyrine, 2mM 3,5-dichloro-2-hydroxybenzenesulfonic acid, and freshly added peroxidase (0.8-1.2 U/mg). After incubation at room temperature for 15 min, absorbance of each sample was measured using a spectrometer at 520nm.

Western blot analysis

For western blot analysis, total proteins were extracted from 50 flies and were homogenized in a 1.5 ml tube containing 250 µl homogenizing buffer (20mM Tris-HCl, 2mM $MgCl_2$, and 0.2 M Sucrose). The extract was centrifuged at 10,000g for 10 minutes at 4°C and the supernatant was collected. Protein concentration was determined using UV spectrophotometric. The protein was boiled at 95°C temperature for 5 minutes after adding 5x loading dye and homogenizing buffer then stored at -80°C. For measurement of SOD1 and SOD2, 50µg total protein was size-fractionated on a12% SDS-PAGE gel at 100v for 70 minutes. For the measurement of CAT and β-actin 50µg total protein was loaded and size-fractionated on a 7% SDS-PAGE gel at 100V for 180 minutes. The protein was then transferred onto membrane. The membrane was incubated for one hour in blocking solution (5% non-fat milk) at room temperature and then in the same solution containing diluted anti-catalase/anti- β-actin/antiSOD1/antiSOD2 antibodies, respectively, at 4°C for overnight. The membrane was then washed in 1 x TBST and incubated for one hour at 4°C in anti-rabbit IGG. The washes were repeated before the membranes were developed.

RESULTS

a. Effect of tea BTE on polytene puffs

As it has been noted earlier, so here, when *D. melanogaster* larval salivary glands were heat shocked at 37°C for 30 min, a small number of genes are induced while most other genes become inactivated. These genes are known as heat shock genes and are located in different chromosomal locations. These sites activated almost immediately after heat shock and known as heat shock puffs, located in the chromosomal position 63BC, 64F, 67B, 70A, 87A, 87C, 93Dand 95D in the 3L and 3R chromosome respectively. These puffs sites produce heat shock RNAs. These heat shock RNAs translated into eight polypeptides in *D. melanogaster* with molecular weights 82, 70, 68, 36, 27, 26, 23, and 22K Dalton respectively.

Our results further revealed that at 24°C, BTE (400µg/ml) treatment failed to activate heat shock puffs except 93D locus and to inhibit chromosomal RNA synthesis other sites. Curiously, when heat stressed salivary glands (37°C) were treated simultaneously with BTE for 30 min, a considerable change in heat shock puffing pattern was noted. For example, the 93D puff of 3R was regressed and some additional puffs were induced. For example, the 74EF puffsite had been induced considerably after treatment of BTE at 37°C. Autoradiographic analysis of salivary gland, exposed to a combination of BTE in Ringer (pH7.2) and heat shock (37°C) also show that such combined treatment caused specific alteration in transcription pattern of the major heat shock loci (Fig. 1). Likewise, when salivary glands were treated with BTE at 4°C , a drastic alteration in the puffing activity was noted. We also observed that exposure of gland to a cold shock of 4°C in presence of BTE induced a number of new puffs at many sites in addition of the 93D sites (data not included). These alterations of puffing activity pattern indicate that BTE induce some alternative metabolic pathways to protect the cell from stressful condition. The results together have been interpreted to have suggested that BTE stimulate the rescue effect of heat shock cells in *Drosophila* by inducing a discrete set of puffs.

b) Effect of dietary supplementation of BTE on expression pattern of SOD, CAT of Oregon R flies

When we examined the effects of the treatment of 200µg/ml BTE containing diet, we noted that the expression patterns of the enzyme SOD (CuZn Sod), and SOD (Mn SOD) of Oregon R males were not changed significantly. In fact, no significant difference in SOD (CuZn Sod), SOD (Mn SOD) activity was noted between the control and BTE treated experiments. However, expression pattern of SODs and CAT were increased significantly with dietary supplementation of 400 µg/ml BTE treated heat shocked flies than the control diet (Fig.2).

DISCUSSION

The present study showed that BTE induces 93D puff at 24°C. However, when salivary gland (37°C) was treated simultaneously with BTE and heat at 37°C for 30 min, most heat shock puffs were induced except 93D puff. These data indicate that apparently, there are some similarities in response of polytene nuclei to the heat shock and BTE treatment during stress condition. Heat shock and BTE induce some common stress puffs considerably. Both treatments also inhibit chromosomal RNA synthesis except the stress puffs. These observations are in agreement with the findings of Cui *et al.*(1999) who also noted that the intracellular defense system is activated by tea polyphenol TF. However, it is not known form the present data how BTE neutralize the cellular stress response by up-regulating the activity of some stress puffs. It is possible that tea polyphenols switch on an alternative pathway through post transcriptional control of heat shock factor and protein- protein interaction in vivo (Gething and Sambrook, 1992).

The aftermath of stress is the production of stress proteins which help to protect the organisms, and for such protein encoding there are some regulatory genes present which are responsible for protecting the macromolecules and other cellular components. Generally, low levels of stress trigger subsequent beneficial effects. This phenomenon, sometimes called 'hormesis' influences stress transient heat shock can extend the life span of flies and worms.

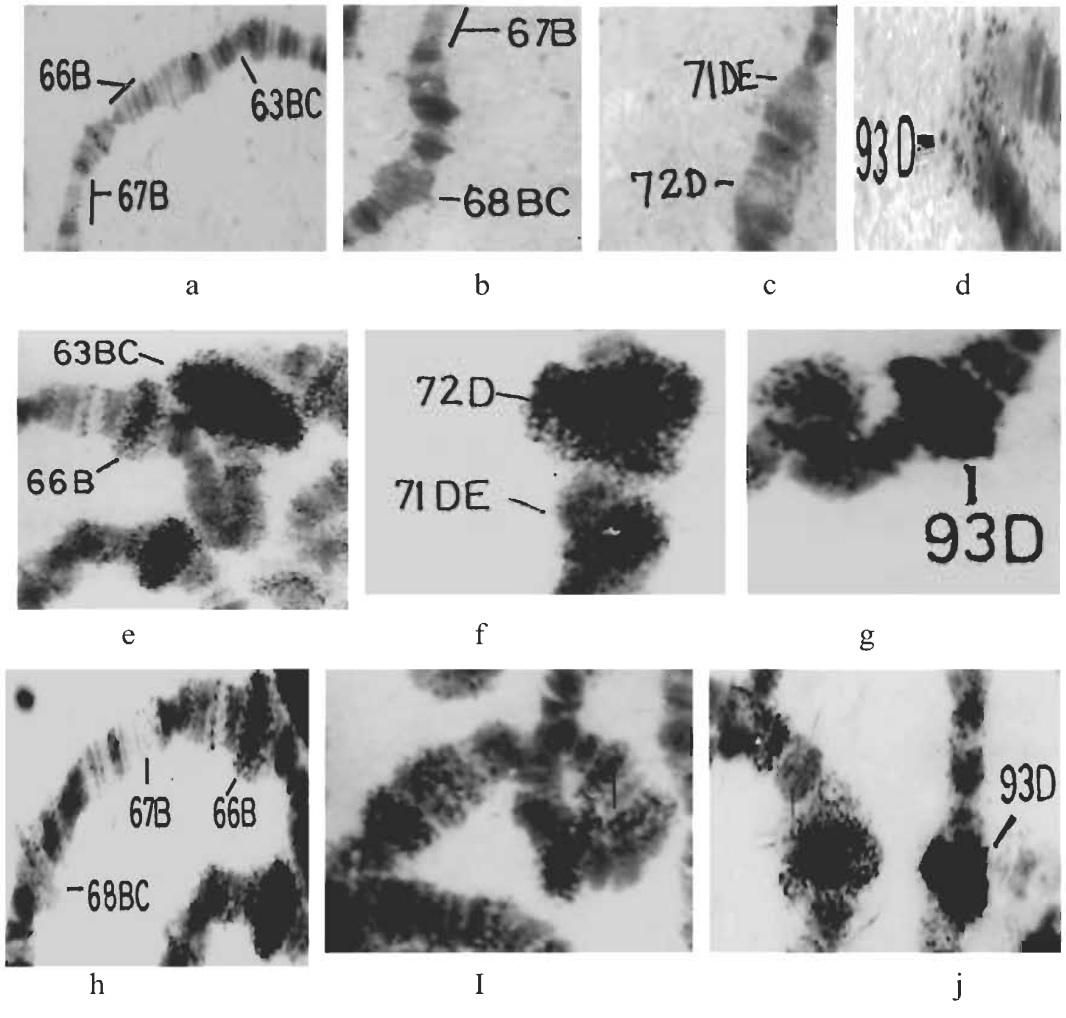

Fig. 1. Puffing and ^{3}H-uridine incorporation pattern of the salivary gland chromosomes of *Drosophila*, (a-d) Puffing activity and ^{3}H-uridine incorporation pattern in control (non-heat-shocked) animals; (e-g) Puffing activity and ^{3}H-uridine incorporation pattern in heat shocked *Drosophila*; (h-j) Puffing activity and ^{3}H-uridine incorporation pattern in heat shock plus BTE treated *Drosophila*. Note that heat shocked puffs were induced in response to heat treatment and some heat shocked puffs were activated by BTE treated animals at 37°C. The puffing pattern and the ^{3}Huridine incorporation pattern of the puffs are indicated in the corresponding figures.

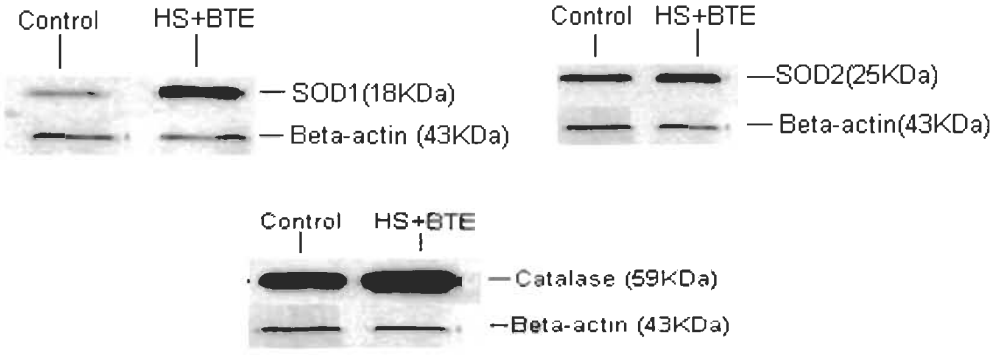

Fig 2: The expression pattern of the SODs and Catalase in relation to βactin (control). Note the effect of dietary supplementation of black tea extract (BTE) on the gene expression of SOD1, SOD2 and Catalase in wild types Oregon R flies after Heat shock.

The results presented in the paper further showed that when the heat stressed flies were supplement with 10mg/ml dietary BTE, we noted that the antioxidant enzymes SOD1, SOD2 and CAT were expressed at higher level than only heat shock response (Fig. 2). This may imply that BTE induce antioxidant enzymes considerably in heat stress D. melanogaster. Precisely, heat stressed Drosophila grown in BTE supplement media, activate the SOD1, SOD2 and CAT enzymes significantly to inhibit oxidative modification in the body (Orr and Shoal, 1993; Ying et al, 2004; Li et al. 2007). The expression patterns of the enzymes were analyzed several times and we obtained similar results. These data together indicate that BTE can induce the antioxidant enzyme activities in response to heat stress. However, the mechanism is not clear at present. Further research in the line may provide valuable insight into molecular mechanism of BTE regulation in stress response of the fruit flies.

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