Effects of Eicosanoid Biosynthesis Inhibitors on Selected Oxidative Stress Biomarkers in the Midgut of *Galleria mellonella* (Lepidoptera: Pyralidae) Larvae[1]

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J. Entomol. Sci. 49(2): 144-155 (April 2014)

Abstract Eicosandoids, or icosanoids, are signaling compounds created by the oxidation of 20-carbon fatty acids. They control many complex physiological and immunological functions in vertebrate and invertebrate animals. This study tested the hypothesis that eicosanoids act in insect antioxidant defense. The effects of 3 eicosanoid biosynthesis inhibitors (EBIs) – dexamethasone, esculetin, and phenidone – on the oxidative stress indicator, malondialdehyde (MDA), and the detoxification enzyme, glutathione S-transferase (GST), was examined in the midgut of larvae of the greater wax moth, *Galleria mellonella* (L.). The larvae were reared on artificial diets supplemented with 0.001, 0.01, 0.1 or 1.0% of the EBIs. Esculetin, which is a lipoxygenase inhibitor, significantly increased MDA content; whereas, GST activity was significantly increased at only the highest concentration tested. Dexamethasone, a phospholipase A_2 inhibitor, significantly increased MDA content and GST activity at concentrations of 0.01, 0.1, and 1.0%. Phenidone, a dual inhibitor of cyclooxygenase and lipoxygenase, increased MDA content, whereas the 0.01 and 0.1% concentrations of phenidone significantly increased GST activity. Our results indicate that antioxidative responses are, at least in part, controlled by a physiological system that includes eicosanoid biosynthesis.

Key Words *Galleria mellonella*, eicosanoid biosynthesis inhibitors, GST, malondialdehyde

Antioxidant systems in insect midgut tissues are of crucial importance in the defense mechanisms against xenobiotics which endogenously produce reactive oxygen species (ROS) in insects. Elevated levels of radicals from xenobiotics, such as plant secondary metabolites, are associated with increased oxidative stress in midgut tissues of lepidopteran larvae and are linked to decreased larval performance. Therefore, larval tissues are believed to be susceptible to reactions with ROS in biological systems causing nutritional depletion through adult emergence (Barbehenn and Stannard 2004, Krishnan and Kodrík 2006, Barbehenn et al. 2008, 2012).

Xenobiotics are associated with the production of free radicals, which react with various biomolecules and impair cell functions (Halliwell and Gutteridge 1999). During the methabolism of xenobotics, such as prooxidant allelochemicals, heavy metals, or pesticides, ROS's are produced. These radicals are scavenged by innate antioxidant defense systems including antioxidant enzymes and various antioxidant compounds. Apparent insufficiencies of antioxidant defense system result in increased ROS's which

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causes oxidative stress (Schafer and Buettner 2001). ROS's are detrimental through reaction with many cellular biomolecules, including proteins, lipids, enzymes, carbohydrates, and DNA (Dean et al. 1997, Beck and Levander 1998). Insects have evolved a complex antioxidant mechanism to overcome the toxic effects of ROS. The antioxidant defense is primarily constituted of the enzymatic action of glutathione peroxidase (GPX), catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase, and glutathione transferases (GSTs) (Barbehenn 2002). This enzymatic complex is capable of inactivating some xenobotics and their derivatives (Li et al. 2007). GSTs are major cellular detoxification enzymes. In insects, GSTs are involved in the transformation of many insecticides, and their over-expression is responsible for development of resistance against those insecticides (Fournier et al. 1992). The detoxification system can also contribute to the defense of insects against entomopathogenic fungal toxins and bacterial and viral infections (Serebrov et al. 2006, Dubovskiy et al. 2008, Büyükgüzel et al. 2007). GSTs exhibit nonselenium-dependent glutathione peroxidase activity and can remove highly reactive electrophilic components, such as lipid hydroperoxide (Storey 1996, Hurst et al. 1998). Lipid peroxidation is initiated by ROS to generate lipid hydroperoxide, such as malondialdehyde (MDA) and trans4-hydroxy-2-nonenal (4-HNE) end products. After exposure to xenobotics, increased MDA levels have been correlated with a variety of tissue and cell membrane damage in animals (Suwalsky et al. 2001).

One of the important growth areas in the physiology of insects is our increasing appreciation of the many information-bearing molecules and their signal transduction systems responsible for organismal integration and homeostasis. Eicosanoids are bioactive products synthesized from certain polyunsaturated fatty acids such as arachidonic acid (AA) (Stanley 2006). Eicosanoids are well known for their important actions in mammalian physiology and disease. Some studies demonstrated that eicosanoids mediate oxidative and antioxidative responses through ROS-mediated damage in mammalian tissues (Hatamoto et al. 2006, Kim et al. 1998). Eicosanoids act in many areas of insect biology, including ion transport, protein trafficking, reproduction, and immune defense, in which they variously exert stimulatory and inhibitory influences (Downer et al. 1997, Miller and Stanley 2001, Dean et al. 2002, Tunaz et al. 2003, Tunaz 2006, Büyükgüzel et al. 2007, Stanley and Shapiro 2007, Durmuş et al. 2008, Figueiredo et al. 2008, Merchant et al. 2008, Stanley et al. 2008). On the basis of this information, Büyükgüzel et al. (2010) first tested the hypothesis that eicosanoids act in antioxidant defense in the hemolymph of insects and in maintaining homeostatic physiology. Those results led to investigation of the role of eicosanoids in antioxidant defense systems of other insect tissues. Reported here are results of eicosanioid biosynthesis inhibitors (EBIs) alteration of oxidative and antioxidative responses as evidenced by increased midgut MDA content and GST activity in the midgut of the greater wax moth, *Galleria mellonella* (L.), larvae.

Materials and Methods

Insects. *Galleria mellonella* larvae used in this study were from a laboratory colony maintained on an artificial diet according to standard methods (Bronskill 1961) at 30 ± 1°C in constant darkness. Fifteen newly-emerged adult females were placed in jars and provided a piece of old honeycomb on the diet for egg deposition and feeding for neonatal larvae. The methods used to prepare and dispense diets into containers, to obtain eggs, and to place larvae on the diets were described in previous studies (Büyükgüzel et al. 2007, Hyršl et al. 2008).

Midgut collection. Last-instar larvae were used to determine the lipid peroxidation product, MDA content, and detoxification enzyme activity in the midgut. Larvae were chilled on ice for 5 min and then surface-sterilized with 95% ethanol. Larvae were dissected longitudinally with an incision starting anterior to the first pair of thoracic legs and extending just posterior to the third pair of abdominal prolegs using dissection scissors. The midguts were removed with fine-tipped forceps under a stereomicroscope (SZ61, Olympus, Tokyo, Japan). Adhering fat body, Malpighian tubules, and gut contents were then removed. Collected midguts were placed into a chilled Eppendorf tube filled with cold homogenization buffer [w/v, 1.15% Potassium Chloride, 25 mM dipotassium hidrogen phosphate (K_2HPO_4), 5 mM ethylendiaminetetraecetic acid (EDTA), 2 mM phenylmethylsulfonyl fluoride (PMSF), and 2 mM dithiothreitol (DTT), pH 7.4], and were immediately frozen and stored at -80°C until used in the assays.

Chemicals. PMSF, DTT, butylated hydroxytoluene, bovine serum albumin, Folin-Ciolcalteu reagent, EDTA, thiobabituric acid (TBA), trichloroacetic acid TCA, K_2HPO_4, glutathione (GSH), 1-chloro-2,4-dinitrobenzene (CDNB), glycerol, ethanol, sodium chloride, phenylthiourea, esculetin (6,7-dihydroxycoumarin), and phenidone (4-methyl-1-phenyl-3-pyrazolidinon) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Dexamethasone [(11β,16α)-9-fluoro-11 - 17,21-trihydroxy-16-methylpregna-1,4-diene-3, 20-dione] was donated by Deva Holding (Levent, Istanbul).

Experimental design. Technical grade esculetin (98%), dexamethasone (98%), and phenidone (97%) were diluted in 1 ml of ethanol (70%) and then further diluted to the target concentrations of 0.001, 0.01, 0.1, or 1.0 g in 100 g of diet, as determined in a previous study (Büyükgüzel et al. 2007). Phenidone was tested at concentrations 0.001, 0.01, or 0.1% because the 1.0% concentration inhibited larval growth and prevented production of seventh-instar larvae. Diluted compounds were then added to the diets as substitutions of water during diet preparation. First-instar larvae were reared to seventh instars on an artificial diet amended with given concentrations of the individual EBIs. Preliminary results showed that ethanol at the volume used did not affect biochemical responses. Furthermore, because ethyl alcohol is less toxic and stimulates food consumption of insects (Norris and Baker 1969), it was used as a solvent. Using standard laboratory rearing conditions, the influence of EBIs esculetin, dexamethasone, and phenidone to selected oxidative stress parameters was determined. The parameters were lipid peroxidation level in terms of MDA content and activity of antioxidant enzyme glutathione S-transferase (GST) in the midgut of the last-instar larvae.

Sample preparation and determination of MDA content and GST activity. Extracts of the midguts collected and stored as previously described were prepared at 4°C by an ultrasonic homogenizer (Bandelin Sonoplus, HD2070, Berlin, Germany) at 50W, 40 - 50s in homogenisation buffer and subsequent centrifugation at 10,000 × g for 10 min. The resulting cell-free extracts were collected for biochemical analysis. Protein concentration was measured according to Lowry et al. (1951) using bovine serum albumin as a standard. Absorbance measurements were made on a Shimadzu UV Spectrophotometer at 595 nm.

Lipid peroxidation was assessed by determination of MDA, an end product of fatty acid peroxidation (Jain and Levine 1995). MDA reacts with TBA to form a colored complex. A colored complex has a maximum absorbance at 532 nm. MDA content was expressed as nmol/mg protein by using 1.56×10^5 $M^{-1}cm^{-1}$ for the extinction coefficient.

GST, (EC 2.5.1.18) activity was determined according to Habig et al. (1974) measuring the formation of the GSH and CDNB conjugate. The increase in absorbance was recorded at 340 nm for 3 min. The specific activity of GST was expressed as nmol GSH-CDNB conjugate formed/min/mg protein using an extinction coefficient 9.6 $mM^{-1}cm^{-1}$. All assays were corrected for nonenzymatic reactions using corresponding substrate in phosphate buffer (50mM, pH 7.0).

Statistical analysis. MDA content and GST activity data were analyzed with a one-way analysis of the variance (ANOVA). The least significant difference (LSD) test was used to separate significant ($P > 0.05$) differences among treatment means (SPSS 1997). Regression analysis also was performed to test the correlation between GST activities and MDA content (SPSS 1997).

Results

In comparison with the controls, MDA content was higher in the midguts of *G. mellonella* larvae fed on a diet containing esculetin with MDA content being 4-fold higher in the midguts of larvae fed on a diet containing 1.0% esculetin (Fig. 1A). Consumption of diets supplemented with the highest esculetin concentration (1%) resulted in increased GST activity than observed in the controls. Activity was about 3-fold higher in those fed on the 1% diet (36.5 ± 6.0 nmol/mg protein/min) than fed the control diet (12 ± 3.5 nmol/mg protein/min) (Fig. 1B). However, feeding on the diet with lower concentrations (0.001, 0.01, 0.1%) of the inhibitor did not significantly affect midgut GST activity.

Increased MDA content also was observed in the midguts of larvae fed on diets containing dexamethasone at concentrations 0.01, 0.1, and 1%. However, MDA content in midguts of larvae fed on diet containing 0.001% dexamethasone did not differ from those fed on diet without dexamethasone (Fig. 2A). GST activity also was significantly higher in midguts of larvae that ingested diets containing 0.01, 0.1, and 1% dexamethasone than in those midguts of insects fed on diets with 0.001% and no dexamethasone. The GST activity increase with dexamethasone was dose-dependent with activity about 4-fold higher with the 1% concentration (64.3 ± 4.7 µmol/mg protein/min) than observed in the controls (16.4 ± 4.2 nmol/mg protein/min) (Fig. 2B).

Ingestion of diets containing phenidone also increased MDA content in the larval midguts. The highest phenidone concentration (0.1%) tested resulted in significantly increased MDA content with an almost 5-fold increase (37.2 ± 5.1 nmol/mg protein) over that of the controls (14.1 ± 1.8 nmol/mg protein) (Fig. 3A). Significantly higher GST activity also was observed in larvae fed on diets containing 0.01 and 0.1% phenidone in comparison with larvae fed on diets containing 0.001% and no phenidone (Fig. 3B).

GST activity also was positively correlated with MDA content ($R^2 = 0.88$, $P < 0.05$) of larvae fed on diets containing esculetin. No significant relationship between MDA content ($R^2 = 0.85$, $P = 0.075$) and GST activity in larvae exposed with phenidone was found.

Discussion

These results support the postulation that eicosanoids are operative in the oxidative and antioxidative responses in insects. Novel findings from this study include: (1)

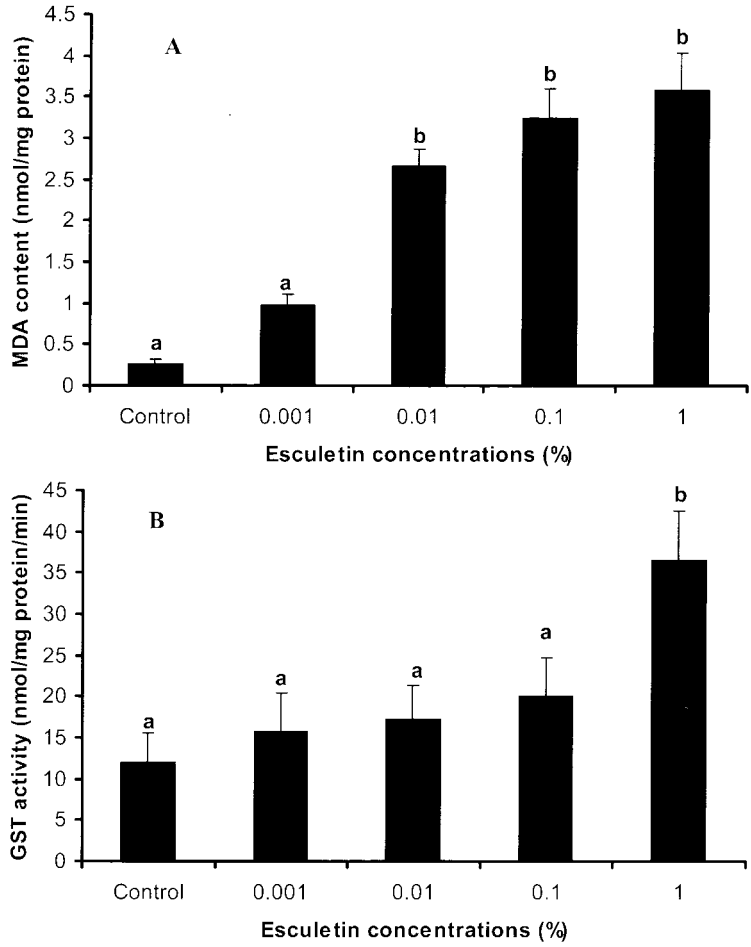

Fig. 1. Effects of esculetin on midgut MDA content (A) and GST activity (B) of *G. mellonella* larvae. Each histogram bar represents the mean of 4 replicates in each of the treatment groups. Vertical bars represent standard deviation. Means followed by the same letter are not significantly different ($P > 0.05$, LSD test).

significantly higher amounts of MDA were produced in insect midguts following dietary exposure EBIs, and (2) PLA_2 (Phospholipase A_2), cyclooxygenase (COX), and lipoxygenase (LOX) inhibitors impaired GST activity, an important antioxidant and detoxification enzyme. These findings extend the known eicosanoid actions in insect antioxidant defense systems and indicate that eicosanoids, at least prostaglandins or lipoxygenase products, act in insect enzymatic antioxidant reactions.

Activities and protein levels of antioxidant enzymes are closely linked with cellular responses to various oxidative stresses. COX and LOX pathways are responsible for the AA metabolism in insects (Nappi et al. 2004) as in vertebrates (Aragno et al.

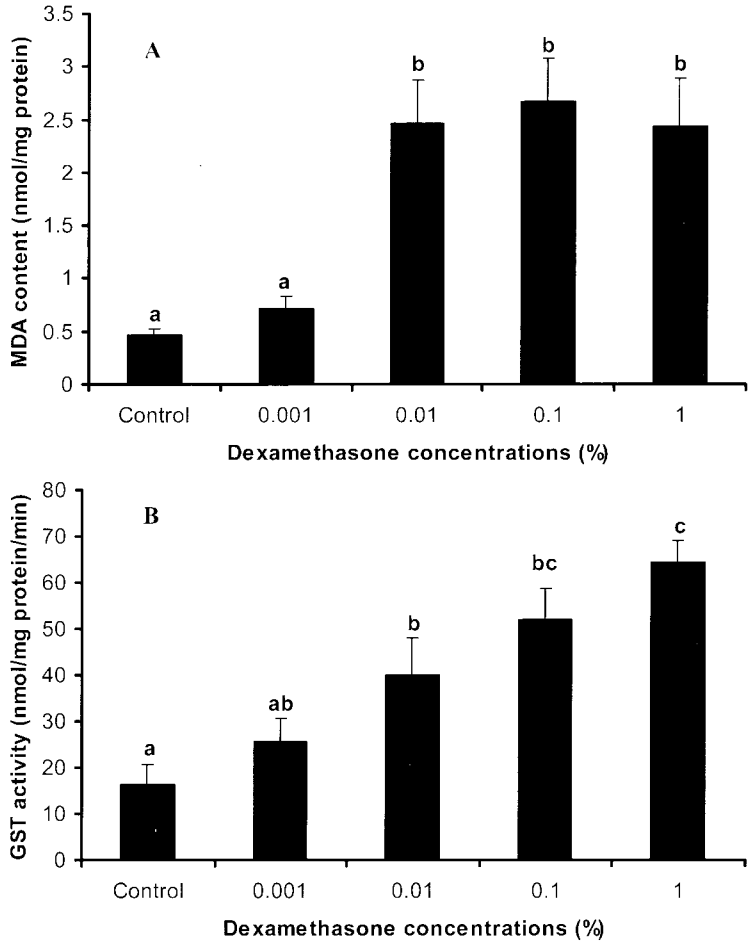

Fig. 2. Effects of dexamethasone on midgut MDA content (A) and GST activity (B) of *G. mellonella* larvae. Each histogram bar represents the mean of 4 replicates in each of the treatment groups. Vertical bars represent standard deviation. Means followed by the same letter are not significantly different ($P > 0.05$, LSD test).

2001). Inhibition of eicosanoids biosynthesis, which play important physiological roles in insects, may lead to production of ROS facilitating the production of lipid peroxidation. Prostaglandins (PGs) were biosynthesized in midguts of different stages of various lepidopteran insects (Büyükgüzel et al. 2002). Eicosanoids have been implicated in the function of oxidative and antioxidative systems during physiological and pathophysiological process of mammals (Kim et al. 2000, Türüt et al. 2008). Beyond their importance in mammals, there is a growing and substantial body of new knowledge on the presence and biological meaning of PGs and other eicosanoids in invertebrates (Stanley 2000, Stanley et al. 2008). Eicosanoids also influence several aspects of

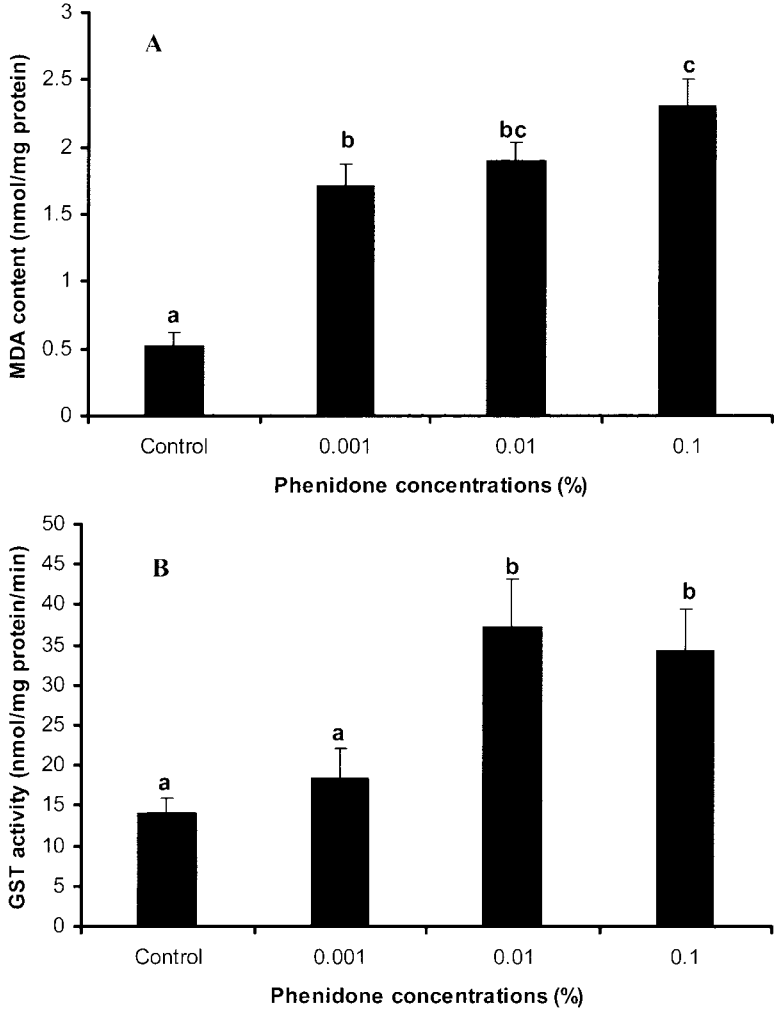

Fig. 3. Effects of phenidone on midgut MDA content (A) and GST activity (B) of *G. mellonella* larvae. Each histogram bar represents the mean of 4 replicates in each of the treatment groups. Vertical bars represent standard deviation. Means followed by the same letter are not significantly different ($P > 0.05$, LSD test).

insect immunity, including cellular defense reaction to bacterial, fungal, and viral infections and parasitization by parasitoids (Stanley and Miller 2006, Büyükgüzel et al. 2007, Durmuş et al. 2008, Stanley and Shapiro 2007). In a marine invertebrate, the decreased glutathione reductase (GR) and CAT activities and unaltered or inhibited SOD were observed following treatment of low prostaglandin E_2 (PGE_2) concentrations, whereas higher PGE_2 concentrations conversely increased all 3 antioxidant

enzymes and inhibited apoptosis (Dolmatova and Zaika 2007). These data are in line with the results reported herein that EBIs treatment led to large alterations in oxidative and antioxidative response as evidenced by increased MDA content and GST activities. The increase in lipid peroxidation level in response to esculetin was not significantly correlated with altered GST activity, but GST appears to be significantly related to changes in MDA content in the larvae that fed on artificial diets containing phenidone and dexamethasone. High dietary concentrations of dexamethasone increased the MDA content and GST activities in this study. These data indicate that MDA contents and GST activity were also increased by high concentrations of phenidone in larval midgut. The increase in the midgut MDA content and GST activity in this study as well as in our previous work (Hyršl et al. 2007) associated with oxidative stress, could be a result of an adaptive metabolic response to the prooxidative challenge caused by inhibition of eicosanoid biosynthesis in *G. mellonella* larvae. Previous studies also showed that the MDA content in the fat body and midgut of the silkworm, *Bombyx mori* L., increased after exposure to lethal doses of phoxim and that GSTs were more inducible in the fat body than in midgut (Yu et al. 2011). Altered oxidative and antioxidative response may reflect an attempt by the larvae to counteract increased production of ROS to maintain homeostasis. This suggestion is confirmed by the findings of Machado et al. (2007) who demonstrated that inhibition of follicle development by aspirin and the rescue of the arrest by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), suggesting that prostaglandins may be involved in physiological homeostasis.

GSTs have been reported as a biomarker for assessing the environmental impact of organic xenobiotics generating oxidative stress (Monteiro et al. 2006, Otitoju and Onwurah 2007). Some studies have demonstrated induction of GST in aphids and house flies exposed to xenobiotics and oxidative agents (Sivori at al. 1997, Francis et al. 2005). Insect GSTs may contribute to antioxidant defense by direct GPx activity preventing and repairing the damage generated by ROS (Singh et al. 2001, Vontas et al. 2001, Ding et al. 2005, Yu et al. 2011). GSTs catalyze essential steps of AA oxygenation in the biosynthesis of $PGF_{2\alpha}$ and prostaglandin E_2 (PGE_2), and some LOX products in mammals (Chang et al. 1987). GST is associated with GSH-dependent prostaglandin synthases (E and D_2 synthases) which are both members of the membrane-associated proteins in eicosanoid and glutathione metabolism in mammals (Ekström et al. 2003). Some prostaglandin (PG) products also induced expression of GSTs (Kawamoto et al. 2000). COX and LOX products mediate cellular and humoral immune responses in *G. mellonella*, in which indomethacin (COX inhibitor) and esculetin (LOX inhibitor) impaired hemocytic phagocytosis and phenoloxidase cascade (Mandato et al. 1997). The findings of the study reported herein infer that inhibition of eicosanoid biosynthesis may result in serious disruption in antioxidant defense as evidenced by altered GST activities in the wax moth larvae. In supporting these results, the studies with indomethacin and dexamethasone indicate that deteriorative cell viability is accompanied by a imbalance of the glutathione-dependent antioxidant enzymes, GST and GPx associated with alterations in eicosanoid biosynthesis in some vertebrates and invertebrates (Hatamoto et al. 2006).

Altered enzyme activities may be attributed to synthesis of aberrant or stress-related proteins in response to oxidative challenges driven by inhibition of eicosanoid biosynthesis. This suggestion is consistent with the results of Stanley et al. (2008) who reported that prostaglandin A_1 (PGA_1) and prostaglandin E_1 (PGE_1) mediate gene expression of some cell protection proteins including GST-like protein, GST subunit 2, catalase, and superoxide dismutase involved in detoxification function and protection

from ROS. ROS may not be only important factor in the oxidative challenge of EBIs, but their inhibitory action on eicosanoid biosynthesis is significant for deterioration of antioxidative defense, especially when the dose of EBIs is low. This is the first demonstration that antioxidative responses in midguts are altered by a physiological system that includes eicosanoid biosynthesis. Altered midgut MDA content and GST activity in the larvae fed on some EBIs in diets may prove of great importance in better understanding the role of eicosanoids in oxidative and antioxidative response in different tissues of *G. mellonella*. This approach is further supported by the results of a recent study that demonstrated eicosanoids act in antioxidant defense in hemolymph of the wax moth mediating oxidative homeostasis (Büyükgüzel et al. 2010). Regulatory functions of eicosanoids that are present at low levels in insect tissues (Stanley 2006) are directed to maintaining a homeostasis of oxidative and antioxidative status as in maintaining homeostatic physiology to crucial events in life cycles (Stanley 2000). Increased MDA content and GST activities in the midgut tissue may be a result of inhibited eicosanoid biosynthesis preventing the function of maintaining oxidative homeostasis. This suggestion is confirmed by results of previous studies that demonstrated that the supplementation of several PG products into artificial diet reversed deteriorative effects of some EBIs on various physiological systems in some tissues of lepidopteran insect larvae (Machado et al. 2007, Miao and Jiang 2003), suggesting that COX and LOX products may be involved in physiological homeostasis in in most insect tissues. However, additional feeding studies using prooxidant nutritonal additives before or concurrently with the exposure of EBIs should be conducted to determine roles of eicosanoids in antioxidative defense against a prooxidant challenge.

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