Prostaglandin Production in Response to a Bacterial Infection in True Armyworm Larvae

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Prostaglandin levels were determined by fluorometric HPLC analysis of hemolymph collected from larvae of the true armyworm, Pseudaletia unipuncta, that had been injected with bacteria. Prostaglandins were extracted and derivatized with the fluorogenic compound 9-anthryldiazomethane and detected by fluorescence-HPLC. One of the prostaglandins produced was identified as prostaglandin F₂α based on HPLC retention time. The chemical identity of prostaglandin F₂α was confirmed by isolation and derivatization followed by gas chromatography mass spectrometry analysis. Larvae injected with heat-killed bacteria, Serratia marcescens, produced about 4 times as much prostaglandin F₂α as larvae injected with saline. In a separate experiment, larvae injected with bacteria and the prostaglandin precursor arachidonic acid produced still higher levels of prostaglandin F₂α. The production of prostaglandin was inhibited with phenidone, a dual cyclooxygenase and lipoxygenase inhibitor. These data indicate that bacterial injections stimulate increased eicosanoid biosynthesis in true armyworms, particularly biosynthesis of prostaglandin F₂α. Our findings add considerable support to the hypothesis that eicosanoids mediate insect cellular immune reactions to bacterial infections. Arch. Insect Biochem. Physiol. 41:225–232, 1999. © 1999 Wiley-Liss, Inc.

Key words: ADAM; fluorescence; Pseudaletia unipuncta; insect immunity; eicosanoid; arachidonic acid

INTRODUCTION

Prostaglandins (PGs) are oxygenated metabolites of arachidonic acid and two other polyunsaturated fatty acids, eicosatrienoic and eicosapentaenoic acids. Although PGs are most widely appreciated with respect to their actions in mammals and other vertebrates, there is increasing interest in the significance of these compounds in invertebrates. PGs act in reproduction, immunity, ion transport, and ecology of many, perhaps all, invertebrate phyla (Stanley-Samuelson, 1994a,b; Stanley and Howard, 1998; Stanley-Samuelson, 1994a,b; Stanley and Howard, 1998;

Abbreviations used: ADAM = 9-anthryldiazomethane; GC = gas chromatography; HPLC = high pressure liquid chromatography; MS = mass spectrometry; PG = prostaglandin.

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Increased research into PG biosynthesis is yielding new insights into invertebrate biology.

With respect to insect immunity, we suggested that eicosanoids mediate cellular defense reactions to bacterial infections in lepidopteran larvae, including true armyworms, *Pseudaletia unipuncta* (Jurenka et al., 1997). Within the insects, this phenomenon has been demonstrated, so far, in several lepidopteran species, two orthopterans, and two hemipterans (Jurenka et al., 1997; Stanley and Howard, 1998; Stanley, 1999; Tunaz et al., 1999; Miller et al., 1999). The evidence that eicosanoids mediate cellular defense reactions is based on three lines of experimentation: (1) treating the experimental insects with pharmaceutical inhibitors of eicosanoid biosynthesis prior to infection attenuated the intensity of defense reactions to bacterial challenges; (2) the influence of the pharmaceuticals was expressed in a dose-dependent manner; and (3) the influence of one eicosanoid biosynthesis inhibitor, dexamethasone, was reversed by injecting arachidonic acid into dexamethasone-treated insects immediately after infection. Whereas these lines of evidence uniformly support the hypothesis that eicosanoids mediate insect immune reactions, there remain important gaps in our information. One of these gaps is the demonstration that bacterial challenges have a measurable influence on hemolymph eicosanoid titers in insects. This is due, in part, to methodological obstacles.

The established methods for detection and quantitation of PGs in biological matrices are bioassay preparations, radioimmunoassays, GC-mass spectrometry (MS), electron-capture GC, and HPLC (Benedetto et al., 1987). There are drawbacks associated with all of these analytical methods. However, HPLC is an efficient and highly effective method of separating PGs and other eicosanoids, but detection remains a problem. Because PGs are present in biological tissues at ng to pg levels, sensitive methods are required to detect them. Among the various HPLC detection methods, fluorogenic derivatization is most sensitive for detecting compounds at pg levels. Several methods to produce fluorescent derivatives of PGs have been reported (Hummert et al., 1996; Kubo and Komatsu, 1986; Osada et al., 1989; Yamaki and Oh-ishi, 1986). However, some fluorescent reagents are highly unstable at room temperatures. Moreover, current fluorometric derivatization methods require complex reaction and clean-up procedures before analysis on HPLC. In this paper, we describe an improved and convenient method for extracting and derivatizing PGs using 9-anthryldiazomethane (ADAM).

PG biosynthesis has previously been recorded from tissues of *P. unipuncta* (Jurenka et al., 1997). However, there is little information on amounts of PGs present in these insect and other invertebrate tissues due to practical difficulties involved in detection of PGs. In this paper, we demonstrate that biosynthesis of certain eicosanoids is increased in response to bacterial infections of true armyworm larvae and we establish the identity of one of these as prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$).

**MATERIALS AND METHODS**

**Chemicals**

The following substances were purchased from commercial sources. PG standards were purchased from Biomol Research Laboratories (Plymouth Meeting, PA); $^3$H-PGE$_2$ (specific activity 6.3 TBq/mmol) from New England Nuclear, (Boston, MA); ADAM from Molecular Probes (Eugene, OR); N$_2$-(trimethylsilyl)trifluoroacetamide (BSTFA) from Pierce, (Rockford, IL); and trimethylchlorosilane, arachidonic acid, and phenidone from Sigma, (St. Louis, MO). All solvents used were HPLC grade.

**Insects**

Sixth instar *P. unipuncta* larvae, reared on standard culture diet, were provided by the Corn Insect Research Unit (USDA, Ames, IA). Last instar larvae were used throughout this study. Approximately 7–8 larvae were used to collect 1 ml of hemolymph.

**Extraction of Prostaglandins**

Larvae were injected with 15 µl of either heat-killed bacteria (*Serratia marcescens*, $10^7$ colony-forming units/ml saline) or saline. Bacteria were injected as described previously (Jurenka et al., 1997) except that the growth media was replaced with phosphate buffered saline (50 mM KH$_2$PO$_4$, 0.15 M NaCl, pH = 7.4). In some experiments, arachidonic acid (10 µg/larvae) or phenidone (26 µg/larvae) in 1 µl of 100% ethanol was
injected into larvae 5 min prior to bacteria injections. Appropriate controls were injected with 1 µl of 100% ethanol. Thirty minutes post injection, larvae were anesthetized by chilling on ice, and hemolymph was collected by bleeding through a cut proleg. Hemolymph was then probe sonicated for 5 s and acidified to pH 4.0 by adding 1 N HCl. PGs were extracted with 500 µl of ethyl acetate three times. The combined ethyl acetate extracts were dried under nitrogen to about 50 µl and applied to a small silicic acid column (2 mm i.d. x 90 mm containing 30 mg of Type 60A, 100–200 mesh silicic acid). The PGs were eluted with the sequential addition of 300 µl of increasingly polar solvents starting with 100% ethyl acetate, followed by ethyl acetate:acetonitrile (1:1, v:v), 100% acetonitrile, acetonitrile: methanol (1:1, v:v), and 100% methanol. PGs were eluted in the acetonitrile:methanol fraction.

Preparation of Fluorescent Derivatives

PG standards or the samples were dried under nitrogen and dissolved in 2 µl ethyl acetate. The reaction was started by adding 2 µl of 0.15% ADAM (w/v) in ethyl acetate, then incubated for 4 h at room temperature. The reaction was carried out in complete darkness to minimize formation of by-products. After 4 h, the samples were diluted to appropriate concentrations and analyzed on HPLC. The amount injected into the HPLC was from a dilution that corresponded to 1/4 to 1/20 of the original volume (approximately 1 ml) of hemolymph collected.

High-Pressure Liquid Chromatography

HPLC was conducted using a Beckman System Gold solvent delivery system (Fullerton, CA) equipped with a Hewlett-Packard 1046A fluorescent detector (Wilmington, DE). The derivatives were analyzed using C18 reverse-phase columns and a solvent system consisting of acetonitrile and water as indicated in individual experiments. Excitation and emission wavelengths were optimized and were found to be 250 nm excitation and 410 nm emission wavelengths for the Hewlett-Packard 1046A detector. In one experiment the HPLC was also interfaced with a flow through radioactivity detector (Radiomatic instruments, Packard Inc.).

Gas Chromatography and Mass Spectrometry

Larvae were injected with 15 µl of bacteria and 1 µl of arachidonic acid (10 µg/µl in 100% ethanol) and 30 min post-injection hemolymph was collected and extracted. The ethyl acetate extract was subjected to silicic acid chromatography and the acetonitrile:methanol fraction was treated with 100 µl of freshly prepared diazomethane in methanol. The methyl esters were purified by HPLC using a 2.1 x 250 mm C18 Inertsil column using a gradient starting at acetonitrile:water 40:60 (v:v) to 100% acetonitrile in 20 min at a flow rate of 300 µl per min. Retention times were determined for standard PG methyl esters using a uv detector set at 195 nm. Retention times corresponding to PGF<sub>2α</sub>, PGE<sub>2</sub>, and PGA<sub>2</sub> were collected, their volume reduced under N<sub>2</sub> and extracted with ethyl acetate. Trimethylsilyl derivatives were made by adding 30 µl of BSTFA containing 1% trimethylchlorosilane and heated at 60°C for 20 min. The reaction was then dried under N<sub>2</sub> and reconstituted in methylene chloride for analysis on GC-MS. A Hewlett-Packard 5890 GC equipped with a DB-5 column (0.25 mm x 30 m) was temperature programmed at 60°C for 1 min, then 10°C/min to 300°C. A Hewlett-Packard 5972 mass selective detector was operated in the scan mode.

RESULTS AND DISCUSSION

Four standard prostaglandins, PGA<sub>2</sub>, PGD<sub>2</sub>, PGE<sub>2</sub>, and PGF<sub>2α</sub>, were derivatized and analyzed using the fluorescent reagent, ADAM (Fig. 1). ADAM specifically reacts with the carboxylic acid group of the PGs via a diazomethane esterification without modifying other functional groups. The derivatized products were separated using gradient programming on reverse phase HPLC and detected using a flow through fluorescence detector. As indicated in Figure 1, the ADAM derivatized prostaglandins were separated with excellent resolution on a reverse-phase C18 column and the in-line fluorescence detector gave excellent sensitivity.

Previous studies on PG derivatization using ADAM have been reported (Yamaki and Oh-ishi, 1986; Yamauchi et al., 1986); however, we have altered the published methods to improve the pro-
cess. First, we utilized a small amount (2 µl of a 0.15% ADAM solution) of unreacted ADAM to minimize unwanted side products. We also reduced the reaction time from overnight to 4 h for similar reasons. These two changes obviated the post-derivatization clean-up steps, which reduced the loss of products. While the formation of the side products was not completely eliminated, the derivatized products could be analyzed on fluorescent HPLC without the lengthy clean-up procedure. To determine that the reduction in the amount and duration of ADAM did not affect the reaction progress in formation of PG esters, we utilized radiolabeled PGE2 to monitor the reaction. In these experiments, ADAM (0.15%; 2.0 µl) and 3H-PGE2 (0.1 µCi) were incubated at room temperature over specified incubation times. After 3 h, about 92% of the 3H-PGE2 was derivatized, and by 4 h 95–98% of the 3H-PGE2 was derivatized. On the basis of these findings, standard conditions for subsequent reactions included 2.0 µl of 0.15% ADAM and 4 h incubation in darkness at room temperature.

The derivatized products can be separated on HPLC, which facilitates the identification and quantitation of several PGs from biological samples in one step. To quantify PGs, we established quantitative curves based on chromatographic peak areas of standards. Figure 2 represents quantitative curves for PGA2, PGD2, PGE2, and PGF2α standards that were processed through the entire extraction and derivatization procedure, thus accounting for extraction and derivatization losses. These curves were linear over the range of 50 to 500 pg, indicating that PG derivatives within this range can be quantitated. Appropriate dilutions were made for the analysis of PGs from the biological source.

Prostaglandins were detected in the hemolymph of true armyworms using the ADAM...
derivatization technique (Fig. 1). In the hemolymph analysis, we found it necessary to include a pre-derivatization clean-up step using a small silicic acid column. This removed possible interfering compounds and resulted in the detection of PGs in the pg range.

PGF$_{2\alpha}$ was the predominant PG present in true armyworm hemolymph. It was present in levels of 4.0 ± 0.9 ng/ml of hemolymph collected from bacteria injected larvae whereas saline injected larvae contained 0.9 ± 0.3 pg/ml hemolymph (means ± S.E. from 4 or 7 experiments, respectively). As indicated in Figure 1, there are also several other putative eicosanoids found in the hemolymph. These eicosanoids did not have similar retention times as the standard PGs used in this study and remain to be identified. Few known PGs are more nonpolar than PGA$_2$ and thus the compounds with longer retention times are most likely to be hydroxylated eicosanoids. The standard 15-hydroxyecosanoate had a retention time of 26 min, which is similar to the two eicosanoids that eluted at 25 and 27.3 min.

To further characterize the products observed by fluorescence HPLC, we injected larvae with arachidonic acid (10 µg/larvae) and bacteria. After 30 min, hemolymph was collected, extracted, and analyzed. We observed a 4-fold increase in abundance of the peak corresponding to PGF$_{2\alpha}$ (Fig. 3, top) over that injected with just bacteria. This indicates that arachidonic acid was converted to PGF$_{2\alpha}$. In our previous study, we had shown that the prostaglandins E$_2$ and F$_{2\alpha}$ were the main PGs produced by arachidonic acid in an in vitro enzyme assay of fat body tissue (Jurenka et al., 1997). In this study, only PGF$_{2\alpha}$ was found in the hemolymph.

PGs isolated from insects have not been routinely characterized by rigorous chemical identification. The structure of PGE$_2$ was confirmed by GC-MS in a study using the reproductive tracts of male house crickets, *Acheta domesticus* (Deste-phano et al., 1974). An enzymatic homogenate was incubated with 100 µg of arachidonic acid and after 1 min PGE$_2$ was extracted and derivatized for analysis by GC-MS. In a study using house flies, 50 µg of arachidonic acid was injected into individual flies to obtain a mass spectrum of PGF$_{2\alpha}$ (Wakayama et al., 1986). Here, we injected larvae with bacteria and arachidonic acid in order to obtain sufficient material for derivatization and GC-MS analysis. As shown in Figure 4, a peak corresponding to PGF$_{2\alpha}$ was found with the proper diagnostic ions, confirming that PGF$_{2\alpha}$ was produced in these larvae. PGE$_2$ and PGA$_2$ were not found in this study of hemolymph PG titers. The retention time for a standard PGE$_2$ is indicated in Figure 4 and it is apparent that a peak is not present (PGA$_2$ data not shown). The total ion scan is identical to published scans for PGF$_{2\alpha}$, except the M-90 ion at 494 was not observed (Pace-Asciak, 1989). To our knowledge, this is the first report identifying a prostaglandin by mass spectral analysis in a lepidopteran insect and only the third report for any insect.

Prostaglandin amounts, as determined by
RIA, have been reported for a few lepidopteran insects. An RIA was used to detect PGE\textsubscript{2} and PGF\textsubscript{2\alpha} with values ranging from 15 pg to 70 pg per reproductive tissue of female cabbage loopers, *Trichoplusia ni* (Hagan and Brady, 1982). Approximately 1 pg of PGE\textsubscript{2} and PGF\textsubscript{2\alpha} was found in whole adult waxmoths, *Galleria mellonella* (Murtaugh and Denlinger, 1982). Murtaugh and Denlinger (1982) also reported various levels of PGE and PGF\textsubscript{2\alpha} in seven different insects, ranging from 1 pg to 550 pg per insect. PGE and PGF\textsubscript{2\alpha} were also measured by RIA in the reproductive

Fig. 4. Partial total ion scan obtained from analysis of trimethylsilyl derivatives by GC-MS of PGs obtained from hemolymph collected from larvae injected with bacteria and arachidonic acid (top). The retention time corresponding to a standard PGE\textsubscript{2} is also indicated. A total ion scan for the compound with a retention time corresponding to PGF\textsubscript{2\alpha} is shown (bottom).
tracts of the house fly, *Musca domestica* (Wakayama et al., 1986). Values ranged from about 1 pg to 20 pg for PGF$_{2\alpha}$ and 1 pg to 5 pg for PGE per reproductive tract. Levels of PGs have been studied in the cricket *Teleogryllus commodus* using quantitation by HPLC of the p-bromophenacyl esters. PGE$_2$ values ranged from 500 pg per spermatheca to 130 ng per head, both from mated females (Stanley-Samuelson et al., 1983).

As indicated by the above discussion, it is apparent that considerable variations in amounts of PGs have been detected from insect sources. Nonetheless, it is not the absolute amounts of PGs that are of interest, but the levels found in relation to a given function. For example, PGs are involved in initiating egg-laying behavior in crickets and thus it was important to find that the level of PGE$_2$ increased from undetectable in spermathecae from virgins to around 500 pg per spermatheca from mated females (Loher et al., 1981). We found that the level of PGF$_{2\alpha}$ was 0.9 ± 0.3 pg/ml hemolymph in insects injected with saline but was 4.0 ± 0.9 ng/ml hemolymph in insects injected with bacteria (means ± S.E. from 4 or 7 experiments, respectively). We also found that the level of PGF$_{2\alpha}$ was decreased by phenidone, a dual cyclooxygenase and lipoxygenase inhibitor (Fig. 3, bottom). In this experiment, larvae were injected with bacteria and ethanol or phenidone in ethanol and eicosanoids analyzed after a 30-min incubation. Lower levels of all the eicosanoids were seen with the phenidone treatment. This study provides direct evidence that the previously used eicosanoid inhibitors (Jurenka et al., 1997) inhibit eicosanoid production.

The significance of the increased levels of PGF$_{2\alpha}$ following infection relates to the finding that PGs are mediators of cellular host defense responses in vertebrates and in invertebrates. In tobacco hornworms and true armyworms, inhibition of PG synthesis severely impairs the insects’ ability to clear bacteria from the hemolymph (Jurenka, et al., 1997; Stanley-Samuelson et al., 1991). From these reports, we proposed that cellular defense reactions in these insects are mediated by eicosanoids. However, it has been argued that results of experiments with eicosanoid biosynthesis inhibitors do not, of themselves, yield more than circumstantial evidence (Stanley-Samuelson, 1994b). Therefore, it is important to observe changes in PG levels after a bacterial infection. We observed here that the level of at least one PG increased in the hemolymph at one time point after injection of bacteria. We have identified one of these PGs as F$_{2\alpha}$, based on the described chromatographic and mass-spectral properties. Several other putative eicosanoids, that remain to be identified, also increased in abundance in response to a bacterial infection (Fig. 1). These results indicate that increased amounts of PGs and other eicosanoids are produced in response to a bacterial infection.

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**LITERATURE CITED**


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