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Prostaglandins A_1 and E_1 influence gene expression in an established insect cell line (BCIRL-HzAM1 cells)

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Abstract

Prostaglandins (PGs) and other eicosanoids exert important physiological actions in insects and other invertebrates, including influencing ion transport and mediating cellular immune defense functions. Although these actions are very well documented, we have no information on the mechanisms of PGs actions in insect cells. Here we report on the outcomes of experiments designed to test our hypothesis that PGs modulate gene expression in an insect cell line established from pupal ovarian tissue of the moth *Helicoverpa zea* (BCIRL-HzAM1 cells). We treated cells with either PGA_1 or PGE_1 for 12 or 24 h then analyzed cell lysates by 2-D electrophoresis. Analysis of the gels by densitometry revealed substantial changes in protein expression in some of the protein spots we analyzed. These spots were processed for mass spectrometric analysis by MALDI TOF/TOF, which yielded *in silico* protein identities for all 34 spots. The apparent changes in three of the proteins were confirmed by semi-quantative PCR, showing that the changes in mRNA expression were reflected in changes in protein expression. The 34 proteins were sorted into six categories, protein actions, lipid metabolism, signal transduction, protection, cell functions and metabolism. The findings support the hypothesis that one mechanism of PG action in insect cells is the modulation of gene expression.

Keywords: Insect cell line; AMHz1 cells; Prostaglandins; 2D electrophoresis; Mass spectrometry; Semi-quantitative PCR

1. Introduction

Prostaglandins (PGs) and other eicosanoids are oxygenated, enzymatic metabolites of arachidonic acid and two other C_{20} polyunsaturated fatty acids. Eicosanoid structures and biosynthetic pathways are described in several reviews and monographs (Stanley, 2000, 2005, 2006). PGs were first discovered in research into human reproductive physiology (von Euler, 1936) and in the ensuing decades a tremendous amount of information on the biological and biomedical significance of PGs in mammals has accumulated. PGs are present and biologically active in virtually all mammalian body tissues and fluids, where they influence a very wide range of physiological events including ion transport, blood pressure, fever and inflammation.

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). In reproductive biology, eicosanoids act in reproduction in mollusks, crayfish, scallops, barnacles and insects (Machado et al., 2007). Eicosanoids influence ion transport processes in several species of mussels, in the locust rectum, in mosquito Malpighian tubules and tick salivary glands (Stanley, 2000). Eicosanoids also influence several aspects of insect immunity, including cellular defense reactions to bacterial, fungal, parasitoid and viral infections (Stanley and Miller, 2006; Büyükgüzel et al., 2007; Durmaş et al., 2007; Stanley and Shapiro, 2007). These reactions are the cellular innate defense reactions of insects, including microaggregation, nodulation and cell spreading. The importance of eicosanoids in mediating insect immune reactions to microbial challenge is highlighted by recent work with the insect pathogenic bacterium, *Xenorhabdus nematophila*. As a part

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of its arsenal of virulence mechanisms, this bacterium secretes factors responsible for disabling eicosanoid-mediated immunity by specifically blocking a phospholipase A_2, the first step in eicosanoid biosynthesis (Stanley and Miller, 2006).

There is a very large corpus of meaningful information on the mechanisms of PG actions in mammalian cells (Coleman et al., 1994; Negishi and Katoh, 2002; Regan, 2003), most of which are mediated by means of G protein coupled, rhodopsin-class receptors. In the case of PGE, for example, there are four receptor subtypes EP1–EP4 (Gobeil et al., 2003). The cellular actions driven by these receptors can lead to short-term changes in homeostatic physiology, such as induction of fever (Ushikubi et al., 1998) [true also for insects, as seen in locusts (Bundey et al., 2003)], smooth muscle contraction (or relaxation) (Moreland et al., 2003) or hemodynamic effects (Audoly et al., 1999). PGs also influence gene expression in many mammalian systems. PGE_2 stimulates expression of the prolactin gene in the leukemic cell line Jurkat via EP3 and EP4 receptors (Gerlo et al., 2004). PGs also act in malignancy growth and metastasis. For example, EP4 signaling through phosphatidylinositol 3-kinase, Erk1 and Erk2 supports growth of CT26 colon carcinoma cells (Hull et al., 2004; Fulton et al., 2006). The potential influence of PGs on mammalian gene expression is emphasized by the revelation of a nuclear PG signaling system in which EP3 receptors are localized in the nuclear membrane. These nuclear receptors influence gene expression via several mechanisms, including Erk-MAP kinase-dependent pathways and NFκB activation (Gobeil et al., 2003). Overall, PGs can influence immediate cell events regulating homeostasis and long-term changes in gene expression in mammalian cells.

Our appreciation of PG action mechanisms in biomedical models is balanced by virtually no information on the topic in invertebrate cells. In their substantive work on the role of PGE_2 in modulating salivary secretions in female ticks, *Amblyomma americanum*, Qian et al. (1997) identified a specific PGE_2 receptor. Further work indicated that the PGE_2 receptor stimulates secretion of protein in salivary glands of female ticks. Yuan et al. (2000) inferred from their results that PGE_2 acts through a G protein coupled EP1 receptor. Aside from this work with tick salivary glands, however, there is no information on the mechanisms of eicosanoid actions in invertebrates.

Two reports suggest that eicosanoids influence gene expression in insect immune reactions. Morishima et al. (1997) reported that induction of the genes encoding the anti-bacterial peptide Cecropin B and the enzyme lysozyme was suppressed in fat bodies of silkworms, *Bombyx mori*, treated with inhibitors of phospholipase A_2, cyclooxygenase (COX) and lipoxygenase (LOX). Expression of the genes for these two proteins was induced by injecting the eicosanoid-precursor AA into silkworm larvae. With respect to a *Drosophila* model, Yajima et al. (2003) showed that LPS-stimulated induction of the gene encoding the anti-bacterial protein, Diptericin, is mediated by the immunedeficiency (imd) pathway, but not by the Toll pathway. In a screen of inhibitory compounds, they also found that inhibitors of PLA_2, including *p*-bromo-phenacyl bromide and dexamethasone, repressed LPS-stimulated gene expression. The authors concluded there is a functional coupling between the imd pathway and pathways responsible for eicosanoid biosynthesis.

Based on these findings, we generated the hypothesis that one mode of PG action in insect cells is their influence on gene expression. To test our hypothesis, we treated an established cell line (BCIRL-HzAM1) with PGA_1 and, separately, with PGE_1. Here we report that, relative to controls, these treatments substantially altered expression of genes encoding a range of proteins.

2. Materials and methods

2.1. Cell treatments

BCIRL-HzAm-1 cells, derived from *Helicoverpa zea* pupae (McIntosh and Ignoffo, 1983) were maintained at 28 °C in EX-CELL 401 medium (SAFC, St. Louis, MO) containing 9% heat-treated fetal bovine serum (Summit Biotechnology, Fort Collins, CO) with penicillin (100 U/ml) and streptomycin (100 g/ml) (HyClone, Logan, UT). Before treating the cells with PGs, they were sub-cultured a minimum of two times in serum-free medium.

The cells were placed into T_{75}'s at 0.9×10^6 cells/ml using serum-free medium. After allowing the cells to attach and begin replicating overnight, cells were then treated with either 0.5% ethanol (vehicle control) or 15 μM PGA_1 or PGE_1 (BIOMOL International, L.P., Plymouth Meeting, PA, or Cayman Chemical Co., Ann Arbor, MI) that had been dissolved in ethanol (final concentration: 0.5% ethanol). After 12 and 24 h at 28 °C, cells were processed for either protein gel electrophoresis or RT-PCR.

2.2. 2D gel electrophoresis

Before harvesting, cells were washed 3X in their flasks with 5 ml CMF-PBS (calcium-magnesium-free phosphate buffered saline) to remove media components. After the final CMF-PBS wash, cells were removed from their flasks and centrifuged at $850 \times g$ for 15 min. Cells were then washed in 1.2 mL 10 mM Tris–HCl, 250 mM sucrose, pH 7.0, to remove the salts, transferred into 1.5 ml tubes (0.5 ml/tube for 2D samples; 0.1 ml for protein assay samples), and centrifuged at $1000 \times g$ for 5 min at room temperature (RT). Once this buffer was decanted, 40 μl 10 mM Tris–HCl buffer, pH 7.0 (containing 1:100 protease inhibitor cocktail, Sigma P 8340) were added to the "2D sample" tubes and 10 μl 10 mM Tris–HCl buffer, pH 7.0 (without protease inhibitor cocktail) were added to the "protein assay" tubes. Samples were snap frozen in liquid N_2 and stored at −80 °C. Protein amounts were

determined using the Pierce Micro BCA Protein Assay Kit (Rockford, IL).

On the day of isoelectric focusing, cells were sonicated 3X (10 s, 35 W) using a Sonifier Cell Disruptor with microtip (Model W185, Misonix, Inc., Farmingdale, NY) and briefly homogenized using microtube pestles. Cell debris was pelleted by centrifugation at 15,000 × *g*, 3 min, 5 °C. The resulting supernatant fluids were subjected to nuclease digestion using 0.5 mg/ml for RNAse A (Type X-11A; Sigma-Aldrich Co., St Louis, MO) and DNAse I (Grade II; Roche Applied Sciences, Indianapolis, IN) in 10 mM Tris–HCl, pH 7.0, for 30 min at 27 °C. Nuclease-treated samples were then centrifuged as above. The supernatant fluids were transferred into 1.5 ml tubes containing 200 µl rehydration solution (9 M Urea, 100 mM DTT, 0.2% Bio-Lyte pH 3-10 ampholyte, 0.001% bromophenol blue) and the tubes were gently inverted. ReadyStrip IPG strips (11 cm, pH 3-10; Bio-Rad Laboratories, Hercules, CA) were rehydrated overnight in the rehydration solution containing the cell lysates (0.6 mg protein/strip). The next day, the IPG strips were subjected to isolectric focusing in the Bio-Rad PROTEAN IEF Cell using the pre-set linear volt ramp program (8000 V and 50 µA/strip max, 35,000 vH). The focused IPG strips were stored at −80 °C. At a later date, IPG strips were equilibrated (15 min/buffer: 6 M Urea, 2% SDS, 0.375 M Tris–HCl [pH 8.7], 20% Glycerol, with 130 mM DTT [Buffer I] followed by 135 mM iodoacetamide [Buffer II]) and subjected to SDS-PAGE, along with Bio-Rad Precision Plus protein standards, using Criterion gels (8–16% Tris–HCl, Bio-Rad). Gels were stained with Coomassie Blue G-250 (BioSafe Stain, Bio-Rad) and analyzed using AlphaImager 2200 software (Alpha Innotech, San Leandro, CA). Gel protein spots were removed using a 1.5 mm spot picker (The Gel Company, San Francisco, CA) and stored at −80 °C.

A minimum of three independent samples were generated per treatment, with at least one gel per replicate. The highest quality gels were used for protein quantitation and mean values are reported in Table 1. Because of the high reproducibility of the 24-h samples, data for 12-h samples were generated from a single set of gels.

2.3. Trypsin digestion

Gel plugs were equilibrated for 15 min in 500 µL 100 mM ammonium bicarbonate at RT with gentle agitation. Plugs were destained 3 times with 500 µL 50/50 (V/V) acetonitrile/100 mM ammonium bicarbonate solution for 15 min at RT with agitation and washed briefly in 500 µL acetonitrile. Gel plugs were then dehydrated for 20 min with 500 µL acetonitrile (RT, with agitation) and rehydrated for 2 h at 4 °C in 5 µL of a 20 µg/mL solution of modified TPCK-treated porcine trypsin (Trypsin Gold, Mass Spectrometry Grade, 17,000 U/mg, cat. # V5280, Promega, Madison, WI) in 40 mM ammonium bicarbonate/10% acetonitrile. Subsequently, the trypsin solution was replaced with 15 µL 40 mM ammonium bicarbonate/10% acetonitrile, and the proteins were digested overnight at 37 °C. The digests were acidified by addition of 4 µL of extraction solvent and were transferred into 500 µL tubes. Each plug was extracted twice with 10 µL 600/300/100 (V/V/V) acetonitrile/water/10% trifluroacetic acid solution for 10 min with gentle agitation at RT. Extracts from one sample were pooled, snap frozen in liquid N_2, and stored at −80 °C.

2.4. MS/MS analysis

Trypsinized protein extracts were lyophilized and reconstituted with 10 µL water and re-dried. A portion of each was mixed with α-cyano-4-hydroxycinnamic acid matrix and applied to the MALDI target. Samples were analyzed in the positive ion mode by reflector MALDI TOF/TOF (4700 MALDI TOF-TOF, Applied Biosystems, Foster City, CA) and MS/MS of the 10 most intense ions (with S/N > 20 per digest) using automated data acquisition. Spectra were processed and batch analyzed in the "Combined MS plus MS/MS" mode with Applied Biosystems GPS Explorer software (vers. 3.0). The initial database search was performed with Matrix Science's search engine (www.matrixscience.com) against the NCBInr Metazoa protein database. Protein identification criteria included protein score, expect level, percent sequence coverage, mass error and a total protein sequence to determine whether peptide sequences were contiguous. Ion scores were generated for individual matched peptides, as well as total ion scores (not shown). These data, combined with observed MW and pI values of the cell-line proteins and with protein data from the species from which the cell line was derived (*Helicoverpa zea*) were used to establish protein identities. For those proteins that did not yield significant matches using Mascot (possibly related to the number of insect protein sequences in the database), manual *de novo* sequence analysis and/or partial sequence tag analysis (i.e., sequences deduced by visual inspection of each spectrum) was performed. Sequences generated from these analyses were used to interrogate NCBI-BLASTp for short, nearly exact, protein matches (using the PAM30 matrix and searching within "Arthropods". *E*-values and frequency of matches to a specific protein were the primary criteria for these determinations.

2.5. Semi-quantitative PCR

Total RNA was isolated from HzAM1 cells treated with PGE_1 and PGA_1 at 12 and 24 h with TRIzol (Invitrogen, CA, USA) according to manufacturer's suggested procedure. Briefly, HzAM1 cells were re-suspended in 500 µl TRIzol and 200 µl chloroform, incubated 10 min and centrifuged at 16,000 × *g* for 15 min at 4 °C. An equal volume of isopropanol was added to the upper aqueous phase. RNA was precipitated by centrifugation for 10 min at 4 °C and washed with 70% ethanol. The RNA was

Table 1
Proteins from HzAM1 cells identified using Mascot

Spot no.	Putative protein with accession no. (*species*)	Mass	pI	MOWSE score[a]	Total ion score[b]	No. peptides[c]	% Protein coverage[d]	Observed peptide mass[e]	*E*-value[f]	Peptide sequence (with highest ion score)[g]
1	Similar to CG9916-PA isoform 1 (cyclophilin) (*Tribolium castaneum*) gi|91076258	17 963	8.5	125	112	3	13	1631.9	2.9e−007	HVVFGTVVEGMDVVK (Oxid., M) (112)
2	Ubiquitin (*Carabus alpestris*) gi|1321735	15 669	5.9	339	139	11	79	1523.8	1.1e−028	IQDKEGIPPDQQR (71)
3	Glutathione-S-transferase-like protein (*Galleria mellonella*) gi|14517793	24 357	6.9	272	210	9	48	2411.2	5.6e−022	INPQHTVPTLVDDGFSLWESR (85)
5	Glucose-3-phosphate dehydrogenase (*Colias meadii*) gi|82754607	33 754	7.2	231	148	13	34	1493.9	7.2e−018	VPVPNVSVVDLTVR (111)
6	GA20688-PA (*Drosophila pseudoobscura*) gi|54637364	52 376	8.5	98	88	4	13	1476.7	0.00013	FFSGFGGQVDFIR (88)
7	Catalase (*Bombyx mori*) gi|51571867	57 092	8.1	173	100	11	22	1837.9	4.5e−012	FSTVGGESGSADTVRDPR (80)
8	Putative glyceraldehyde-3-phosphate dehydrogenase (*Oncometopia nigricans*) gi|53830712	35 753	8.3	207	133	11	27	1493.9	1.8e−015	VPVPNVSVVDLTVR (107)
9	Bmsqd-1 (*Bombyx mori*) gi|784909	31 036	8.4	143	133	4	11	1851.0	4.5e−009	LFVGGLSWETTDKELR (77)
10	Cu/Zn superoxide dismutase (*Plutella xylostella*) gi|53148457	15 527	6.3	135	113	3	43	2031.0	2.9e−008	TLVVHADPDDLGAGGHELSK (113)
11	Abnormal wing disc-like protein (*Choristoneura parallela*) gi|21435082	17 142	6.8	188	137	8	48	1757.9	1.4e−013	QMLGATNPADSLPGTIR (Oxid., M) (70)
12	Cellular retinoic acid binding protein (*Manduca sexta*) gi|3115357	14 875	5.7	392	233	14	68	1824.8	5.7e−034	FKPGEEFDEERADGAK (74)
13	Actin-depolymerizing factor 1 (*Bombyx mori*) gi|95103010	17 227	6.2	626	468	16	89	2280.1	2.3e−057	YIQATDLSEASQEAVEEKLR (123)
15	Similar to *Drosophila melanogaster* Jafrac1 (*Drosophila yakuba*) gi|38047571	21 938	5.5	204	152	6	35	1431.8	3.6e−015	GLFIIDDKQNLR (79)
16	Heat shock protein HSP20.4 (*Bombyx mori*) gi|49036077	20 415	6.5	236	156	11	61	1745.9	2.3e−018	RYALPEGAAPETVESR (78)
17	Bmsqd-1 (*Bombyx mori*) gi|784909	31 036	8.4	222	196	8	21	1851.0	5.7e−017	LFVGGLSWETTDKELR (95)
20	LOC496165 (*Xenopus laevis*) gi|56540962	34 945	7.8	130	116	3	10	1790.0	9.1e−008	LLYDLADQLNAAVGASR (116)
21	Acyl-coenzyme A dehydrogenase (*Bombyx mori*) gi|103058047	47 506	7.1	179	142	11	33	1097.5	1.1e−012	YAAGFLNEGR (74)
22	Arginine kinase (*Bombyx mori*) gi|82658675	40 308	5.9	390	215	25	57	1761.9	9.1e−034	ETQQQLIDDHFLFK (58)
23	(Predicted) similar to T21B10.2b isoform 2 (enolase) (*Tribolium castaneum*) gi|91090948	37 443	7.6	226	145	14	42	1764.9	2.3e−017	AAVPSGASTGVHEALELR (80)
25	Heat shock 70 kDA protein 9B (mortalin-2) (*Gallus gallus*) gi|57524986	73 432	6.1	320	229	16	27	1694.9	9.1e−027	NAVITVPAYFNDSQR (98)
26	HSC 70 (*Trichoplusia ni*) gi|1495233	72 104	5.5	674	348	34	54	1691.8	3.5e−062	STAGDTHLGGEDFDNR (79)
27	Transitional endoplasmic reticulum ATPase TER94 (*Bombyx mori*) gi|95102992	89 781	5.3	195	97	3	43	1194.4	3e−014	GVLFYGPPGCGK (46)
29	Heat shock cognate 70 protein (*Spodoptera frugiperda*) gi|27260894	73 178	5.2	820	560	39	51	1888.0	9.1e−077	VTHAVVTVPAYFNDAQR (111)
30	Heat shock protein 60 (*Culicoides variipennis*) gi|2738077	61 983	6.4	134	58	18	24	1375.7	3.5e−008	GYISPYFINSSK (41)
31	Actin 5 (*Aedes aegypti*) gi|67782283	42 194	5.3	694	480	26	75	1790.9	3.6e−064	SYELPDGQVITIGNER (109)
32	14-3-3 protein, zeta isoform (*Bombyx mori*) gi|114050901	28 097	4.9	578	334	21	66	1209.6	1.4e−052	YLAEVATGETR (78)
33	Tyrosine 3-monooxygenase/tryptophan 5-monoxygenase activation protein epsilon polypeptide (*Bombyx mori*) gi|95102932	29 767	4.7	423	278	15	55	1404.7	4.5e−037	NVSDNELTVEER (75)
34	Ubiquitin-like protein SMT3 (*Bombyx mori*) gi|87248605	10 359	5.3	89	49	3	31	1331.7	0.0012	VLGQDNAIVQFK (49)
36	Calmodulin (*Renilla reniformis*) gi|115518	16 671	4.0	146	92	5	39	1739.9	2.3e−009	VFDKDGDGFISAAELR (92)

[a]MOWSE protein scores >72 are significant ($p<0.05$).
[b]Ion scores >43 are significant ($p<0.05$).
[c]The number of peptide sequences identified by Mascot that contributed to the MOWSE score.
[d]The percent of the protein sequence that is accounted for by the matching peptide sequences.
[e]The observed peptide mass as reported by Mascot.
[f]*E*-values that were generated by Mascot (searched within "Metazoa").
[g]Sequences of the peptides with the highest ion scores. The individual ion scores are shown in brackets.

dissolved in diethyl pyrocarbonate (DEPC)-treated water and quantified by optical density measurement at 260 nm.

For cDNA synthesis and PCR amplification, the total RNA was first treated with DNase. The first-strand cDNA was synthesized from total RNA using Oligo(dT) primers and superscript II reverse transcriptase (Invitrogen) following the manufacturer's protocol. The cDNA was amplified using the gene specific primers (primer for protein spot #12: 5′-CGTGGGCCTGATCACCCGCA-3′ and 5′-GTCATCACGGCTTTCATCTC-3′; primer for protein spot #13: 5′-ACGAGGAGATCAAGAAGGACA-3′ and 5′-GAGTACAACATCTTCTTCTT-3′; primer for protein spot #34: 5′-ATGGCTGATGAAAAGAAGG-3′ and 5′-GTCTGCTGTTGGTAAAC-3′). The PCR products were subjected to electrophoresis in 1.0% agarose gel. To normalize the cDNA, the primers for the housekeeping gene *rp* 21 (5′-ATGACGAACTCCAAGGGTTA-3′, 5′-ATAGGGGATGGGAGCCAATA-3′) were also used to amplify cDNA from the treated samples.

3. Results

Following 12- and 24-h exposure to 0.5% EtOH (control), PGA_1 or PGE_1, total cell protein preparations from HzAM1 cells were separated by 2D electrophoresis. Representative 2D protein gels of cell lysates are displayed in Fig. 1 (for 12-h exposure to EtOH, PGA_1 and PGE_1) and Fig. 2 (for 24-h exposure). Analysis of densities of

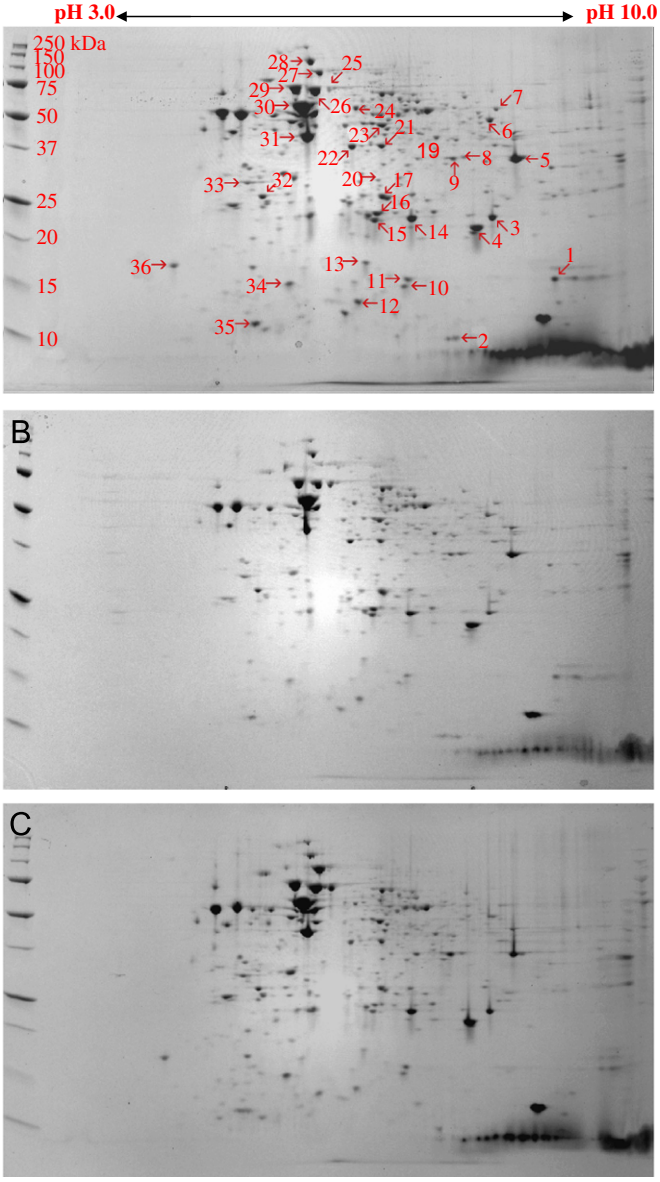

Fig. 1. Representative gels showing PGs influence protein expression in HzAM1 cells. Cells were exposed to (A) 0.5% ethanol (vehicle control), (B) 15 μM PGA1, (C) 15 μM PGE1 for 12 h and then analyzed on 2D electrophoresis as described in M&Ms. The 36 protein spots indicated in this image were selected for extraction and MS/MS analysis.

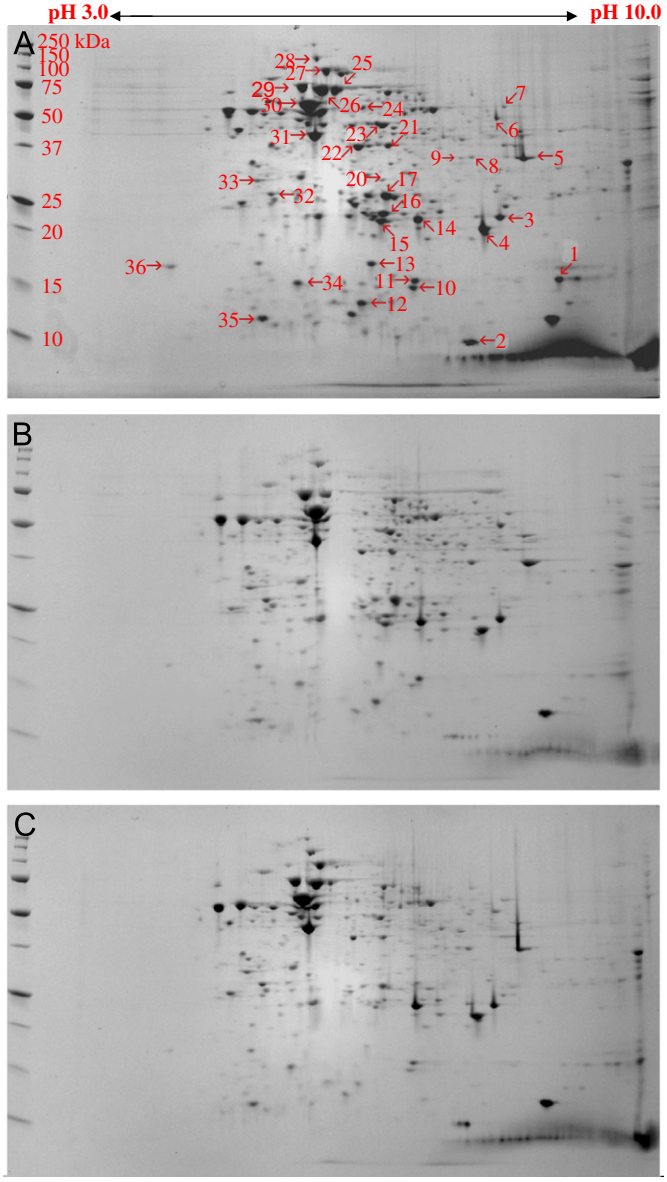

Fig. 2. PGs influence protein expression in HzAM1 cells. Cells were exposed to (A) 0.5% ethanol (vehicle control), (B) 15 μM PGA1, (C) 15 μM PGE1 for 24 h and then analyzed on 2D electrophoresis as described in M&Ms. These are representative gels, with three or more independent replicates.

approximately 500 spots revealed at least 30 spots with substantial changes in densities after 12- or 24-h exposures to the PGs (Table 3). These spots were selected for in-gel digestion and analysis by MALDI-TOF MS/MS. Following 12-h exposures to PGA_1 we recorded approximately two-fold or greater increases in expression of four proteins (spots 4, 8, 20 and 25). After 24-h, expression of only one protein (spot 8) remained elevated by 2.5-fold. Following 12-h PGE_1 treatments, we recorded two-fold or greater increases in expression of seven proteins (spots 9, 18, 24, 25, 27, 28 and 33); of these, #25 increased expression by 17-fold. Again, by 24-h expression of only one protein was two-fold higher than controls (spot 8).

The PG treatments exerted down-regulating actions in expression of most proteins that changed expression (Table 3). Following 12-h exposures, we registered 50% or greater decreases in expression of seven proteins (for PGA_1) and three proteins (for PGE_1). After 24-h expo-

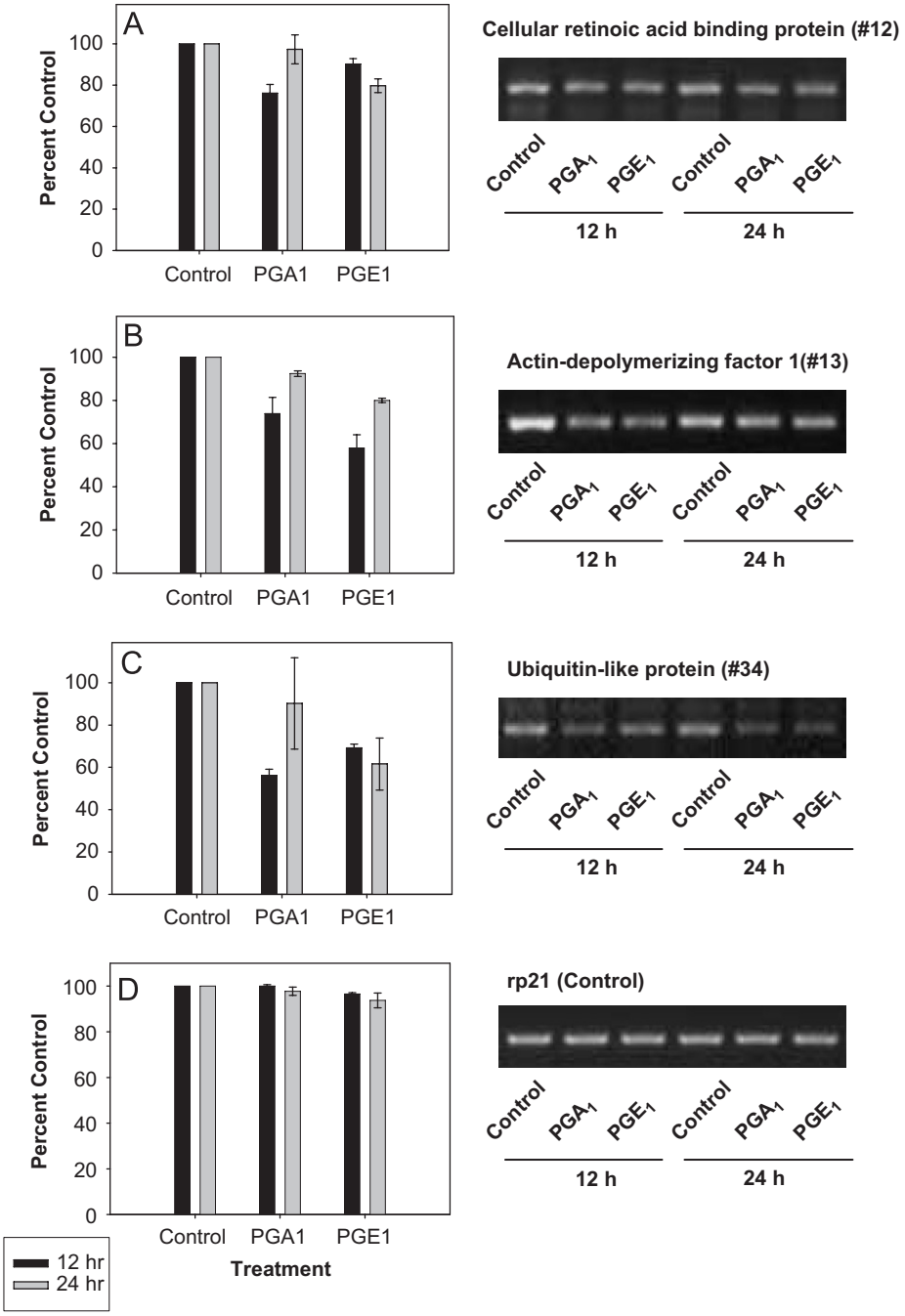

Fig. 3. PGs influence mRNA levels in HzAM1 cells. Gene-specific primers were generated based on sequence data from selected proteins and used to determine mRNA levels using semi-quantitative PCR. Separate cultures of HzAM1 cells were exposed to either 0.5% ethanol (vehicle control), 15 μM PGA1 or 15 μM PGE1 for either 12 or 24 h. The figures on the left show mRNA levels as proportions of control values and figures on the right show corresponding representative agarose gels. Histogram bars represent means ± SE. (A) Primer 12; (B) primer 13, (C) primer 34, (D) primer rp21.

Table 2
Proteins from HzAM1 cells identified by *de novo* sequence analysis

Spot no.	Putative protein with accession no. (*species*)	Protein mass	pI	No. peptides[a]	*E*-value[b]	Peptide sequence[c]
4	Mn superoxide dismutase (*Bombyx mori*) gi|112983802	24 227	8.8	3	8e−07	KHTLPELPYEYSALE[d]
14	Glutathione S-transferase, subunit 2 (*Orthosia gothica*) gi|1044967	23 437	>9.0[e]	1	0.78	FNLNGLAEPLR
24	Mitochondrial processing peptidase alpha subunit (*Aedes aegypti*) gi|108882534	60 086	6.6	1	8e−06	FGQFCTAGVVLDSGPR
28	(predicted) similar to CG2918-PA (*Tribolium castaneum*) gi|91080263	103 445	5.1	3	0.13	TVPQLQVLGVGFDR
35	Thioredoxin-like protein (*Manduca sexta*) gi|6560635	11 753	4.8	3	0.005	VEEFSGANVDKLR

[a]The number of sequences deduced using *de novo* sequence analysis that resulted in the same protein identity.
[b]*E*-values were generated by BLASTp searches for short, nearly exact, protein matches (using PAM30, within "Arthropods"). These are the values of the peptides with the most significant *E*-value.
[c]Sequences were determined by manual *de novo* analysis from observations of the original spectra. Only the peptide sequences with the most significant *E*-values are shown.
[d]Deduced partial sequence tag (therefore Lys/Arg residues not shown on C-terminal).
[e]The pI for GSH from *O. gothica* has not been reported. Additionally, this identity was based only on a partial sequence, therefore the pI could not be estimated.

sures, nine proteins (PGA_1) and six proteins (PGE_1) declined in expression by 40% or more.

We designed primers to several randomly selected protein spots based on the protein databank search of MALDI-TOF MS/MS data and confirmed the quantitative changes registered in proteins 12, 13 and 34 by semi-quantitative PCR (Fig. 3). These experiments show that the changes we recorded in mRNA levels are reflected in changes in the corresponding proteins.

Identifications of each of the 34 proteins by protein databank matches of MALDI-TOF MS/MS data are presented in Tables 1 and 2. These proteins are sorted by functional category in Table 3, which shows six broad categories of protein function, protein actions, lipid metabolism, signal transduction, cell protection, cell functions and energetics/metabolism. These are addressed in Section 4.

4. Discussion

The data reported in this paper support our hypothesis that one mechanism of PG action in insect cells is their influence on gene expression as registered by changes in cellular protein profiles. We recorded measurable differences in expression of at least 30 proteins, with substantial, multi-fold differences in expression of a sub-set of these proteins. Although PGs exert observable physiological and behavioral actions in invertebrate systems (Stanley, 2000), we considered the possibility that the effects of any PG treatment on gene expression could result from adventitious processes. We controlled for this possibility by deliberate PG selection, as noted just below. We infer that the influence of PGs on insect cell gene expression is expressed via physiological signal transduction mechanisms rather than other, non-specific, processes.

In this first analysis, we selected PGE_1 and PGA_1 to assess the influence of PGs on gene expression. On the biomedical background, PGs are categorized into two groups, the conventional PGs (PGE, PGD, PGF, PGI) and the cyclopentenone PGs (PGA, Δ^{12}-PGJ). The conventional PGs exert their cellular actions by interactions with specific G protein coupled receptors (Tsuboi et al., 2002; Sugimoto and Narumiya, 2007), some of which are located on the nuclear envelope (Bhattacharya et al., 1998). PGA and other cyclopentenone PGs do not have cell surface receptors. These PGs are actively transported into cells where they combine with various proteins to influence gene expression. Both conventional and cyclopentenone PGs influence gene expression in mammalian cells.

We note situations in which PGE_1 and PGA_1 influenced expression of different proteins and in different ways. For example, after 12-h exposures, PGA_1 inhibited expression of protein 33 by >50% and PGE_1 up-regulated its expression by nearly two-fold. Similarly, 12-h PGA_1 treatment stimulated expression of protein 4 by approximately two-fold, and PGE_1 down-regulated expression by 80%. These two PGs act through different intracellular mechanisms and such differences are to be expected. More to the point, these differences document specificity in PG action, supporting our view that the PGs influence gene expression through physiological mechanisms.

The broad pattern seems to be an up-regulation (by two- or more fold) of a few proteins (four for PGA_1 and seven for PGE_1) coupled with down-regulation of most of the proteins we studied after 12 h PG treatments. The picture changed after 24-h PG treatments, continuing down-regulation of most proteins and up-regulation of a few proteins. Let us turn to a few selected proteins for further discussion.

Protein 25 is identified as a heat shock protein, one of the 12 proteins we placed in the Protein Action Category. Protein 25 expression was up-regulated by more than 3.5-fold after 12-h PGA_1 treatments and more than 17-fold after 12-h PGE_1 treatments. Several genes involved in protein

Table 3
Quantity and function of proteins identified by MS/MS from HzAM1 cells treated with 15 μM PGA1 or PGE1

Category	Spot no.	% of control (12/24 h)[a]				Putative protein name	Function
		PGA_1		PGE_1			
Protein action	1	60.7	15.3	10.7	17.0	CG9916-PA isoform 1	Accelerates protein folding
	2	132.6	36.5	75.8	100.1	Ubiquitin	Protein degradation
	16	103.3	50.0	65.6	23.0	Heat shock protein 20.4 kDa	Folding and assembly of proteins
	24	81.0	47.7	399.9	26.6	Mitochondrial processing peptidase alpha subunit	Processes all proteins entering mitochondria
	25	374.7	8.4	1767.9	32.4	Heat shock protein 70 kDa (9B; mortalin-2)	Folding and assembly of proteins
	26	74.2	23.1	178.0	87.9	Heat shock cognate 70 kDa	Folding and assembly of proteins
	28	134.2	104.9	241.7	161.0	Heat shock protein 70 kDa (CG2918-PA)	Folding and assembly of proteins
	29	100.0	87.1	149.2	41.9	Heat shock cognate 70 kDa	Folding and assembly of proteins
	30	101.7	115.0	147.7	67.4	Heat shock protein 60 kDa	Folding and assembly of proteins (can assist in virus production)
	34	33.0	63.1	94.5	63.4	Ubiquitin-like protein, SMT3	Modulation of protein activities
Lipid metabolism	12	73.1	75.8	92.5	43.2	Cellular retinoic acid binding protein	Fatty acid (unsaturated) binding
	21	87.2	84.0	155.8	51.0	Acyl-coenzyme A dehydrogenase	Metabolizes short-chain fatty acids
Signal transduction	32	90.4	80.8	136.3	51.0	14-3-3 protein, zeta isoform	Binds proteins containing (mainly) phosphothreonine/phosphoserine motifs (e.g., protein kinase C)
	33	46.3	106.4	184.2	70.5	Tyrosine 3-monooxygenase/ tryptophan 5-monoxygenase activation protein epsilon polypeptide	First enzyme in catecholamine biosynthesis: converts tyrosine to DOPA
	36	10.0	41.9	115.0	150.4	Calmodulin	Calcium binding molecule
Protection	3	41.0	162.8	97.2	126.1	Glutathione-S-transferase-like protein	Detoxification/metabolism
	4	193.9	87.4	20.5	104.1	Mn superoxide dismutase	Protects from reactive oxygen species
	7	81.4	123.2	81.4	138.0	Catalase	Protects from reactive oxygen species
	10	42.6	100.3	107.1	25.5	Cu/Zn superoxide dismutase	Protects from reactive oxygen species
	14	65.2	155.2	127.1	144.5	Glutathione S-transferase, subunit 2	Detoxification/metabolism
	15	77.3	63.9	70.7	39.7	Similar to *Drosophila melanogaster* Jafrac1 (Thioredoxin peroxidase)	Protects from peroxides; possible role in signal transduction (via H_2O_2 or superoxide ions)
Cell function	9	135.1	63.76	305.9	60.6	Bmsqd-1 (a heterogeneous nuclear ribonucleoprotein; hnRNP)	Nuclear RNA-binding protein: forms complexes with RNA polymerase II transcripts (involved in activities such as transcription, nuclear pre-mRNA processing, cytoplasmic mRNA translation and turnover)
	13	58.0	42.8	34.8	33.5	Actin-depolymerizing factor 1	Cell movement and cell division
	17	125.6	81.5	92.8	34.0	Bmsqd-1	See above
	27	16.9	19.3	332.6	70.6	Transitional endoplasmic reticulum ATPase TER94	Membrane fusion, organelle biogenesis
	31	83.6	93.9	126.0	58.3	Actin 5	Cell motility, chromosome segregation, macromolecule transport, endo-/ exocytosis
Energetics and/or metabolism	11	36.3	72.4	88.9	58.5	Abnormal wing disc-like protein	Catalyses conversions of NDP to NTP
	23	70.7	103.6	135.8	42.4	Similar to T21B10.2b isoform 2 (enolase)	Catalyses dehydration reaction important in glucolysis and gluconeogenesis
	5	74.4	105.4	71.0	76.1	Glucose-3-phosphate dehydrogenase	Glycolysis (sixth step)
	6	84.0	82.6	76.0	62.3	Acetyl-CoA hydrolase/transferase (GA20688-PA)	Acetyl CoA metabolism
	8	196.2	249.5	153.8	94.3	Glucose-3-phosphate dehydrogenase	Glycolysis (sixth step)
	20	200.0	77.9	150.0	104.4	LOC496165	Electron transport (FAD binding)
	22	60.4	37.7	53.0	66.6	Arginine kinase	Part of the ATP buffering system: releases ATP from phoshoarginine
	35	38.4	51.3	127.0	55.5	Thioredoxin-like protein	Electron carrier, important for cell redox homeostasis

[a]Percent change in protein density between cells treated with PGs versus those treated with 0.5% ethanol (vehicle control).

folding were influenced to a greater or lesser extent by PG treatments, including proteins 1, 16, 25, 26 and 28. This is consistent with the biomedical background, in which PGs are thought to play major signaling roles in stress responses, including induction of HSP genes (Negishi and Katoh, 2002). Protein 1 is a putative cyclophilin, proteins involved in protein folding. Expression of this protein was severely reduced, by approximately 10-fold, following 12-h treatment with PGE_1 and 24-h treatment with both PGs. Turning to another insect cell line, 12-h PGA_1 treatments stimulated biosynthesis of several proteins in a cell line established from the mosquito *Aedes albopictus* (C6/36). By comparing the influence of PGA_1 and heat treatments on protein synthesis, the authors concluded PGA_1 stimulates expression of stress proteins, based on 1D gels, in this insect cell line (Barbosa and Rebello, 1998).

Our experimental PG treatments influenced expression of genes encoding proteins involved in the signal transduction category, #32, 33 and 36. PGE_1 treatments resulted in approximate two-fold increases in expression of protein 33, from which it can be inferred that one or more PGs exert their actions on insect cells via influencing signal transduction mechanisms.

PGs also act in cellular protection mechanisms, particularly in gastrointestinal cytoprotection (Mozsik et al., 2007). It is not surprising to note that PGs influenced expression of genes involved in protection. The 12-h PGA_1 treatments resulted in up-regulation of protein 4 and down-regulation of proteins 3 and 10, all acting in protection from reactive oxygen species or other potential toxins. The 12-h PGE_1 treatments resulted in down-regulation of protein 4. The 24-h PGA1 treatments resulted in an approximate 50% reduction in protein 10% and 50% increase in protein 14. However, the PGE_1 treatments substantially decreased expression of proteins 10 and 15.

PGE_1 treatments increased expression of proteins connected to cell functions. After 12-h exposures, proteins 9 and 27 were increased three-fold. Particularly for protein 9, putatively a nuclear RNA-binding protein, PG treatments could influence cell actions in several areas of insect physiology.

Our PG treatments influenced expression of genes for several proteins involved in cellular metabolism, including proteins 11, 20, 22 and 35. We recorded substantial changes in protein 8, two-fold for PGA_1 treatments at 12-h exposure and 2.5-fold increase at 24-h exposure. PGE_1 treatments resulted in similar increases in expression, about 1.5-fold at 12- and 24-h. This is a likely glyceraldehyde-3-phosphate dehydrogenase, an enzyme in the glycolytic pathway. Specifically, this enzyme catalyzes synthesis of 1,3-bisphosphoglycerate, a relatively high energy intermediate that drives synthesis of ATP. We note that protein 5 also is a putative glyceraldehyde-3-phosphate dehydrogenase, which was not substantially changed in expression.

The HzAM1 cells used in these experiments were established from pupal ovarian tissue of the lepidopteran *Helicoverpa zea* (McIntosh and Ignoffo, 1983). The established HzAM1 cell line is heteroploid, a situation typical of cell lines established from vertebrate and invertebrate sources. While established insect cell lines have been crucial for advancing understanding of insect cell/virus interactions, this is the first study of the influence of PGs on gene expression in insect cells in culture. As is true for most analyses of protein expression, we are not yet able to comment on the meaning of changing expression in all proteins. Given recent work (Büyükgüzel et al., 2007; Durmaş et al., 2007) showing that eicosanoids mediate at least some insect/virus interactions, continued proteomic research will yield important new insights into the mechanisms responsible for allowing baculoviruses to replicate in some insect cell lines, but not others.

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