Eicosanoids mediate induction of immune genes in the fat body of the silkworm, *Bombyx mori*

Isao Morishima*, Yoshiaki Yamano, Kenji Inoue, Noriyuki Matsuo

Department of Biochemistry and Biotechnology, Faculty of Agriculture, Tottori University, Tottori 680, Japan

Received 2 October 1997; revised version received 27 October 1997

Abstract The expression of cecropin and lysozyme genes is induced in response to bacterial peptidoglycan in the fat body of the silkworm, *Bombyx mori*. Specific inhibitors of either phospholipase A_2, cyclooxygenase or lipoxygenase significantly inhibit the induction of the immune genes both in vivo and in cultured fat body as detected by means of Northern hybridization. Arachidonic acid injected into the larvae induces the expression of the cecropin and lysozyme genes. The findings support the idea that eicosanoids mediate some process leading to the expression of immune genes in the fat body following recognition of peptidoglycan as a signal for invading bacteria.

Key words: Eicosanoid; Cecropin; Lysozyme; Peptidoglycan; Silkworm; Insect immunity

1. Introduction

Many insects respond to a microbial infection by the production of humoral immune proteins including a set of antibacterial proteins such as cecropin, attacin and lysozyme [1,2]. Cecropins are a family of highly potent bactericidal peptides with 35–37 amino acid residues [3]. Most of the antibacterial genes are normally silent and are induced upon bacterial infection, except for lysozyme, which is constitutively synthesized in normal insects and up-regulated upon infection [1,4,5]. These immune genes are simultaneously induced in response to bacterial cell wall components such as peptidoglycan or lipopolysaccharide [5–7]. We have shown in the silkworm, *Bombyx mori*, that peptidoglycan fragments of a particular structure are recognized as a signal molecule and elicit the synthesis of antibacterial proteins in the fat body [8,9]. However, it is not yet known how the signal from the invading bacteria is transmitted to the immune genes, or what molecules mediate the signal transduction in the fat body cells.

Stanley-Samuelson et al. [10] found in the tobacco hornworm, *Manduca sexta*, that eicosanoids regulated bacterial cleaning from the hemocoelom, and suggested that eicosanoids mediated cellular immune responses in insects as in mammals. They have also shown that nodule formation is one of the cellular defense mechanisms mediated by eicosanoids [11–13]. With use of the inhibitors of eicosanoid biosynthesis, both cyclooxygenase and lipoxygenase products are suggested to be involved in nodulation responses to bacterial infections [11–13] and phagocytosis [14]. Recent findings on the roles of eicosanoids in insect defense responses are, however, limited only to the cellular responses. A possibility that eicosanoids may also influence other aspects of insect immune responses is as yet unexplored.

We are interested in the molecular mechanisms which lead to the induction of the immune genes following recognition of an external signal molecule such as bacterial cell wall peptidoglycan. During the screening of the inhibitors for this process, we found that eicosanoid biosynthesis inhibitors significantly repressed the induction of the expression of the cecropin and lysozyme genes elicited by peptidoglycan. We report here that eicosanoids mediate the induction of immune genes in the fat body of the silkworm.

2. Materials and methods

2.1. Insect

Silkworm (*Bombyx mori*) strain C108 was aseptically reared on an artificial diet (Nihon Nohsan Kogyo, Yokohama) at 27°C as previously described [9]. Larvae on day 3–4 of the fifth instar were used for the experiments.

2.2. Injection of insect and isolation of fat body

Soluble peptidoglycan (SPG) was prepared from *Bacillus licheniformis* cell wall as previously described [8]. All inhibitors and arachidonic acid were purchased from Sigma Chemicals. Test larvae were anesthetized with CO_2 gas and at first injected with inhibitor (in 10 μl of DMSO) or DMSO alone for control. After 30 min at room temperature, the larvae received a second injection with SPG (10 μg in 10 μl of insect Ringer [15]) or Ringer alone, and were further incubated at 27°C for 1 h. The fat body was excised, rinsed in ice-cold Ringer and frozen on dry ice.

2.3. In vitro culture of fat body

Fat body was excised from naive larvae and cultured as previously described [9]. Approx. 50 mg of the tissue was preincubated for 1 h at 27°C in 200 μl of Grace's medium (Gibco), then transferred to a fresh medium and further incubated for 15 h with gentle shaking. To test the effect of eicosanoid biosynthesis inhibitors, the inhibitor was added to the culture, and after 30 min incubation at 27°C, SPG was added at a concentration of 50 μg/ml. After further incubation for 3 h, the fat body was collected by brief centrifugation, briefly washed with ice-cold Ringer and frozen on dry ice.

2.4. Isolation of RNA and dot blot hybridization

Total RNA was isolated from frozen fat body by the guanidinium isothiocyanate-phenol-chloroform method [16]. The quantity of RNA was determined spectrophotometrically, and the quality was checked by electrophoresis on a formaldehyde-agarose gel. An aliquot (1 μg) of RNA was spotted onto a Hybond N^+ membrane (Amersham) using Biodot blotter (Bio-Rad). The membrane was hybridized as previously described [17] with [^{32}P]cDNA of cecropin B (pBCB-6) [17] or lysozyme (kind gift from Dr. Paul T. Brey of the Pasteur Institute) [18]. The membrane was then cut into small pieces, slightly larger than the area of the dot, and the radioactivity trapped on the membrane was counted in a liquid scintillation counter.

*Corresponding author. Fax: (81) (857) 31-5360.
E-mail: moris@agr.tottori-u.ac.jp

Abbreviations: SPG, soluble peptidoglycan; DMSO, dimethyl sulfoxide; BPB, *p*-bromophenacylbromide; NDGA, nordihydroguaiaretic acid; PLA_2, phospholipase A_2

PII S0014-5793(97)01418-X

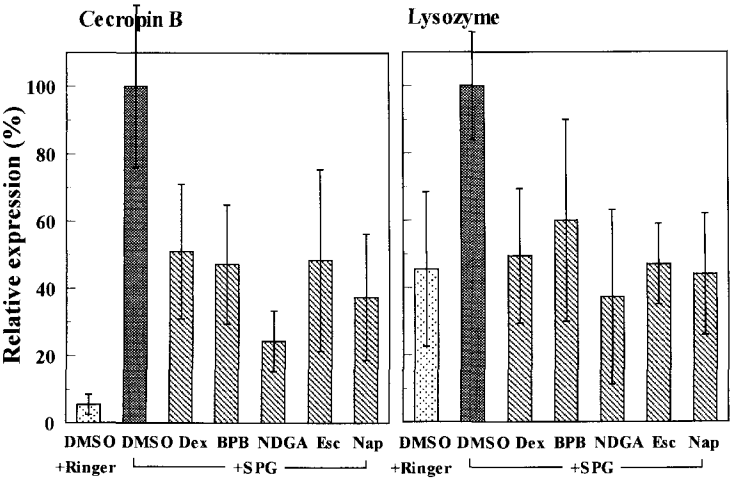

Fig. 1. Effect of treating silkworm larvae with eicosanoid biosynthesis inhibitors on cecropin and lysozyme gene induction in response to SPG. The larvae were at first injected with 100 μg (in 10 μl DMSO) of either dexamethasone (Dex), BPB, NDGA, esculetin (Esc) or naproxen (Nap). For control larvae, 10 μl of DMSO was injected. After keeping the larvae at room temperature for 30 min, the larvae then received a second injection of either insect Ringer or 10 μg of SPG (in 10 μl of Ringer). Total RNA was isolated from the fat body 60 min after the second injection, and analyzed by dot blot hybridization with cecropin B or lysozyme cDNA as a probe. The mRNA levels are shown as a percentage of the levels in the control larvae treated with DMSO and SPG. Bars represent the mean ± S.D. ($n = 4$ or 5).

3. Results

3.1. Inhibition of immune gene induction in larvae

To study the effect of eicosanoid biosynthesis inhibitors on SPG-induced immune gene expression, silkworm larvae were first injected with either the phospholipase A_2 (PLA_2) inhibitor dexamethasone, p-bromophenacylbromide (BPB), the cyclooxygenase inhibitor naproxen, the lipoxygenase inhibitor nordihydroguaiaretic acid (NDGA) or escuretin, then challenged with SPG (Fig. 1). In the control larvae which had been treated with vehicle alone, cecropin B mRNA was barely detectable, in contrast to lysozyme mRNA which is constitutively synthesized in normal fat body [5]. Both cecropin B and lysozyme genes were induced by the treatment of the larvae with SPG. All the inhibitors injected prior to the treatment with SPG greatly depressed the SPG-induced expression of both cecropin B and lysozyme genes.

3.2. Inhibition of immune gene induction in cultured fat body

When isolated fat body from the silkworm is incubated in Grace's medium, the cecropin and lysozyme genes are activated and the antibacterial proteins are secreted into the medium without addition of any elicitor, possibly triggered by dissection stress [9]. The stimulated expression of the genes without added elicitor was, however, gradually depressed and fell to the zero-time levels during 15 h of incubation (results not shown). In the experiments to detect the induction of immune genes in vitro, therefore, we preincubated the fat body for 15 h prior to the addition of inhibitors, and the mRNA was isolated after 3 h of incubation with SPG. As shown in Fig. 2, after 15 h of preincubation, the fat bodies still kept the ability to respond to added SPG. BPB and NDGA inhibited the induction by SPG of cecropin B and lysozyme gene expression. Indomethacin also dose-dependently inhibited the induction of the genes (Fig. 3). Treatment with the inhibitors abolished the SPG-induced stimulation of lysozyme gene expression, the expression at a constitutive level was, however, not affected by the inhibitors even at the highest dose of 1 mM, though cecropin gene expression was totally abolished (Figs. 2 and 3).

3.3. Induction of immune genes by arachidonic acid

The inhibitor experiments strongly suggested that the arachidonic acid metabolites mediated the induction of immune gene expression in the fat body. To test the direct action of arachidonic acid metabolites on the induction, silkworm larvae were treated with arachidonic acid, and the cecropin and lysozyme mRNA levels in the fat body were examined. As shown in Fig. 4, the treatment with arachidonic acid induced

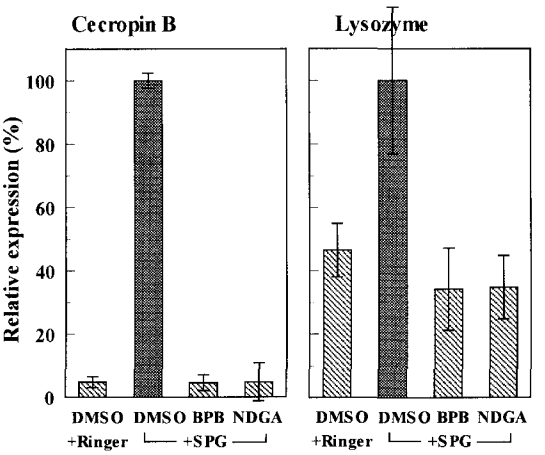

Fig. 2. Effect of eicosanoid biosynthesis inhibitors in cultured fat bodies on cecropin and lysozyme gene induction in response to SPG. Fat bodies were isolated and cultured as described in Section 2. To the culture, BPB or NDGA (in 10 μl of DMSO) was added at a concentration of 1 mM. For control, 10 μl of DMSO was added. After 30 min incubation at 27°C, 10 μg of SPG (in 10 μl of Ringer) or Ringer (10 μl) was added to the culture. After further incubation for 60 min, total RNA was isolated and analyzed by dot blot hybridization with cecropin B or lysozyme cDNA as a probe. The mRNA levels are shown as a percentage of the levels in the control culture treated with DMSO and SPG. Bars represent the mean ± S.D. ($n = 3$).

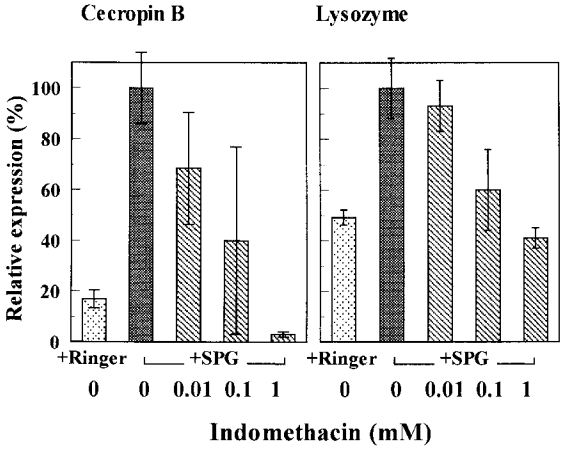

Fig. 3. Dose-dependent effect of indomethacin in cultured fat bodies on cecropin and lysozyme gene induction in response to SPG. The experimental conditions were identical to those described in Fig. 2, except that the indicated concentration of indomethacin (in 10 μl of DMSO) was added to the fat body culture. The mRNA levels are shown as a percentage of the levels in the control culture treated with DMSO and SPG. Bars represent the mean ± S.D. ($n = 3$).

cecropin and lysozyme gene expression, though the levels were somehow lower than that induced by SPG. The increase in cecropin gene expression by arachidonic acid was significant ($P > 0.05$) compared to the control.

4. Discussion

The data presented in this paper support the hypothesis that eicosanoids mediate immune gene expression in response to bacterial infection in the silkworm, *Bombyx mori*. Eicosanoids are known to mediate cellular immune reaction in insects [19]. Inhibition of either PLA_2, cyclooxygenase or lipoxygenase decreases bacterial clearance and nodule formation in lepidopterans [10,11,13] and in a beetle [12]. Activation of the prophenoloxidase cascade, which is considered to be involved in the early phase of nodulation, and phagocytosis are also regulated by eicosanoids [14]. These immune responses mediated by eicosanoids so far reported are all early events at the cellular level requiring no new gene products.

Our data clearly show that eicosanoid biosynthesis inhibitors repress the induction of the cecropin and lysozyme genes elicited by peptidoglycan, and arachidonic acid, the direct product by PLA_2, induces expression of the cecropin and lysozyme genes in the larval fat body. Effects of pharmacological inhibitors of eicosanoid metabolism in insects have been extensively studied by Stanley-Samuelson and his colleagues, and the effectiveness and specificity of the inhibitors have also been proved in insect tissues [19,20]. Our findings strongly suggest a novel function of eicosanoids in insects: eicosanoids regulate not only the cellular responses but humoral immune reaction including induction of the genes. In mammals, eicosanoids are known to play an important role in inflammatory responses in various tissues: arachidonic acid is released from murine macrophage cell membranes upon bacterial infection [21], and prostaglandin production is stimulated by LPS in murine Kupffer cells [22]. Recent studies on insect immunity have indicated that the innate immune systems in insects and mammals share control mechanisms [23,24]. The findings presented in this paper suggest that

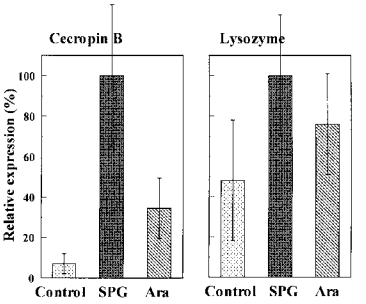

Fig. 4. Induction of cecropin and lysozyme genes by arachidonic acid injected into the silkworm larvae. The larvae were injected with either 10 μl of DMSO (control), 10 μg of SPG in 10 μl of Ringer or 100 μg of arachidonic acid in 10 μl of DMSO (Ara). The fat bodies were excised 4 h after the injection, and total RNA was analyzed as in Fig. 1. The mRNA levels are shown as a percentage of the levels in the control larvae treated with SPG. Bars represent the mean ± S.D. ($n = 5$).

both insects and mammals also share eicosanoids as a mediator in the regulation of acute phase reactions.

In the present study, both cyclooxygenase and lipoxygenase inhibitors repressed the immune gene induction. The inhibition pattern is similar to that previously found with the inhibition of nodulation [11–13] and the prophenoloxidase cascade [14], although it is not possible to determine which metabolites of arachidonate play a dominant role in the immune responses. Multiple eicosanoid metabolites may be intricately involved in the regulation of insect immune responses. In the cecropia silkmoth fat body, the attacin gene is induced by arachidonic acid [25]. This is the only case so far reported to show involvement of eicosanoids in the regulation of insect immune gene expression. In a *Drosophila* blood cell line, in contrast, neither arachidonic acid nor dexamethasone has any effect on induction of the cecropin gene [26]. The role of eicosanoids in immune response may be different in lepidopteran and dipteran insects. Further experiments should clarify this apparent contradiction.

Acknowledgements: We are grateful to Dr. Paul T. Brey, Institut Pasteur, for providing us silkworm lysozyme cDNA. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

References

[1] Boman, H.G. (1994) in: Phylogenetic Perspectives in Immunity: The Insect Host Defense, pp. 3–17, R.G. Landes Co., Austin, TX.
[2] Hultmark, D. (1993) Trends Genet. 9, 178–183.
[3] Boman, H.G., Faye, I., Gudmundsson, G.H., Lee, J.Y. and Lidholm, D.A. (1991) Eur. J. Biochem. 201, 23–31.
[4] Sun, S.C., Åsling, B. and Faye, I. (1991) J. Biol. Chem. 266, 6644–6649.
[5] Morishima, I., Horiba, T., Iketani, M., Nishioka, E. and Yamano, Y. (1995) Dev. Comp. Immunol. 19, 357–363.
[6] Samakovlis, C., Kimbrell, D.A., Kylsten, P., Engström, Å. and Hultmark, D. (1990) EMBO J. 9, 2969–2976.
[7] Taniai, K., Furukawa, S., Shono, T. and Yamakawa, M. (1996) Biochem. Biophys. Res. Commun. 226, 783–790.
[8] Morishima, I., Yamada, K. and Ueno, T. (1992) Insect Biochem. Mol. Biol. 22, 363–367.
[9] Iketani, M. and Morishima, I. (1993) Insect Biochem. Mol. Biol. 23, 913–917.
[10] Stanley-Samuelson, D.W., Jensen, E., Nickerson, K.W., Tiebel,

K., Ogg, C.L. and Howard, R.W. (1991) Proc. Natl. Acad. Sci. USA 88, 1064–1068.
[11] Miller, J.S., Nguyen, T. and Stanley-Samuelson, D.W. (1994) Proc. Natl. Acad. Sci. USA 91, 12418–12422.
[12] Miller, J.S., Howard, R.W., Nguyen, T., Nguyen, A., Rosario, R.M.T. and Stanley-Samuelson, D.W. (1986) J. Insect Physiol. 42, 3–12.
[13] Jurenka, R.A., Miller, J.S., Pedibhotla, V.K., Rana, R.L. and Stanley-Samuelson, D.W. (1997) J. Insect Physiol. 43, 125–133.
[14] Mandato, C.A., Diehl-Jones, W.L., Moore, S.J. and Downer, R.G.H. (1997) J. Insect Physiol. 43, 1–8.
[15] Jungreis, A.M., Jatlow, P. and Wyatt, G.R. (1973) J. Insect Physiol. 19, 225–233.
[16] Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156–159.
[17] Yamano, Y., Matsumoto, M., Inoue, K., Kawabata, T. and Morishima, I. (1994) Biosci. Biotech. Biochem. 58, 1476–1478.
[18] Lee, W.J. and Brey, P.T. (1995) Gene 161, 199–203.
[19] Stanley-Samuelson, D.W. and Pedibhotla, V.K. (1996) Insect Biochem. Mol. Biol. 26, 223–234.
[20] Stanley-Samuelson, D.W. (1994) J. Insect Physiol. 40, 3–11.
[21] Narumi, S. and Hamilton, T.A. (1990) Immunopharmacology 19, 93–101.
[22] Decker, K. (1990) Eur. J. Biochem. 192, 245–261.
[23] Hultmark, D. (1994) in: Phylogenetic Perspectives in Immunity: The Insect Host Defense, pp. 184–188, R.G. Landes Co., Austin, TX.
[24] Hoffmann, J.A. and Reichhart, J.M. (1997) Trends Cell Biol. 7, 309–316.
[25] Sun, S.O. and Faye, I. (1995) Eur. J. Biochem. 231, 93–98.
[26] Samakovlis, C., Åsling, B., Boman, H.G., Gateff, E. and Hultmark, D. (1992) Biochem. Biophys. Res. Commun. 188, 1169–1175.