A newly established *in vitro* culture using transgenic *Drosophila* reveals functional coupling between the phospholipase A_2-generated fatty acid cascade and lipopolysaccharide-dependent activation of the immune deficiency (imd) pathway in insect immunity

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Innate immunity is the first line of defence against infectious micro-organisms, and the basic mechanisms of pathogen recognition and response activation are evolutionarily conserved. In mammals, the innate immune response in combination with antigen-specific recognition is required for the activation of adaptive immunity. Therefore, innate immunity is a pharmaceutical target for the development of immune regulators. Here, for the purpose of pharmaceutical screening, we established an *in vitro* culture based on the innate immune response of *Drosophila*. The *in vitro* system is capable of measuring lipopolysaccharide (LPS)-dependent activation of the immune deficiency (imd) pathway, which is similar to the tumour necrosis factor signalling pathway in mammals. Screening revealed that well-known inhibitors of phospholipase A_2 (PLA_2), dexamethasone (Dex) and *p*-bromophenacyl bromide (BPB) inhibit LPS-dependent activation of the imd pathway. The inhibitory effects of Dex and BPB were suppressed by the addition of an excess of three (arachidonic acid, eicosapentaenoic acid and γ-linolenic acid) of the fatty acids so far tested. Arachidonic acid, however, did not activate the imd pathway when used as the sole agonist. These findings indicate that PLA_2 participates in LPS-dependent activation of the imd pathway via the generation of arachidonic acid and other mediators, but requires additional signalling from LPS stimulation. Moreover, PLA_2 was activated in response to bacterial infection in *Sarcophaga*. These results suggest a functional link between the PLA_2-generated fatty acid cascade and the LPS-stimulated imd pathway in insect immunity.

Key words: host defence, innate immunity, nuclear factor κB (NF-κB), tumour necrosis factor (TNF).

INTRODUCTION

Innate immunity is the front line of self-defence against pathogen infection [1]. The innate immune system uses germline-encoded pattern-recognition receptors to recognize conserved molecular structures that are present on the surface of pathogens but absent in the host, such as lipopolysaccharides (LPS), peptidoglycans and β-1,3-glucans [2]. Genetic and molecular studies of model organisms reveal a striking conservation between the mechanisms that regulate insect host defence and the mammalian innate immune response [3]. In response to microbial infection, *Drosophila* secrete several antimicrobial peptides into the haemolymph from the fat body, the functional equivalent of the mammalian liver, by two distinct signalling pathways, Toll and immune deficiency (imd), which are similar to the Toll-like receptor/interleukin 1 receptor signalling pathways and the tumour necrosis factor (TNF) receptor signalling pathway, respectively, in mammals [4–6]. The Toll pathway regulates the induction of an antifungal peptide gene, *Drosomycin*, in response to Gram-positive bacterial infection and fungal infection [7,8]. A pattern-recognition receptor, peptidoglycan-recognition protein (PGRP)-SA, is required for this response to Gram-positive bacterial infection [9], whereas the induction of *Diptericin*, an antibacterial peptide gene, in response to Gram-negative and other Gram-positive bacterial infections is almost completely regulated by the imd pathway via the Rel/nuclear factor κB (NF-κB)-like transcriptional factor Relish [8,10,11]. Another PGRP family member, PGRP-LE, is a pattern-recognition receptor for diaminopimelic acid-containing peptidoglycans and activates the imd pathway [12]. Moreover, PGRP-LC participates in the recognition of LPS and is required for activation of the imd pathway [13–15]. Diaminopimelic acid-containing peptidoglycans are cell-wall components of bacteria that activate the imd pathway, and LPS are cell-wall components of Gram-negative bacteria that also activate the imd pathway [8,12]. The induction of *Attacin*, an antibacterial peptide gene, is thought to be regulated by both the imd and Toll pathways [4,5].

In contrast with innate immunity, adaptive immunity is mediated by highly specific antigen receptors that are generated by somatic cell gene rearrangement [2]. These receptors are found only in higher vertebrates in which interaction between adaptive immunity and innate immunity produce a complex immune system. Innate immunity has an instructive role in the adaptive immune response to antigens by inducing co-stimulatory molecules and cytokines, indicating that activation of the antigen-specific adaptive response is coupled to pathogen infection through innate immunity [16,17]. Therefore, from a pharmaceutical point of view, innate immunity is a good target for the

Abbreviations used: BPB, *p*-bromophenacyl bromide; Dex, dexamethasone; GFP, green fluorescent protein; imd, immune deficiency; LPS, lipopolysaccharide; NF-κB, nuclear factor κB; PGRP, peptidoglycan-recognition protein; PLA_2, phospholipase A_2; RT-PCR, reverse transcriptase PCR; TNF, tumour necrosis factor.

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development of immune regulators, such as immune activators and immune suppressors. Here, we established an *in vitro* culture based on the innate immune response of *Drosophila*. The established system can be used to evaluate the effects of samples on the LPS-dependent and the imd-mediated antibacterial response. Using this system, we demonstrated a functional link between the phospholipase A_2 (PLA_2)-generated fatty acid cascade and the LPS-stimulated imd pathway.

EXPERIMENTAL

Fly strains

Drosophila stocks were raised on standard cornmeal/agar medium at 25 °C. Oregon R flies were used as a standard wild-type strain. The transgenic strains *Diptericin-lacZ* (*Dpt-lacZ*) and *Drosomycin-GFP* (*Drs-GFP*; where GFP is green fluorescent protein) were described previously [18,19]. *imd*, $Relish^{E38}$ and J4 are described elsewhere [10,11,20]. The flesh fly, *Sarcophaga peregrina*, was reared at 27 °C. The larvae were synchronized at pupariation as described previously [21].

In vitro culture of the transgenic larvae

All procedures were performed in LPS-free conditions. The third-instar *Dpt-lacZ* larvae were treated with 70 % ethanol for 20 min and washed with LPS-free water and LPS-free saline. The abdominal cavity of the larva was opened using fine pincettes in LPS-free saline. Individual larvae were cultured in the presence or absence of LPS (*Escherichia coli* O55:B5; Sigma, St. Louis, MO, U.S.A.) with Schneider's *Drosophila* medium (100 μl; Gibco-BRL, Invitrogen Corp., Carlsbad, CA, U.S.A.) containing 20 % fetal calf serum (Gibco-BRL) and 1 % antibiotics/antimycotics (Gibco-BRL) in each well of a 96-well plate at 25 °C for 12 h. Fatty acids, dexamethasone (Dex) and *p*-bromophenacyl bromide (BPB) were dissolved in DMSO and added to the culture medium. For each condition, six separate females were cultured to produce six replicates.

β-Galactosidase assay

The cultured individual larvae were sonicated with 200 μl of reaction buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl and 1 mM $MgCl_2$) using an Ultrasonic Processor (Misonix, New York, NY, U.S.A.). After centrifugation (10000 ***g***) at 4 °C for 10 min, the β-galactosidase activity of the resulting supernatants was determined using Galacton-plus (Tropix, Bedford, MA, U.S.A.) as a substrate. After incubation with a substrate for 1 h, chemiluminescence was measured using Emerald II (Tropix) as an enhancer with a luminometer (Belthold, Wildbad, Germany). Standard curves were made using β-galactosidase (Wako, Osaka, Japan) after determination of the units of the enzyme with *o*-nitrophenyl β-D-galactopyranoside as a substrate. The activity was standardized against total amounts of protein, which were determined by protein assay (Bio-Rad, Hercules, CA, U.S.A.) using BSA as a standard. To eliminate larvae that had an activated immune response during breeding or had no response during *in vitro* culture for technical reasons, the highest and the lowest values were eliminated from the six individual values of each sample, and the means ± S.D. of the four remaining samples were calculated.

Quantitative reverse transcriptase PCR (RT-PCR)

Total RNA was isolated from 20 larvae using Trizol reagent (Gibco-BRL) and dissolved in 20 μl of RNase-free water. Total RNA (1 μg) was used in 20 μl of reverse transcription reaction with ReverTraAce reverse transcriptase (Toyobo, Osaka, Japan) and oligo(dT)15 primer (Promega, Madison, WI, U.S.A.). First-strand cDNA (0.05 μl) was used as the template for quantitative RT-PCR. Real-time PCR was performed with a LightCycler (Roche Diagnostics, Mannheim, Germany). PCR specificity was confirmed based on the molecular mass of the PCR products and melting-curve analysis for each data point. The copy numbers of RNA coding the genes of interest were standardized against those of the RNA coding rp49 in each sample. Primers were as follows: *Diptericin*, 5′-GTTCACCATTGCCGTCGCCTTAC-3′ and 5′-CCCAAGTGCTGTCCATATCCTCC-3′; *Drosomycin*, 5′-TTGTTCGCCCTCTTCGCTGTCCT-3′ and 5′-GCATCCTTCGCACCAGCACTTCA-3′; *Attacin*, 5′-GTGGTGGGTCAGGTTTTCGC-3′ and 5′-TGTCCGTTGATGTGGGAGTA-3′; *rp49*, 5′-AGATCGTGAAGAAGCGCACCAAG-3′ and 5′-CACCAGGAACTTCTTGAATCCGG-3′. Efficiency was as follows: *Diptericin*, 1.79; *Drosomycin*, 1.78; *Attacin*, 1.80; *rp49*, 1.86. Correlation coefficients of dilution curves: *r* values were −1.00 in each experiment.

Measurements of PLA_2 activity of *Sarcophaga*

PLA_2 activity was assayed with L-3-phosphatidylcholine-1,2-di-[1-^{14}C]oleoyl as a substrate according to the method reported previously [22]. Immune challenge was performed by pricking adult flies with a fine needle dipped into a concentrated culture of *E. coli* K-12. The flies were homogenized with ice-cold 100 mM Tris/HCl buffer, pH 9, and centrifuged at 1500 ***g*** for 10 min at 4 °C. The resulting supernatants were used for the PLA_2 assay. Protein was determined by protein assay (Bio-Rad) using BSA as a standard.

RESULTS

Establishment of an *in vitro* culture capable of quantifying the LPS-dependent activation of the imd pathway in innate immunity

In *Drosophila*, the activation of humoral immune responses was monitored by the expression of two reporter genes, *Dpt-lacZ* and *Drs-GFP*, which were constructed with the promoter regions of *Diptericin* and *lacZ* [18] and of *Drosomycin* and the *GFP* gene [19], respectively. To establish a pharmaceutical screen for the regulators of innate immunity, we investigated whether these reporter genes were activated by addition of LPS or peptidoglycan into the medium in which the transgenic larvae carrying these reporter genes were cultured. *Dpt-lacZ* was activated *in vitro* by the addition of LPS into the culture medium in an LPS dose-dependent manner, reaching a plateau at 10 μg/ml (Figure 1A). There was no significant activation of the other reporter gene, *Drs-GFP*, in the presence of either LPS or peptidoglycan (results not shown). Quantitative RT-PCR analyses revealed that the endogenous *Diptericin* gene was strongly induced by adding LPS into the medium, whereas there was only weak induction of *Drosomycin* and *Attacin* (Figures 1B–1D). The LPS dose-dependent activation of endogenous *Diptericin* coincided with the activation of *Dpt-lacZ*. The time-course of *Dpt-lacZ* activation coincided with that of endogenous *Diptericin* activation in the culture (Figures 1E and 1F). The expression of the reporter gene was induced in the fat body of *Dpt-lacZ* larvae after *E. coli* infection *in vivo* (Figure 1H). Similar staining was observed in the fat body of LPS-stimulated larvae but not in the fat body of non-stimulated control larvae *in vitro* (Figures 1I and 1J). These results indicate that the expression of *Dpt-lacZ* reflects the expression of endogenous *Diptericin in vitro*.

The *Diptericin* gene is almost completely regulated by the imd pathway via the Rel transcription factor Relish [10,11], whereas

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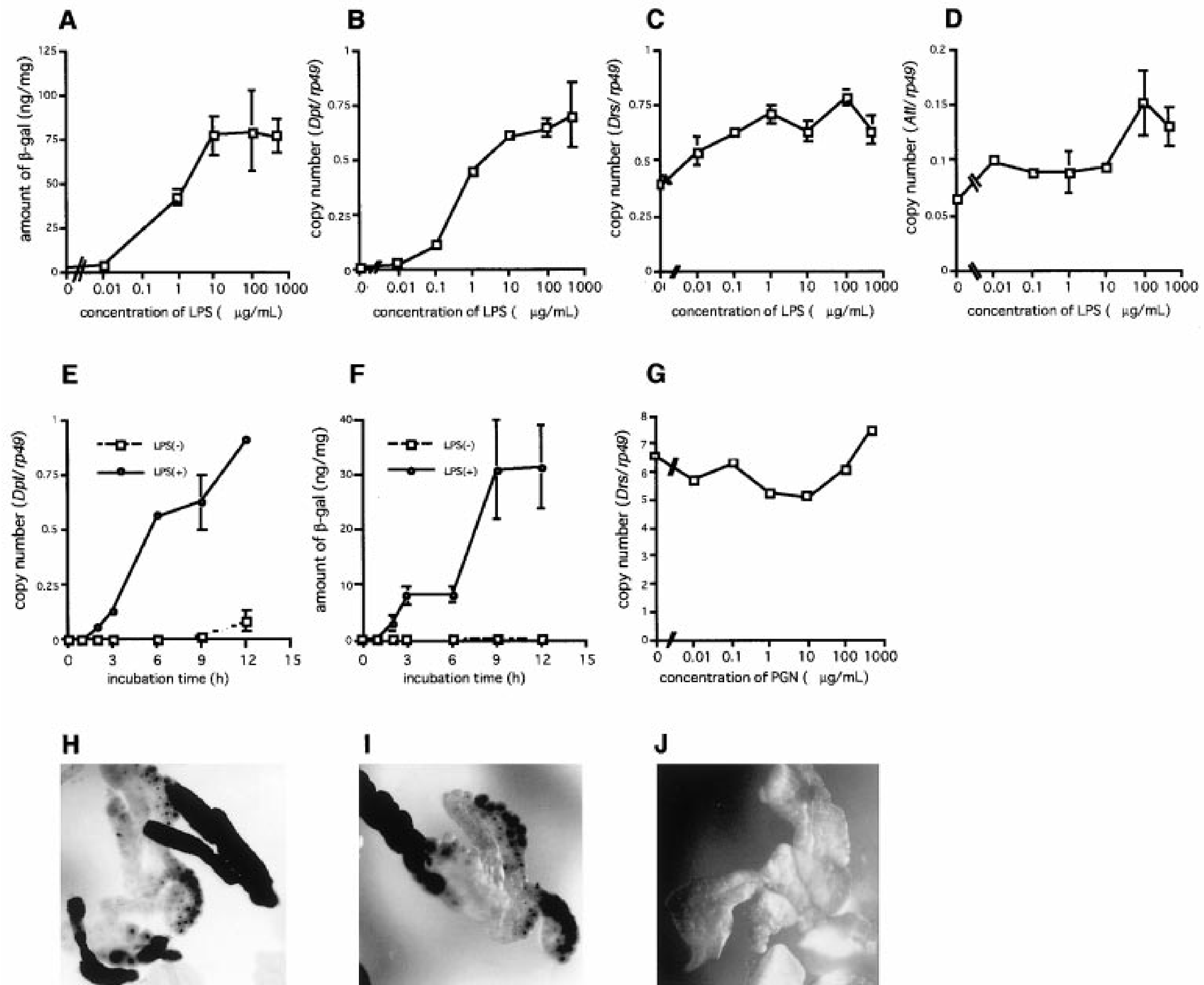

Figure 1 Quantification of LPS-dependent activation of the antibacterial response *in vitro*

(**A**) Induction of *Dpt-lacZ* in response to the addition of LPS into the culture medium. In the culture, endogenous *Diptericin* gene (**B**) was also activated in response to LPS, but significant induction of *Drosomycin* (**C**) and *Attacin* (**D**) was not observed. The amounts of mRNA of *Diptericin*, *Drosomycin*, *Attacin* and the *rp49* internal control were quantified by real-time RT-PCR in each sample. The time-course of *Dpt-lacZ* activation (**F**) also coincided with that of endogenous *Diptericin* activation in the culture (**E**). Oregon R (**E**) and *Dpt-lacZ* (**F**) larvae were cultured in the absence (□) and presence (○) of LPS (10 μg/ml). (**G**) Addition of peptidoglycan (PGN) of *Staphylococcus aureus* (Fluka) into the medium failed to induce *Drosomycin* expression. (**H**) Expression of the reporter gene was induced in the fat body of *Dpt-lacZ* larvae after *E. coli* infection *in vivo*. Similar staining was observed in the fat body of LPS-stimulated larvae (**I**), but not in the fat body of non-stimulated control larvae *in vitro* (**J**). Bars indicate S.D. The results were confirmed by at least three independent experiments.

Drosomycin is predominantly regulated by the Toll pathway [7]. The lysine-containing peptidoglycan, a cell-wall component of many Gram-positive bacteria, also failed to induce the expression of *Drosomycin in vitro* (Figure 1G). Therefore, differential induction of the antimicrobial genes *in vitro* suggests that the imd pathway was specifically activated in the culture. In *imd* or *Relish* loss-of-function backgrounds [10,11], the LPS-stimulated expression of *Diptericin* was abolished *in vitro* (Figure 2A). Moreover, the LPS-stimulated expression of *Diptericin* was observed in a J4 deletion [20] of Rel factor genes in the Toll pathway, *dorsal* and *Dorsal-related immunity factor* (Figure 2A). These results indicate that the imd pathway-dependent antibacterial responses were reconstructed in the culture, but that the Toll-pathway-dependent antimicrobial responses were not. The LPS-stimulated expression of *Dpt-lacZ* was also abolished in an *imd* loss-of-function background (Figure 2B), indicating that the established *in vitro* system was capable of quantifying the LPS-dependent activation of the imd pathway.

Functional link between PLA_2-generated fatty acid cascade and LPS-stimulated imd pathway in insect immunity

Systematic screening using the culture indicated that the well-known inhibitors of PLA_2, Dex and BPB, inhibit LPS-dependent activation of the imd pathway in a dose-dependent manner (Figures 3A and 3B). PLA_2 has a crucial role in liberating free fatty acids and lysophospholipids from membrane phospholipids, thereby initiating the production of biologically active lipids, which mediate inflammatory reactions in mammals [23]. To elucidate whether inhibition of the LPS-dependent activation of the imd pathway observed with PLA_2 inhibitors was due to

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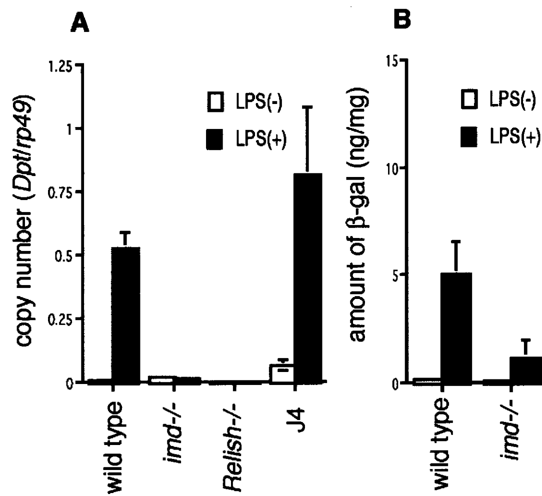

Figure 2 LPS-dependent activation of antibacterial response is mediated by the imd pathway but not by the Toll pathway

(**A**) Induction of the *Diptericin* gene by the addition of LPS into the medium in various mutant backgrounds. (**B**) The LPS-stimulated induction of *Dpt-lacZ* is also abolished in *imd* mutants. Oregon R (**A**) and *Dpt-lacZ* larvae (**B**) were cultured in the absence (□) and presence (■) of LPS (10 μg/ml). wild type, wild-type background; *imd−/−*, *imd/imd*; *Relish−/−*, *Relish*^{E38}/*Relish*^{E38}; J4, J4/J4. Bars indicate S.D. The results were confirmed by at least two independent experiments.

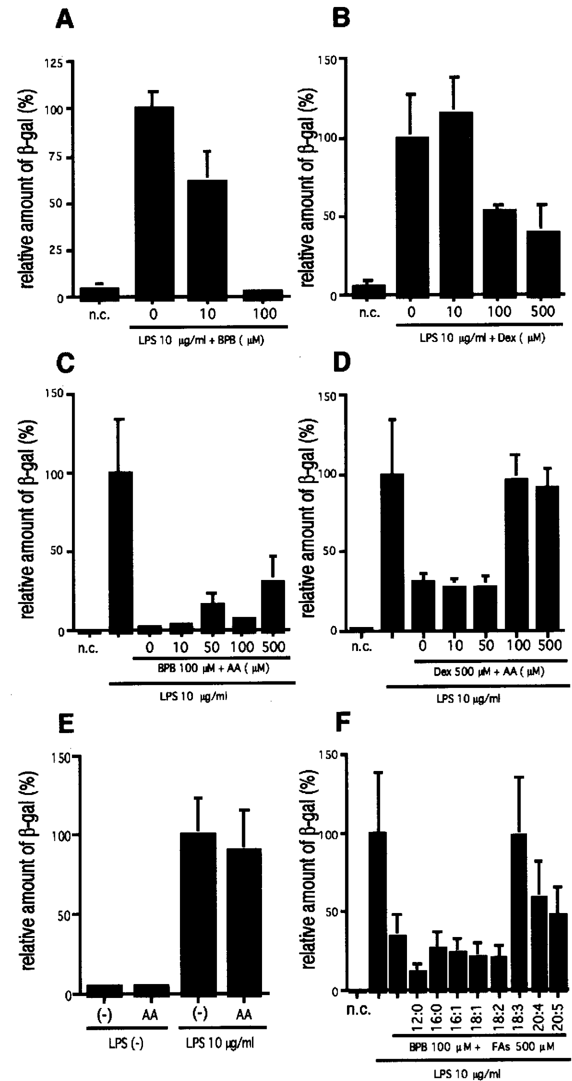

Figure 3 Inhibition of imd/Relish-mediated antibacterial response by the inhibition of PLA_2 and rescue of the inhibition by exogenous fatty acids

Dose-dependent inhibition of the imd/Relish-mediated antibacterial response by the addition of PLA_2 inhibitors, BPB (**A**) and Dex (**B**). *Dpt-lacZ* larvae were cultured in the absence (n.c.) and presence of LPS (10 μg/ml). Concentrations of inhibitors (μM) are indicated. Exogenous arachidonic acid (AA) rescued the inhibitory effects of BPB (**C**) and Dex (**D**); concentrations of arachidonic acid (μM) are indicated. (**E**) Arachidonic acid failed to activate the imd pathway when used as the sole agonist; −, no arachidonic acid. (**F**) The inhibitory effects of BPB were rescued by the addition of γ-linolenic acid (18:3), eicosapentaenoic acid (20:5) and arachidonic acid (20:4), but not by addition of the other fatty acids (FAs) lauric acid (12:0), palmitic acid (16:0), palmitoleic acid (16:1), oleic acid (18:1) and linoleic acid (18:2). Bars indicate S.D. The results were confirmed by at least two independent experiments.

reduced arachidonic acid levels and thus whether arachidonic acid is the effective component produced by PLA_2, we examined the effects of exogenous addition of arachidonic acid on LPS-dependent activation of the imd pathway in the presence of these inhibitors. Exogenous arachidonic acid rescued the inhibitory effects of the inhibitors (Figures 3C and 3D), suggesting that arachidonic acid, rather than other PLA_2-generated products such as lysophospholipids and platelet-activating factor, participated in the LPS-dependent activation of the imd pathway. Arachidonic acid, however, did not activate the imd pathway when used as the sole agonist, indicating that arachidonic acid participated in the imd pathway, but required additional signalling from LPS stimulation (Figure 3E).

To determine the fatty acid specificity of the rescue activity, we examined the effects of seven other fatty acids on the inhibitory effect of BPB on activation of the imd pathway. γ-Linolenic acid, a precursor of arachidonic acid, completely rescued inhibition of LPS-stimulated activation of the imd pathway by BPB, and eicosapentaenoic acid partially rescued the inhibition to a similar extent as arachidonic acid (Figure 3F). Eicosapentaenoic acid and arachidonic acid are eicosanoid precursors. The addition of other fatty acids, lauric acid, palmitic acid, palmitoleic acid, oleic acid and linoleic acid, did not rescue the inhibition. These results suggest that arachidonic acid metabolites or related lipid mediators specifically participated in the LPS-dependent activation of the imd pathway.

In mammals, PLA_2 is activated in response to various stimuli such as cytokines, hormones, antigens and endotoxins. The activation is mediated by transcriptional regulation and cell-specific intracellular signalling that involves G-protein, increases in intracellular Ca^{2+} and the activation of kinases such as mitogen-activated protein kinase and protein kinase C [24]. We investigated whether PLA_2 is activated in response to bacterial infection in insects. PLA_2 was transiently activated by *E. coli* infection in *Sarcophaga* (Figure 4), suggesting that PLA_2 participated in the antibacterial response in insects.

DISCUSSION

We established an *in vitro* culture based on the antibacterial response of *Drosophila*. The expression of a reporter gene, *Dpt-lacZ*, reflected the LPS-dependent activation of the imd pathway in the *in vitro* system. In the *in vitro* culture, the mammalian

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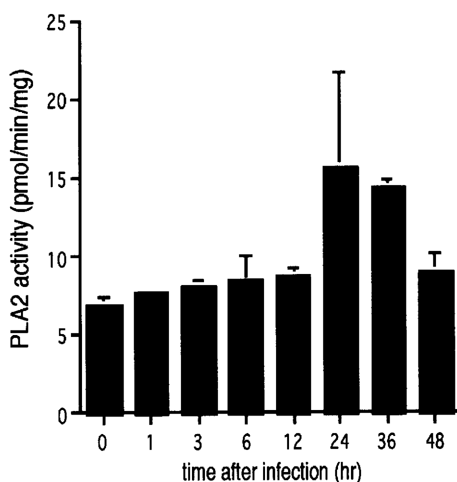

Figure 4 Induction of PLA_2 after *E. coli* infection in *Sarcophaga*

PLA_2 activity was measured over time after *E. coli* infection in *Sarcophaga*. Means ± S.D. of triplicates are shown.

inflammatory response suppressors, Dex and BPB, inhibited the LPS-dependent activation of the imd pathway in insects. In combination with the loss-of-function and the gain-of-function mutations in the *Drosophila* immune response, the culture system has an advantage over conventional cell-line culture in identifying the target molecules that the chemicals affected.

LPS-dependent activation of the imd pathway was observed in the culture, whereas activation of the Toll pathway was not induced in response to the Lys-containing peptidoglycans or LPS. These results are consistent with the conclusions that membrane PGRP-LC participates in the LPS recognition and is required for activation of the imd pathway, and circulating PGRP-SA has affinity for Lys-containing peptidoglycans and is required for activation of the Toll pathway [9,13–15,25]. Because the haemolymph is lost in the culture, the signalling cascades present in haemolymph, including the circulating pattern-recognition receptors such as PGRP-SA and PGRP-LE, are not functional in the *in vitro* system.

We demonstrated a functional link between the PLA_2-generated fatty acid cascade and LPS-dependent activation of the imd pathway in *Drosophila*. In the silk moth, using the PLA_2 and cyclo-oxygenase inhibitors, eicosanoids are suggested to mediate the induction of cecropin, an antibacterial peptide, and lysozyme genes in response to the soluble peptidoglycan *in vivo* and *in vitro* fat body culture [26]. Eicosanoids also mediate cellular immune reactions to bacterial infections in other insects [27–30]. Therefore, the PLA_2-generated fatty acid cascade might have a fundamental role in humoral and cellular insect immunity. Because the fat body is surrounded by the basement membrane [31], which is a physiological barrier to chemicals, relatively high doses of inhibitors and fatty acids might be required to produce effects in these experiments, including those of the present study.

In mammals, PLA_2 is involved in TNF-mediated NF-κB activation via the generation of arachidonic acid and other mediators [32]. To participate in the activation of NF-κB, PLA_2 requires additional signals from the TNF receptor, probably to initiate activation of the inhibitory κB ('IκB') kinase (IKK) complexes [33]. In the present study, we demonstrated that in *Drosophila* PLA_2 participates in the LPS-dependent activation of the imd pathway via the generation of arachidonic acid and other mediators, but requires additional signals from LPS signalling. The imd pathway is similar to the TNF signalling pathway, including IKKs in mammals [5,6]. These findings suggest some evolutionary conservation of the link between the PLA_2-generated fatty acid cascade and activation of Rel/NF-κB signalling in innate immunity.

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