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Interactions between the endocrine and immune systems in locusts

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Abstract. The prophenoloxidase cascade in the haemolymph of mature adult *Locusta* migratoria migratorioides (R. & F.) is activated in response to injection of laminarin, a β-1,3 glucan. Co-injection of adipokinetic hormone-I (*Lom*-AKH-I) and laminarin prolongs the activation of the enzyme in a dose-dependent manner. However, injections of bacterial lipopolysaccharide (LPS) do not activate prophenoloxidase unless AKH is co-injected, when there is a dose-dependent increase in the level of phenoloxidase that persists in the haemolymph for several hours. Even when AKH is co-injected, the highest levels of phenoloxidase activity are always greater after injection of laminarin than after LPS, and these two immunogens must activate the prophenoloxidase cascade by quite distinct pathways. In the present study, interactions between the endocrine and immune systems have been examined with respect to activation of prophenoloxidase and the formation of nodules: injection of LPS induces nodule formation in adult locusts. With LPS from *Pseudomonas* aeruginosa, nodules form exclusively in dense accumulations in the anterior portion of the abdomen on either side of the dorsal blood vessel associated with the dorsal diaphragm. With LPS from *Eschericia coli*, however, fewer nodules are formed but with a similar distribution, except that occasionally some nodules are aligned additionally on either side of the ventral nerve cord. Co-injection of *Lom*-AKH-I with LPS from either bacteria stimulates greater numbers of nodules to be formed. This effect of co-injection of AKH on nodule formation is seen at low doses of hormone with respectively only 0.3 or 0.4 pmol of *Lom*-AKH-1 increasing the number of nodules by 50%. Injections of octopamine, or 5-hydroxytryptamine do not mimic either of the actions of *Lom*-AKH-I described here. Co-injection of an angiotensin converting enzyme (ACE) inhibitor, captopril, reduces nodule formation in response to injections of LPS but has no effect on the activation of phenoloxidase. Co-injection of an inhibitor of eicosanoid synthesis, dexamethasone, with LPS influences nodule formation (with or without AKH) in different ways according to the dose of dexamethasone used, but does not affect activation of prophenoloxidase. Eicosanoid synthesis is important for nodule formation, but not for the activation of the prophenoloxidase cascade in locust haemolymph.

Keywords: adipokinetic hormone; captopril; dexamethasone, eicosanoid synthesis; 5-hydroxytryptamine; immune response; laminarin; lipopolysaccharide; *Locusta migratoria*; LPS; nodule; octopamine; phenoloxidase

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Introduction

The insects' defence systems against potential pathogens involve both cellular and humoral responses (see Lavine & Strand, 2002). Proteins present in the plasma or haemocytes bind to polysaccharides of bacteria or fungi and initiate cellular mechanisms involving phagocytosis and encapsulation, where haemocytes engulf and/or entrap foreign bodies or invading microbes, and aggregate to form nodules (see Ratcliffe *et al.*, 1995). The humoral responses include activation of a prophenoloxidase cascade (Ashida & Brey, 1998), and the eventual synthesis by the fat body of antimicrobial peptides to fight infection in the haemolymph (see Gillespie *et al.*, 1997).

In mammals, eicosanoids and hormones such as corticosterone regulate aspects of the immune response, and eicosanoids are also an important component of the cellular response in insects (Stanley-Samuelson *et al.*, 1991, 1997; Miller *et al.*1996, 1999; Bedick *et al.*, 2000; Miller & Stanley, 2001; Dean *et al*., 2002). Furthermore, injections of biogenic amines, ecdysteroids, and opiate peptides all exert effects on insect immune responses (Gillespie *et al*., 1997). However, there are several lines of evidence that suggest the regulation of lipid transport mechanisms in insects could be an additional important point of interaction between the endocrine and immune signalling systems (see Goldsworthy *et al*., 2002). Thus, because adipokinetic hormones (AKHs) regulate lipid transport in locusts (Goldsworthy, 1983; Goldsworthy *et al*., 1997; Goldsworthy & Joyce, 2001), the potential of these neurohormones to regulate immune responses triggered by polysaccharides from bacteria or fungi has been tested in locusts.

Goldsworthy *et al*. (2002) showed for the first time that while the phenoloxidase cascade is activated in the locust *in vivo* by injection of microbial cell wall components such as laminarin (mainly β,1-3 glucan), co-injection of adipokinetic hormone-I (*Lom*-AKH-I, AKH) prolongs this response to laminarin (Fig. 1) in a dose-dependent manner (Fig. 2). In the case of injections of bacterial lipopolysaccharide (LPS) from *Escherichia coli*, co-injection of AKH brings about an activation of prophenoloxidase activity that is not seen in the absence of the hormone (Fig. 1) but, again, the effect of the hormone is dose-dependent

(Fig. 2). It is intriguing that even when AKH is co-injected, the maximal activity of phenoloxidase seen after injection of laminarin is higher than after injection of LPS.

Although injection with LPS in the absence of AKH does not activate the prophenoloxidase cascade in normal (fed, non-AKH-injected) locusts (see Fig.1), this does not mean that the immune system does not respond at all to LPS when it is injected alone. Indeed, locusts form nodules in response to injections of bacterial products or LPS (Hoffmann *et al.*, 1974; Gunnarsson & Lackie, 1985; Gunnarsson, 1988; Brookman *et al.*, 1989b; Goldsworthy & Chandrakant, 2002). In a number of different insect species, inhibition of arachidonic acid metabolism by dexamethasone compromises some cellular responses to bacterial infections, including nodule formation (Stanley-Samuelson *et al.*, 1991, 1995; Miller *et al.*1996, 1999; Bedick *et al.*, 2000; Miller & Stanley, 2001). The present study investigates whether signalling molecules like AKH, octopamine, 5-hydroxytryptamine, and eicosanoids influence LPS-induced nodule formation and prophenoloxidase activation in locusts.

Materials and methods

Insects

Locusta migratoria migratorioides (R. & F.) were reared under crowded conditions at 30°C in a LD 12:12h photocycle, and fed daily with fresh grass and wheat seedlings supplemented with bran. All experiments were conducted on adult males between 12 and 25 days after adult emergence.

Injection of materials into the haemolymph of locusts

Injections of test materials were made using plastic pipette tips within the bore of which a short length of stainless steel needle was held by friction. Using these, 20 µl of saline containing test materials were injected into the haemocoel by inserting the needle between two abdominal terga and expelling the sample using an automatic pippettor.

Stock solutions of *Lom*-AKH-I were made up in 80% methanol (20 pmol/µl) and quantified by measuring the tryptophan fluorescence in an LS50B Fluorimeter (Ex, 280 nm; Em, 348 nm) and calibrating against a standard solution of tryptophan. Unless otherwise stated, a

standard dose of 20 pmol of *Lom*-AKH-I was used. All chemicals were purchased from Sigma Chemical Co., except for dexamethasone (Calbiochem) and *Lom*-AKH-I (Novabiochem). The commercial preparations of LPS used were prepared by phenolic extraction from *E. coli*, serotype 0111:B4 (L 2630, Sigma) and *Pseudomonas* aeruginosa, serotype 10 (L 9143, Sigma). All stock solutions of LPS were made in a simple locust saline (7.5 g NaCl and 0.375g KCl/litre); LPS from *E. coli* at 8 mg/ml and from *Pseudomonas* at 4 mg/ml; and, unless otherwise stated, 100 or 8 µg of LPS were injected respectively into each locust. Stock solutions of dexamethasone in 95% ethanol (25 mg/ml), octopamine (50 x 10^{-3} M), captopril (0.2 x 10^{-3} M), and 5-hydroxytrptamine (50 x 10^{-3} M) were stored at –15 ºC until needed, and then diluted in saline just before injection.

Samples of haemolymph

Haemolymph was obtained from locusts 3 h after injection of test materials: without cooling or anaesthesia, a small puncture was made in the arthrodial membrane at the base of a hind leg. A calibrated capillary tube was used to take up 5 µl of haemolymph, and this was blown immediately into plastic 1.5 ml centrifuge tubes containing 95 µl of phosphate buffer (10 mM, pH 5.9).

Phenoloxidase activity

For the original data presented here, phenoloxidase activity was measured by a slight modification of the procedure described by Goldsworthy *et al.* (2002). Briefly, 5 µl of fresh whole haemolymph were blown into 95 µl of phosphate buffer. After centrifugation (10000 x g, at 4°C for 5 min), 40 µl of the supernatant was pipetted into a well of a microtitre plate and 160 µl of *L*-dopamine (3mg/ml phosphate buffer) added as substrate (instead of the *L*-dopa used previously). This change in substrate was adopted because of the higher solubility of *L*-dopamine in aqueous media: the change did not alter the qualitative aspects of the results obtained, compared with previous studies in this laboratory, but did generate rates of enzyme activity *c*. twice those obtained with L-dopa. Phenoloxidase activity was assessed as the initial linear increase in absorbance at 492 nm over 30 min using a Labsystems Multiskan Bichromatic platereader. Enzyme activity is expressed in absorbance units (au) at 492 nm per minute per microlitre of haemolymph.

Nodule formation

Insects were killed by decapitation, and a mid-ventral longitudinal incision allowed the abdomen to be pinned out for examination under a binocular microscope. The numbers of nodules in the first abdominal segment were counted 24 h after injection of LPS.

Results

The dose-response relationships for LPS and AKH and their effects on nodule formation
In locusts injected with LPS from *E. coli*, nodules formed almost exclusively in the anterior region of the abdomen, associated with the dorsal diaphragm and concentrated on either side of the heart but, sometimes, smaller numbers of nodules were seen on the ventral diaphragm, concentrated along either side of the nerve cord. After injection of LPS from *Pseudomonas*, larger numbers of nodules formed than with LPS from *E. coli* (Figs. 3 and 4), and they were always associated exclusively with the dorsal diaphragm. In mature adult male locusts, the numbers of nodules formed were linearly related to the amounts of LPS from *E. coli* or *Pseudomonas* injected (Fig. 3). The preparation of LPS from *Pseudomonas* was very much more potent and had a higher efficacy than that from *E. coli* in stimulating nodule formation. Nevertheless, co-injection of *Lom*-AKH-I with either preparation of LPS increased the numbers of nodules in a dose-dependent manner. Figure 4 shows that classical sigmoidal hormonal dose-response relationships were established, with similar ED_{50} values for both preparations (Fig 4). Small numbers of nodules were found in saline-injected animals (see Figs 5B and 6B) but nodules were usually absent in non-injected locusts.

Effect of dexamethasone on nodule formation and activation of prophenoloxidase by LPS
Dexamethasone was co-injected with LPS from *E. coli* at two different concentrations both in the presence and absence of *Lom*-AKH-I. Phenoloxidase activity was measured after 3 h, and nodule formation after 24 h. The higher concentration of dexamethasone inhibited nodule formation whereas the lower concentration appeared to be stimulatory. These effects were observed even in the presence of AKH (Fig 5B). Dexamethasone had no effect on the activation of prophenoloxidase (Fig. 5A).

Effects of captopril on nodule formation and activation of prophenoloxidase by LPS

When captopril was co-injected with LPS from *E. coli*, the activation of prophenoloxidase was unaffected 3 h later compared with when LPS was injected alone or in the presence of AKH (Fig. 6A). Captopril did, however, inhibit nodule formation in both the presence and absence of AKH (Fig 6B).

Effect of octopamine and 5-hydroxytrptamine on nodule formation and activation of prophenoloxidase by LPS

Co-injection of octopamine or 5-hydroxytrptamine with LPS from *E. coli* had no significant effect on the production of nodules, nor was there any facilitation of the phenoloxidase response as seen with AKH-I (Table 1).

Discussion

Injected adipokinetic hormone (*Lom*-AKH-I) prolongs or facilitates respectively the activation of prophenoloxidase in the haemolymph of adult locusts injected with the immunogens laminarin or bacterial lipopolysaccharide (Goldsworthy *et al.*, 2002). It seems clear from Figures 1 and 2 that there must be quite separate mechanisms/pathways for the effects of laminarin and LPS, otherwise it would be difficult to explain the differences in locust sensitivity to these immunogens in the absence of injected AKH, or in the maximum responses in the presence of the hormone. Indeed, it is known that distinct specific binding proteins for these (and other immunogenic) substances can be extracted from insect haemolymph (Koizumi *et al.*, 1997, 1999; Chen *et al*., 1999; Ma & Kanost, 2000; Werner *et al*., 2000).

Goldsworthy *et al.* (2002) suggested that there is a direct link between the phenoloxidase responses described here and the well-known lipid mobilisation response. This is consistent with the suggestion by Wiesner *et al.* (1997), Halwani and Dunphy (1999), Halwani *et al.* (2000), and Dettloff *et al.* (2001) that in *Galleria* a component of the lipid mobilisation system, apolipophorin-III (ApoLp-III), is involved in anti-bacterial defence. The involvement of ApoLp-III in the immune system of locusts will be the subject of a separate report (L.M. Mullen and G.J. Goldsworthy, in preparation), but it is likely that AKH could be exerting its effect at least partly through its effects on ApoLp-III metabolism. A lipid-associated form of ApoLp-III is induced by the action of AKHs, and this may be necessary for the activation of prophenoloxidase by LPS (see Dettloff *et al.*, 2001). This might help to explain the responses

to preparations of LPS in the presence of AKH, but the fact that laminarin induces a rapid phenoloxidase response in the absence of AKH, and the effect of AKH is to delay the return of phenoloxidase activity to pre-injection levels, suggests that AKH may exert more than one effect on the prophenoloxidase activation system. The detailed mechanisms by which AKH is affecting the pathways responsible for activation of prophenoloxidase remain to be elucidated.

Goldsworthy *et al*., (2002) found that a commercial preparation of LPS from *Pseudomonas* was not able to activate prophenoloxidase in adult male locusts even when co-injected with LPS. Subsequently it was realised that this 'inactive' preparation had been extracted by TCA precipitation, and that a second preparation extracted in phenol from *Pseudomonas* was active in the phenoloxidase assay but, as with other phenolic extraction preparations from a range of Gram negative bacteria, only when co-injected with *Lom*-AKH-I. In fact, in this study the phenol-extracted LPS from *Pseudomonas* is both more effective and more potent than one from *E. coli*. Perhaps these differences relate to the fact that *Pseudomonas* is a pathogen of locusts, whereas *E. coli* is not. However, different batches of commercial LPS extracted by the same procedure from the same microbial source can vary considerably in their potency to cause either nodule formation or prophenoloxidase activation (unpublished observations). Furthermore, the commercial preparations of LPS used here were not subjected to any further purification before use. Thus the differences between various preparations of LPS should be interpreted with caution.

The present study shows that exogenous AKH can potentiate a further component of the locust's immune response: co-injection of *Lom-AKH-I* increases the formation of nodules in response to injections of LPS. In response to injection of laminarin or LPS, nodules form closely associated with the dorsal diaphragm in a pattern that reflects the distribution of haemopoietic tissue as described by Ogel (1959). In *Locusta*, circulating haemocytes may not play the major part in the phagocytosis of foreign materials: injected particles of Indian ink or bacteria, for example, are phagocytosed by the reticular cells of the haemopoietic tissue (Ogel, 1959; Hoffmann, 1970, 1973; Hoffmann *et al.*, 1974). It seems that as a result of massive uptake by the reticular cells of foreign material, such as the LPS in this study, nodules formed in a highly defined pattern showing the distribution of the reticular cells. With LPS derived from *E. coli* a slight difference between the pattern of the distribution of reticular cells described by Ogel (1959) and the distribution of nodules observed in this study

is the association of some nodules with the ventral diaphragm on either side of the ventral nerve cord: perhaps there are small numbers of previously unrecognised reticular cells in these latter sites. Although this possibility remains to be investigated, it may not offer a satisfactory explanation, because while LPS derived from *Pseudomonas* appears to be more potent and more effective overall than that from *E. coli*, nodules are not observed in these ventral locations with the LPS from *Pseudomonas*.

Co-injection of *Lom*-AKH-I with LPS from either *E. coli* or *Pseudomonas* enhances the numbers of nodules formed in a dose-dependent manner, and the ED_{50} of the hormone is similar for the two preparations of LPS tested (0.3-0.4 pmol *Lom-AKH-I*). Nodule formation in response to injection of LPS is therefore as sensitive to *Lom*-AKH-I as is lipid mobilisation in adult locusts, which shows an ED_{50} of 0.8 pmol of *Lom*-AKH-I (see Goldsworthy *et al.*, 1997). Thus the effects of AKH on nodule formation could be of physiological significance.

Injection of inhibitors of eicosanoid synthesis, such as dexamethasone, reduces nodule formation in a number of insects, and has led to the suggestion that products of cyclo- and lipoxygenase may regulate some aspects of the insect immune response (Miller *et al.*, 1994; 1996; 1999; Bedick *et al.*, 2000; Miller & Stanley, 2001; Dean *et al*., 2002). The present study provides evidence to support this for nodule formation in the locust, but suggests that these messengers are not involved in activation of the prophenoloxidase cascade. As suggested by Brookman *et al.* (1989ab), there is not a direct relationship between activation of the prophenoloxidase cascade and nodule formation: nodule formation can be inhibited by injection of dexamethasone without affecting activation of the prophenoloxidase cascade. The apparent stimulation of nodule formation at low concentrations is unexpected, and requires further investigation before any explanation can be put forward.

Lamango & Isaac (1993,1994) identified and characterised a peptidyl dipeptidase in the brain and haemolymph of the housefly, *Musca domestica*, that resembles mammalian angiotensin-converting enzyme (ACE). This enzyme appears to have widespread occurrence in insects and is involved in peptide processing in the nervous system (Lamango & Isaac, 1997) as well as other functions associated with its presence in the haemolymph. The activation of prophenoloxidase involves a protease cascade (Ashida & Brey, 1998), and so the testing of captopril, a specific inhibitor of ACE, was rather speculative, and gave an unexpected result in that it was nodule formation, rather than the activation of prophenoloxidase, that was

inhibited, although nodule formation must involve a localised activation of prophenoloxidase associated with melanisation of the nodules.

The interactions between the locust endocrine and immune systems are summarised in Figure 7. Important differences exist in the factors that influence the two immune responses studied here. Nodule formation in response to injection of LPS is sensitive to captopril and dexamethasone, whereas neither inhibitor affects activation of prophenoloxidase. Starvation for between 1-3 days in locusts has no effect on nodule formation in response to injection with LPS, but has a marked stimulatory effect on the phenoloxidase response to LPS injection (Opoku-Ware & Goldsworthy, 2002). Thus starvation in locusts can mimic to some extent the effect on prophenoloxidase activation of co-injection of AKH with LPS, presumably because it brings about hyperlipaemia (Jutsum *et al*., 1975), but it has no effect on nodule formation (G.J. Goldsworthy & K. Opoku-Ware, unpublished observations). Furthermore, nodule formation in response to injection with LPS occurs in V^{th} instar hoppers and adults of all ages, whereas activation of prophenoloxidase cannot be demonstrated in V^{th} instar hoppers in response to LPS, and these age-related changes in responsiveness correlate with changes in the concentration of ApoLp-III in the haemolymph during development (Mullen and Goldsworthy, 2002). Some previous studies have implicated other messengers, such as octopamine and 5-hydroxytryptamine, in stimulating nodule formation in *Periplaneta* (Baines *et al*., 1992) and *Galleria* (Dunphy & Downer, 1994) but in the present study these messengers were found to have no effect on either nodule formation or prophenoloxidase activation when co-injected with LPS into locusts. The elucidation of interactions between the immune and endocrine systems is at a very early stage, and it can be predicted with confidence that the picture that evolves as more studies are undertaken will become more complex, as other messengers, and other components of the immune system are examined.

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Table 1. The effects of co-injection of various messenger molecules on nodule formation and activation of prophenoloxidase activity in response to injected LPS.

	Amount injected	Nodules per segment Mean ± SE (*n*)	Phenoloxidase activity Au_{492}/min/µl Mean ± SE (*n*)
Saline		12 ± 12 (10)	0.003 ± 0.001 (10)
LPS[1]	100 µg	144 ± 46 (10)	0.008 ± 0.002 (10)
LPS + *Lom*-AKH-I	20 pmol	210 ± 39 (10)	0.022 ± 0.002 (10)
LPS + octopamine	9.5 µg[2]	131 ± 23 (10)	0.008 ± 0.003 (10)
LPS + 5-hydroxytryptamine	19.4 µg[2]	118 ± 18 (10)	0.001 ± 0.001 (10)

When tested alone, none of the substances (apart from LPS) caused nodule formation or activated phenoloxidase activity to a greater extent than injection of saline.

[1]The total volume injected was always 20 µl, and the amount of LPS (from *E. coli*) was constant at 100 µg.

[2]This was equivalent to 1 µl of stock solution injected per locust.

Figure Legends

Figure 1. Changes in the phenoloxidase activity of the haemolymph of *Locusta* with time after injection with immune stimulators: A; 15 µg of laminarin, or 15 µg of laminarin and 20 pmol of *Lom*-AKH-I. B. 100 µg of LPS (*E. coli*), or 100 µg of LPS and 20 pmol of *Lom*-AKH-I. Vertical lines represent ± SEM, n ≥ 10. Data re-plotted from Goldsworthy *et al.* (2002). Note that L-dopa was used as substrate in these assays, which gives lower rates of phenoloxidase activity than those measured in the present study using L-dopamine as substrate.

Figure 2. A. Dose response relationships for the effects of *Lom*-AKH-I on the phenoloxidase activity in the haemolymph of *Locusta* 3 h after co-injection with 15 µg of laminarin (A) or 100 µg of LPS from *E. coli* (B). Curve fitting and estimation of ED_{50} values were undertaken as Hill-plots in FigP (Biosoft). Vertical lines represent means ± SE, n ≥ 10. Data re-plotted from Goldsworthy *et al.* (2002). Note that L-dopa was used as substrate in these assays.

Figure 3.The effects of increasing doses of preparations of LPS from two different bacterial species on nodule formation in adult male *Locusta migratoria*. Two groups of locusts were studied. In each group, 20 pmol of *Lom*-AKH-I were co-injected with increasing concentrations of the LPS preparation under test. Each data point represents the nodules counted (Mean ± SE) 24 h after injection for between 5 and 10 locusts at each concentration of LPS.

Figure 4. Dose response relationships for the effects on nodule formation of co-injection of *Lom*-AKH-I with two different preparations of LPS: from *E. coli* (100 µg injected) and from *Pseudomonas* (8 µg injected). Curve fitting and estimation of ED_{50} values were undertaken as Hill-plots in FigP (Biosoft). Data points and vertical lines represent means ± SE, $n \geq 5$.

Figure 5. The effects of administration of dexamethasone on nodule formation and activation of prophenoloxidase by LPS. Two doses of dexamethasone were tested:
Dex^1 was 125µg, and Dex^2 was 12.5µg. LPS (100 µg) was from *E. coli* and was injected both in the presence and absence of 20 pmol of *Lom*-AKH-I. Phenoloxidase was measured

3 h after injection, and nodule formation was assessed 24 h later in the same locusts. Bars and vertical lines are the means ± SE of 10 observations.

Figure 6. The effects of administration of captopril (5µl of 200µM stock solution: 0.1 µg) on nodule formation and activation of prophenoloxidase by LPS. The LPS (100 µg) was from *E. coli* and was injected both in the presence and absence of 20 pmol of *Lom*-AKH-I. Phenoloxidase was measured 3 h after injection, and nodule formation was assessed 24 h later in the same locusts. Bars and vertical lines are the means ± SE of 10 observations.

Figure 7. A summary of the interactions between the immune and endocrine systems as described in this study. For completeness, laminarin has been included in this scheme (see Goldsworthy *et al*., 2002; Mullen & Goldsworthy, 2002) but the data are not presented here.

Figures

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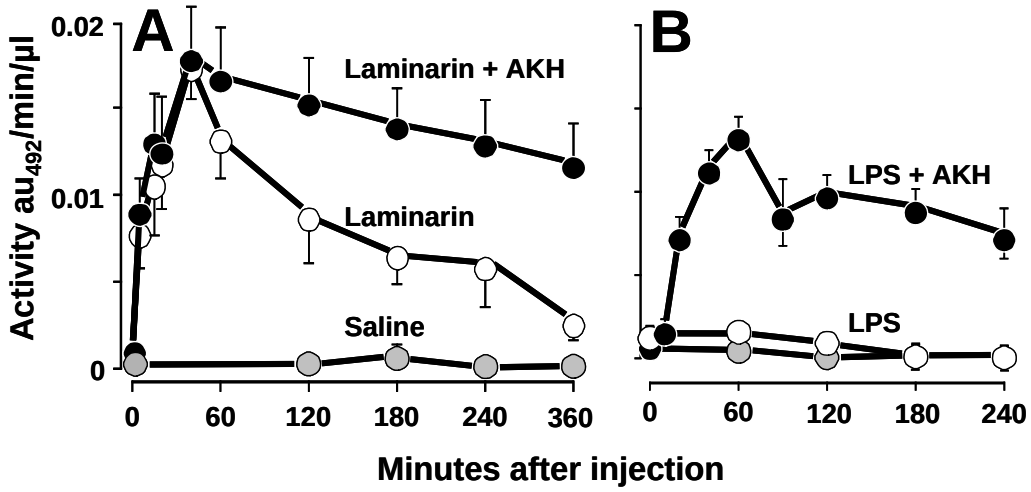

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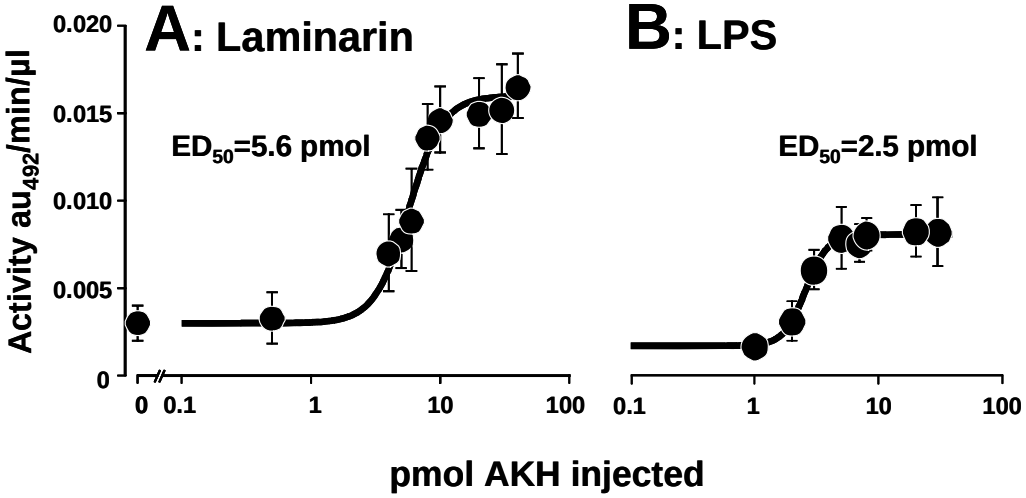

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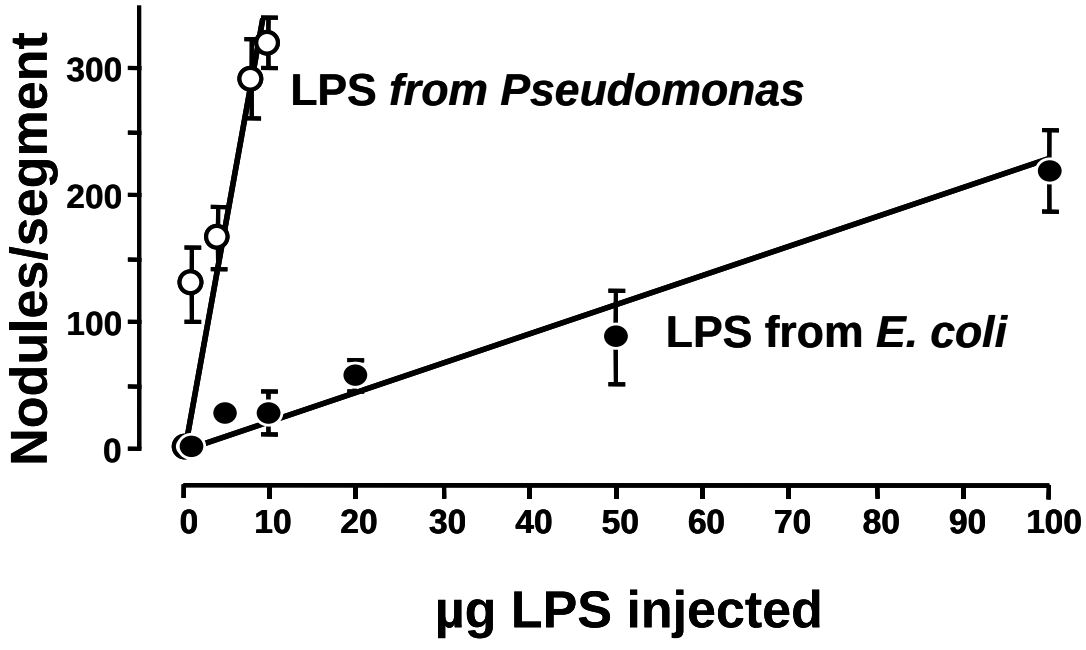

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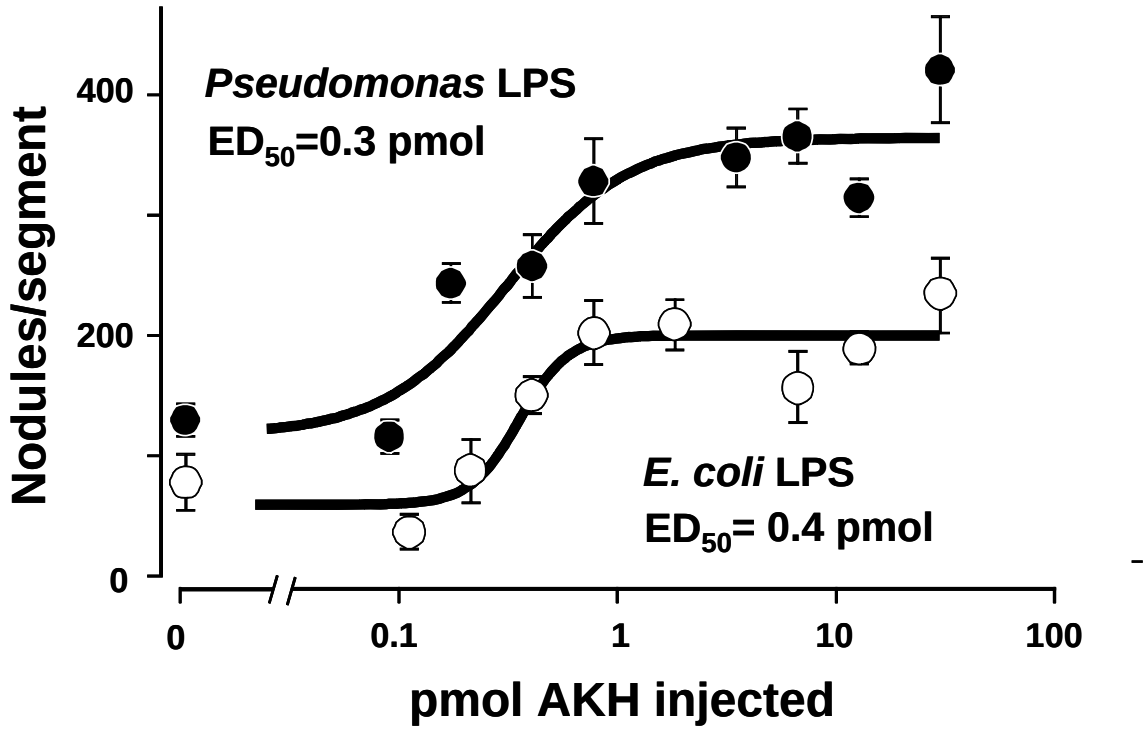

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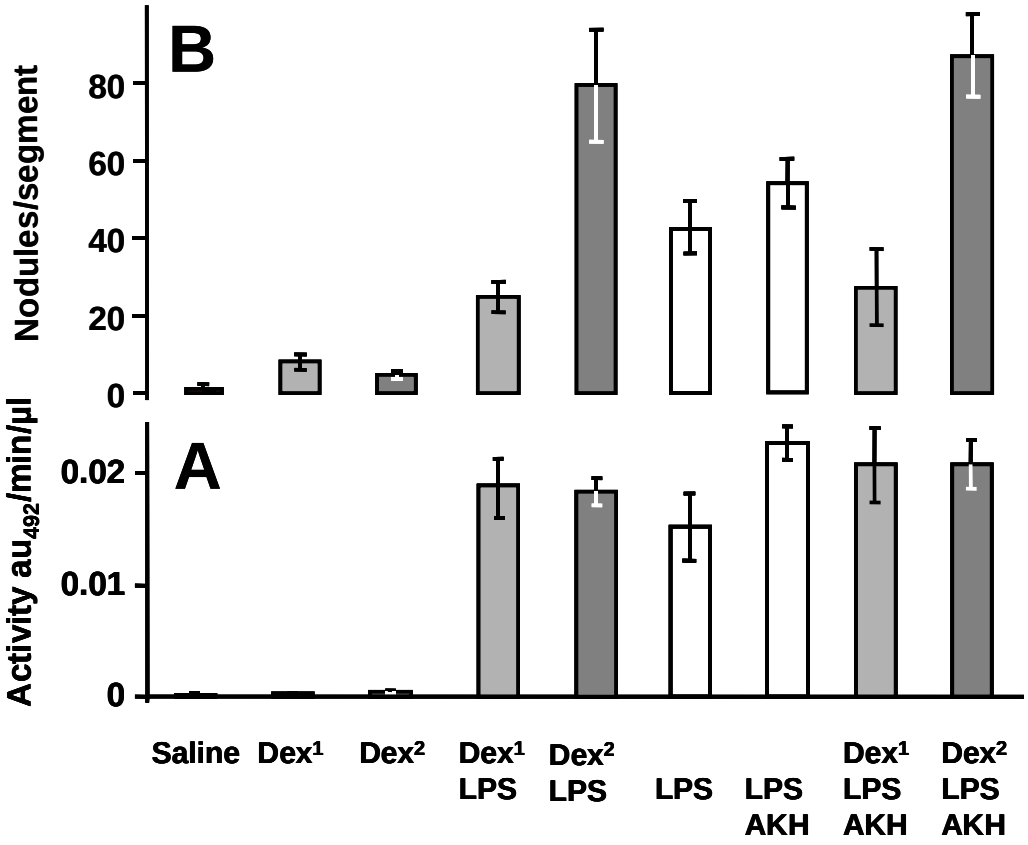

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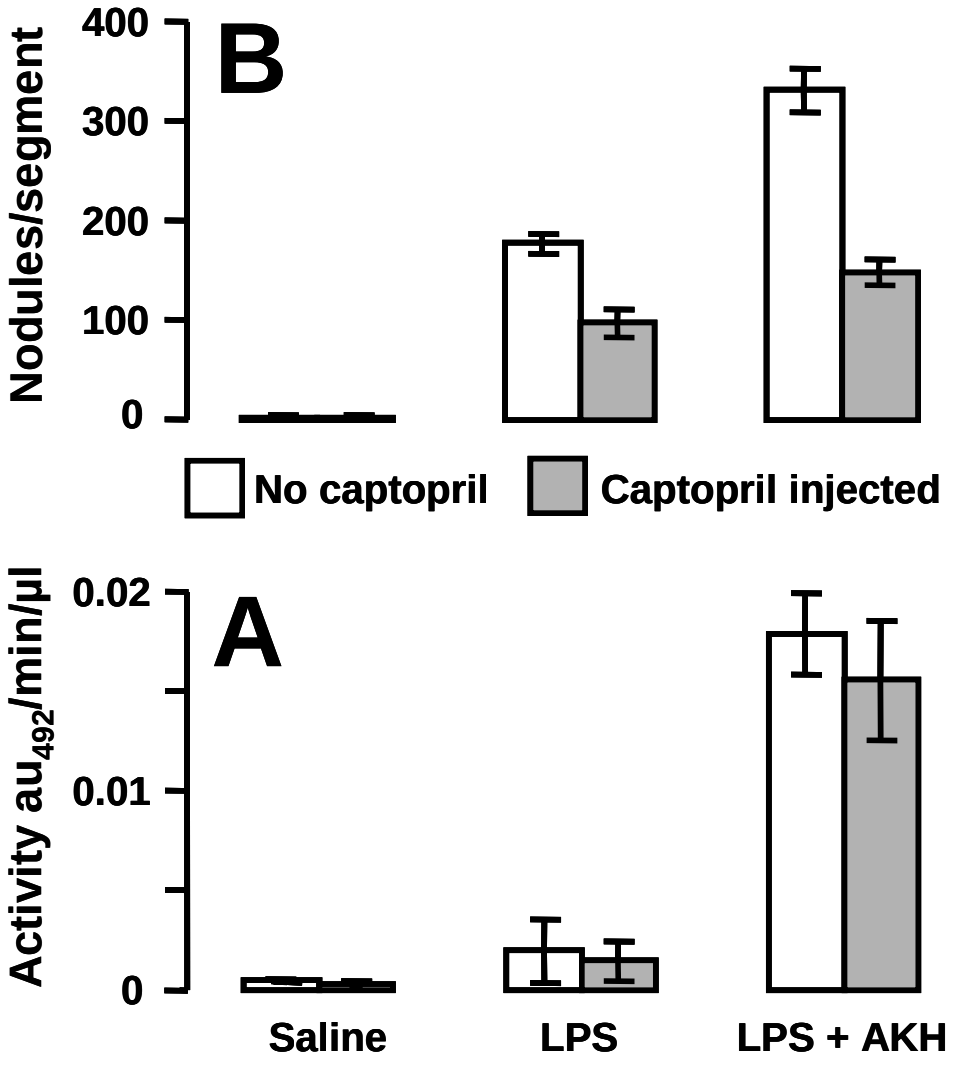

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