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# OCCURRENCE OF NOVEL TYPES OF NITRIC OXIDE SYNTHASE IN THE SILKWORM, *BOMBYX MORI*+

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Nitric oxide (NO) synthase activity was detected in fat body and the Malpighian tubles of the silkworm, *Bombyx mori*. Main NO synthase activity in the fat body was $Ca^{2+}$/calmodulin-dependent, inducible by bacterial lipopolysaccharide (LPS) and required NADPH, FAD, FMN, dithiothreitol (DTT) and tetrahydrobiopterin ($BH_4$) as cofactors for the full expression of the activity. The Malpighian tubles contained two types of NO synthase. One was $Ca^{2+}$-independent, calmodulin-dependent and constitutive and the other was $Ca^{2+}$-dependent and constitutive. The former NO synthase required the same cofactors as fat body NO synthase. The activity of Malpighian tuble NO synthases increased dramatically at the end of the last instar period, just prior to spinning. These results indicate that *B. mori* contains new types of NO synthase, suggesting the wide distribution and different characteristics of this enzyme among vertebrates and invertebrates.



Since the discovery in 1987 that vascular endothelial cells can synthesize NO from L-arginine, numerous evidence of NO synthase has been accumulated from different mammals (1,2). One NO synthase is induced after activation of macrophages, endothelial cells and a number of other cells by cytokines and/or endotoxin, and is cytosolic and $Ca^{2+}$-independent. Once these cells are activated, NO is synthesized for long periods. Another type of enzyme reported is constitutive, $Ca^{2+}$/calmodulin-dependent and releases NO for short periods in response to receptor or physical stimmulation (1).

On the other hand, evidence for production of NO in invertebrates is limited to a horse-shoe crab, *Limulus polyphemus* (3) and a blood sucking bug, *Rhodnius prolixus* (4). In the case of L. polyphemus, NO production was observed in hemocytes and the synthesis of NO results in down-regulation of the aggregatory function of these cells. The hematophagous insect, *R. prolixus* was found to have a NO-containing hemeprotein in the salivary glands (4) and this nitrosylhemeprotein plays a role as a vasodilator and platelet antiaggregating

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substance during blood feeding (5). The NO synthase activity in the salivary glands of this insect was dependent on $Ca^{2+}$, calmodulin, FAD, NADPH and $BH_4$ and converts arginine to citrulline while producing vasorelaxing activity, indicating the similarity of the activity to the vertebrate constitutive NO synthase (6).

In our efforts to understand the self-defense mechanism in insects, we examined whether NO synthase activity can be detected in the silkworm, *Bombyx mori*. In this paper, we present evidence that two *B. mori* tissues, fat body and the Malphigian tubles, contain NO synthase activity. The enzyme activity from these tissues showed different characteristics and some of them were distinct from those of mammalian NO synthase.

## MATERIALS AND METHODS

***Materials***: LPS from *Salmonella entertidis* was obtained from Sigma. L-[U-$^{14}$C] arginine was from Moravek Biochemical. W-5, W-7 and trifluoperazine were purchased from Wako. Other reagents (analytical grade) were purchased from Sigma or Wako.

***Injection of insects and preparation of tissue extracts***: Silkworms, *Bombyx mori* (Tokai x Asahi), were reared on an artificial diet (Nihonnosanko) at 25°C. The day 4 fifth instar larvae were injected with either various doses of *S. enteritidis* LPS suspended in 0.75% NaCl solution (insect saline) or insect saline only as a control through an abdominal leg. At various time periods after injection, larvae were anesthetized with ether for 5 min and fat body, the Malpighian tubles, midgut, silkgland and trachea were excised. These tissues were washed once with cold insect saline and then the saline was drained off using a filter paper. Hemolymph of the larvae was collected by cutting off an abdominal leg into a test tube on ice, which contains 2 μg/ml aprotinin. The tissue samples were stored at -110°C until used. Tissues were homogenized at 0°C with a Physcotron (Nichionirika) in 5 volumes of 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM DTT, 0.4 mM p-amidinophenyl methanesulfonyl fluoride (p-APMSF), 10 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor and 2 μg/ml aprotinin. The homogenates were then centrifuged at 11,270 x g for 30 min (for midgut) or for 10 min (for other tissues). The supernatants were stored on ice for up to 2 h before use.

***NO synthase assay***: NO synthesis was measured by the conversion of L-[U-$^{14}$C] arginine to [U-$^{14}$C] citrulline as described by Salter *et al.* (1991). Briefly, 15 μl of tissue extract was added to 10 ml plastic test tube preincubated at 25°C which held 100 μl of 50 mM potassium phosphate buffer, pH7.2 containing 60 mM L-valine, 1 mM NADPH, 1.2 mM L-citrulline, 10 μM FAD, 0.24 mM $CaCl_2$, 100 μM $BH_4$ and L-[U-$^{14}$C] arginine (150,000 dpm). After incubation for 10 min at 25°C, the reaction was terminated by the addition of $H_2O$/Dowex-50w (200-400, 8% cross-linked, $Na^+$ form) (1:1 v/v). Two ml distilled $H_2O$ was then added to the resin, mixed and left to settle for 10 min (7). The supernatant was removed and the radioactivity of [$^{14}$C] citrulline was measured by liquid-scintillation counting. The activity of the $Ca^{2+}$-dependent NO synthase was determined from the difference of the produced [$^{14}$C] citrulline of control samples and of samples containing 1 mM EGTA while $Ca^{2+}$-independent NO synthase was determined from the difference of the activity between samples containing 1 mM EGTA and samples containing 1 mM $N^{\omega}$-nitro-L-arginine (L-NNA). For the analysis of effects of cofactors, tissue extract was centrifuged at 11,270 x g for 20 min and the supernatant was applied onto a Sephadex G-25 (1 ml) (Pharmacia) column to remove low molecular mass components.

## RESULTS

### *Induction of NO synthase activity by LPS in fat body*

The optimal assay conditions for NO synthase activity *in vitro* were examined with crude homogenates of the tissue samples. Cofactors such as NADPH, $BH_4$ and FAD were found to be required for the full expression of NO synthase activity (data not shown). Under the optimal conditions, NO synthase activity was confirmed to increase proportionately with the incubation time up to 90 min at 25°C (data not shown). Significant NO synthase activity was detected in fat body and the Malpighian tubles but not in the other tissues

such as hemocytes, midgut, silkgland and trachea, suggesting that NO synthase in *B. mori* is expressed tissue specifically (data not shown).

The time course of NO synthase activity after LPS injection was observed in fat body. Fat body of fifth instar larvae (fourth day) was excised at the incremental times after LPS injection, homogenized and used as an enzyme source for *in vitro* NO synthase reaction. As is shown in Fig. 1, main NO synthase activity in fat body was $Ca^{2+}$-dependent. Addition of L-NNA, an analogue of L-arginine, suppressed NO synthase activity totally *in vitro*, suggesting a specific reaction of NO synthase (data not shown). The $Ca^{2+}$-dependent NO synthase activity in fat body started to increase at 3 h, reached a maximum level at 9 h after LPS injection and gradually decreased thereafter.

***Effects of LPS dose on NO synthase activity***

Effects of LPS dose on NO synthase activity in fat body and the Malpighian tubles were investigated. The silkworm larvae were injected with 0, 0.5, 2, 10 or 20 μg *S. enteritidium* LPS suspended in 20 μl insect saline and fat body and the Malpighian tubles were excised 9 h after the injection. In the fat body, NO synthase activity increased in a dose-dependent manner up to 10 μg (Fig. 2A) and EGTA and L-NNA strongly supressed the activity (data not shown). This result confirmed again that major NO synthase in the fat body is inducible and $Ca^{2+}$-dependent. In the Malpighian tubles, both $Ca^{2+}$ -dependent and -independent NO synthetase activities were detected even in the larvae which were not treated with LPS, however, these activities were not increased significantly by injection of LPS at any doses (Fig. 2B), suggesting that two types of NO synthases from the Malpighian tubles are consitutive enzymes.

***Effects of calmodulin antagonists on NO synthase activity***

The calmodulin antagonists, W-5, W-7 and trifluoperazine were used to examine whether or not NO synthase in *B. mori* is calmodulin dependent. Different concentration of these antagonists were added to the *in vitro* reaction mixture and incubated for 15 min at 25°C. As shown in Figure 3, fat body NO synthase activity was significantly inhibited

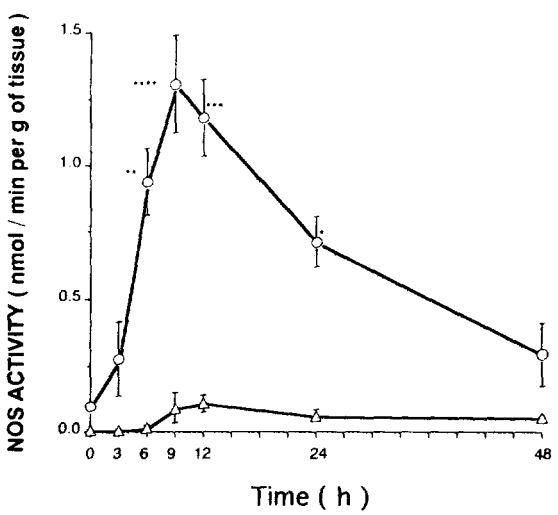


**Fig. 1. Time course of NO synthase activity induction by LPS in fat body.** The data shown are mean ±SEM from four experiments except 9 h where n=6. $Ca^{2+}$-dependent (open circle) or $Ca^{2+}$-independent (open triangle) NO synthase activity was measured from fat body removed at the time indicated after injection of LPS (10 μg/larva), by conversion of L-[U-$^{14}$C] arginine to [U-$^{14}$C]citrulline. *, **, and *** indicate significant differences from the value at time 0 at $p<0.05$, $p<0.01$, and $p<0.025$, respectively (Student's t test).

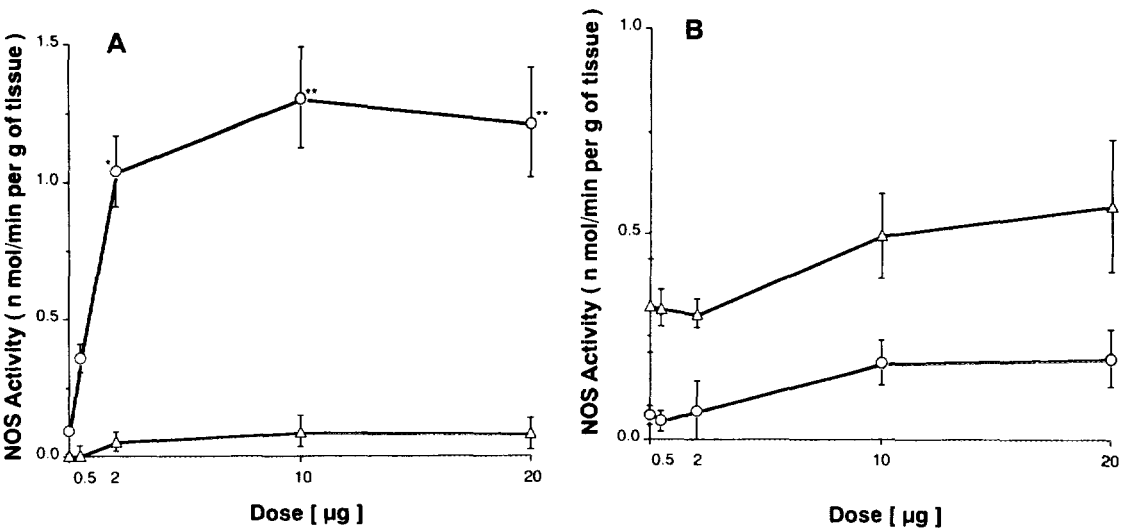


**Fig. 2. Effects of LPS dose on the induction of NO synthase activity in fat body or Malpighian tuble.** The data shown are means ± SEM from four experiments except 10μg where n=6. $Ca^{2+}$-dependent (open circle) or $Ca^{2+}$-independent (open triangle) NO synthase activity was measured with the samples from fat body (panel A) and Malpighian tuble (panel B) excised 9 h after LPS or insect saline injection, by conversion of L-[U-$^{14}$C] arginine to [U-$^{14}$C] citrulline. * and ** indicate significant differences from the control activity at $p<0.025$ and $p<0.005$, respectively.

by W-5, W-7 and trifluoperazine and recovery of the activity was observed by the addition of calmodulin (Fig. 3A). Malpighian tuble $Ca^{2+}$-independent NO synthase was also inhibited in a dose-dependent manner by these antagonists and a recovery similar to that observed in the major fat body NO synthase was observed with the addition of calmodulin (Fig. 3B). These results indicate that major NO synthase in fat body and $Ca^{2+}$-independent NO synthase in the Malpighian tubles are calmodulin-dependent.

***Requirement of cofactors for NO synthase activity***

NO synthase samples from fat body or the Malpighian tubles were partially purified by gel filtration through a Sephadex G-25 column to remove endogenous cofactors of this enzyme. NO synthase activity was investigated with the enzyme samples *in vitro* in the presence of different possible cofactors. The results are shown in Fig. 4. Fat body $Ca^{2+}$-dependent or the Malpighian tubles $Ca^{2+}$-independent NO synthase required NADPH, FAD, FMN, DTT and $BH_4$ as cofactors for the full expression of the enzyme activity.

***Naturally increased NO synthase activity in the Malpighian tubles in the last instar stage***

The activity change of $Ca^{2+}$-dependent or -independent NO synthase in the Malpighian tubles was observed in the different developmental stages of fifth instar larvae (Fig. 5). A dramatic increase in enzyme activities was detected both in $Ca^{2+}$-dependent and $Ca^{2+}$-independent NO synthase at the day 7 fifth instar larvae. This suggests that NO synthase plays an important role in the Malpighian tubles and that the activity is regulated in a development specific manner.

## DISCUSSION

Our results demonstrate that *B. mori* fat body and the Malpighian tubles contain different types of cytosolic NO synthase activity. Fat body contains two types of NO

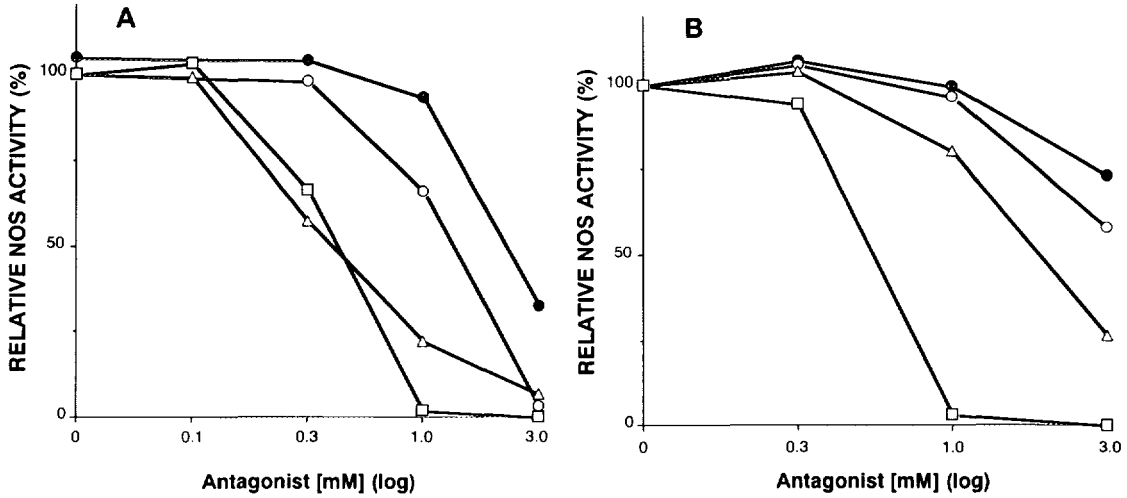


**Fig. 3. Inhibition of NO synthase activty by calmodulin antagonists, W-5, W-7 and trifluoperazine.** Fat body (Panel A) or Malpighian tuble (Panel B) was exised from the larvae injected with 10μg LPS 9 h before the dissection. To exclude the influence of $Ca^{2+}$-dependent NO synthase in Malpighian tuble sample, 1 mM EGTA was included in reaction mixture. Different concentrations of W-5 (open circle), W-7 (open triangle) and trifluoperazine (open square) were added to the reaction mixture. The recovery of NO synthase activity in the presence of W-5 was also investigated by the addition of 1 μM calmodulin to the reaction mixture (closed circle). Data are the means of triplicate experiments.

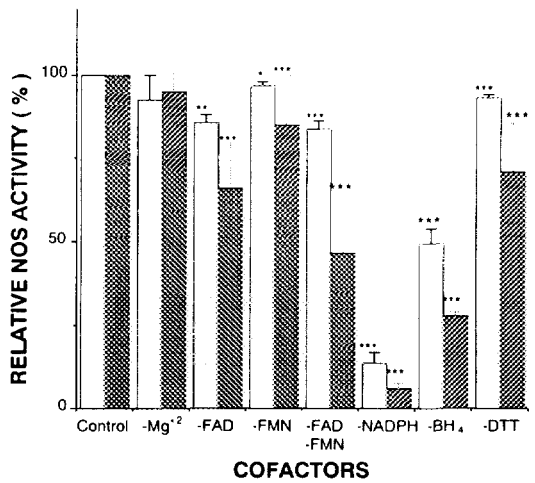


**Fig. 4. Effects of omission of the possible cofactors from the enzyme reaction mixture on NO synthase activity.** Values of relative NO synthase activity were expressed as percentage against the value obtained from the reaction mixture containing all cofactors. Reaction mixture contained 50 mM potassium phosphate buffer, pH 7.2, 60 mM L-valine, 1.2 mM L-citrulline, 1 mM NADPH, 10 μM FAD, 10 μM FMN, 1.2 mM $MgCl_2$, 0.24 mM $CaCl_2$, 100 μM tetrahydrobiopterin, 1 mM DTT, L-[U-$^{14}$C] arginine (150,000 dpm) and 15 μl tissue extract partially purified by passing through Sephadex G-25 column. Enzyme activity was determined in the absence of each cofactor indicated. In this experiment, DTT was omitted from the extraction buffer. To exclude the influence of $Ca^{2+}$-dependent NO synthase in Malpighian tuble, 1 mM EGTA was included in enzyme reaction mixture. The bars indicate mean + SEM of enzyme activity in fat body (open column, n=3) and Malpighian tuble (dashed column, n=3). *, **, and *** indicate significant differences from the control activity at $p<0.05$, $p<0.01$, and $p<0.005$, respectively.

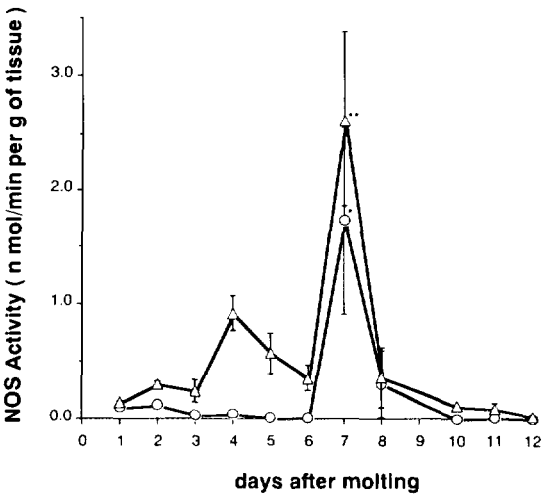


**Fig. 5. NO synthase activity in Malpighian tuble at various developmental stages of the fifth instar larvae.** The data shown are means ± SEM from four experiments. The activity of $Ca^{2+}$-dependent (open circle) or $Ca^{2+}$-independent NO synthase (open triangle) was measured with Malpighian tuble samples excised from the larvae at the indicated day after molting. * and ** indicate significant differences from the activity at day 1 at $p<0.05$ and $p<0.025$, respectively (student's t-test).

synthase. The major one is inducible by LPS and $Ca^{2+}$/calmodulin-dependent. The Malpighian tubles have two different types of NO synthase. One of them is constitutive, $Ca^{2+}$-independent and calmodulin-dependent and the other is constitutive and $Ca^{2+}$-dependent.

In mammalian systems, six types of functionally distinct NO synthase have been reported (2). The vascular endothelium contains a constitutive and $Ca^{2+}$/calmodulin-dependent and cytosolic or particulate NO synthase (8-12). Neural cells contain a constitutive, $Ca^{2+}$/calmodulin-dependent and cytosolic NO synthase (13-20) and macrophage NO synthase is inducible, $Ca^{2+}$/calmodulin-independent and cytosolic (22-26). Liver contains NO synthase which is inducible, $Ca^{2+}$-independent, calmodulin-dependent or -independent and cytosolic (7, 27-29). NO synthase in rabbit chondrocyte is inducible, $Ca^{2+}$-dependent, calmodulin -independent and cytosolic (30). Human chondrocyte NO synthase is inducible, $Ca^{2+}$/calmodulin-independent and cytosolic (31). From the comparison of these NO synthase properties with those of *B. mori* NO synthase, it is evident that *B. mori* contains novel types of NO synthase, suggesting the divergence of the enzyme's characteristics aquired during the evolution.

Contrary to the results in fat bodies, the NO synthase activity in the Malpighian tubles increases dramatically during the final stage of the fifth instar. At 7 or 8 days after molting in the fifth instar, the silkworm usually starts to spin a cocoon. The results suggest that the increase of NO synthase activity in the Malpighian tubles might have a strong relationship with the life cycle of *B. mori*. At the end of the fifth instar stage, the concentration of ecdysone which plays an important role in insect metamorphosis starts to increase. Therefore, the gene expression of Malpighian tuble NO synthase might be regulated hormonally. The activity of hemocytin, a *B. mori* lectin, also increases at the late stage of fifth instar without LPS stimmulation (32). The lectin and NO synthases of this insect might play a role in self-defense reactions and moreover, these substances might also relate to the insect metamorphosis. Concerning such dual functions of an insect hemolymph protein, sapecin, an antibacterial protein from *Sarcophaga perigren*

(the flesh fly), was reported to play roles both in self-defence and in the morphogenesis of leg imaginal discs as growth factor (31, 33). Interestingly, the regulation of the gene expression of mammalian constitutive nNOS (type I NO synthase) (34) and eNOS (type III NO synthase) (2) is also subject to control, though by different stimmuli from cytokins and bacterial components that induce iNOS (type II NO synthase) (2). $Ca^{2+}$-dependent NO synthase in the brain and in a variety of peripheral tissues is substantially increased during pregnancy. These changes are accompanied by significant increases in both eNOS and nNOS mRNAs in skeletal muscle. Thus the $Ca^{2+}$-dependent eNOS and nNOS are subject to induction by estrogen (35).

At present, the biological significance of NO synthase in *B. mori* remains obscure. However, both the possibility of the NO synthase gene regulation by ecdysone during the metamorphosis of insects and the observation of the gene regulation of mammalian eNOS and nNOS by estrogen during pregnancy suggest that NO synthase has something to do with the morphological and the physiological change during the development. Further analysis of the relationship between the metamorphosis and the increase of the NO synthase activity would provide a clue to understand as yet unknown role of this enzyme in the development of animals.

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