Modulators of the Insect Cellular Immune Response

by

Craig Anthony Mandato

A thesis
presented to the University of Waterloo
in fulfilment of the
thesis requirement for the degree of
Doctor of Philosophy
in
Biology

Waterloo, Ontario, Canada, 1998

The University of Waterloo requires the signatures of all persons using or photocopying this thesis. Please sign below, and give address and date.

Abstract

This thesis is intended to add to the growing body of knowledge pertaining to the immune systems of insects and to explore the hypothesis that the invertebrate immune system serves as a template of the vertebrate innate response. Insects are capable of mounting an effective defense reaction consisting of both cellular and humoral components. The focus of this thesis is on aspects of the insects' cellular immune response, the main effector of which is the insect blood cell or hemocyte.

Biogenic amines, such as octopamine, have previously been shown to enhance both phagocytosis and nodule formation in insects. Physiological levels of octopamine elicit increases in inositol trisphosphate, cAMP, and intracellular calcium in hemocytes. Since these second messengers are putative regulators of cytoskeletal organization in a variety of cell types, the effects of octopamine on locomotory behaviour, polarity, and the cytoskeleton of hemocytes from the Greater Wax Moth, *Galleria mellonella*, were analyzed. Morphological changes were reflected in alterations of the hemocyte cytoskeleton and were consistent with changes in the relative concentration of F-actin. These data suggest that one mechanism by which octopamine modulates hemocyte activity is via reorganization of the actin cytoskeleton.

Eicosanoids are biologically active, oxygenated metabolites derived from C20 polyunsaturated fatty acids, and have been implicated as regulators of bacterial clearance from the insect hemocoel (Stanley-Samuelson *et al*, 1991). I further refined our understanding of the action of eicosanoids by demonstrating their regulatory roles in key

aspects of the cellular immune responses: phagocytosis, prophenoloxidase activity, nodulation and cell spreading.

Nitric oxide (NO) is a gaseous signalling molecule which has a variety of physiological roles in vertebrates, including regulation of the innate vertebrate immune response. In this thesis I have shown the first biochemical, molecular, and physiological evidence for the presence and role of nitric oxide synthase (NOS) in insect hemocytes. Furthermore, I demonstrate possible cross-talk between the eicosanoid and NOS pathways, a feature in common with vertebrate immunocytes. These findings suggest a conservation of immunomodulatory strategies between vertebrates and invertebrates, and have both theoretical and practical implications as discussed herein.

Acknowledgements

I would like to thank Roger G. H. Downer for his guidance, advice and encouragement throughout my studies in his lab. I consider it a true privilege to have worked in his laboratory.

I am also very grateful to my supervisor, Bill Diehl-Jones, who made this work possible. His patience, counsel and support, both in and out the of laboratory, were much more than any student could hope for. It was his contagious enthusiasm for research that has had the strongest influence on me, and for this I cannot express my gratitude adequately.

I am indebted to Dr. Bill Taylor, who assumed the responsibility of being my co-supervisor in the midst of my research. I would also like to thank my committee members, Drs. Neils Bols, Jack Carlson, and Guy Guillemette for their helpful suggestions and discussions during this study and I give special thanks to Dr. John Steele for acting as my external examiner.

I am very grateful to Susan Moore for her patience, vigilance and tutelage as we made our way through the morass of molecular biology. Everything I know about molecular biology I attribute to her proficient instruction.

I would like to give a special thanks to Sheryl Farrar for her constant encouragement and support throughout the course of my studies.

Dedication

This thesis is dedicated to my innate parents, Tony and Jan Mandato, and my adaptive parents, Bill and Charlene Diehl-Jones.

Table of Contents

Chapter 1: Introduction

Chapter 2: Monoaminergic Regulation of Hemocyte Activity

Table of Contents: *Chapter 2*

Chapter 3: The Effects of Eicosanoid Biosynthesis Inhibitors on Hemocytic Immune Function

Chapter 4: Identification and Characterization of a Novel Nitric Oxide Synthase from Insect Hemocytes

Table of Contents: *Chapter 4*

Chapter 5: The Relationship Between Nitric Oxide and Eicosanoids

Chapter 6: Conclusions

Appendices

List of Tables

List of Figures

List of Abbreviations Used in this Thesis

5-HT	5-hydroxytryptamine
ANOVA	analysis of variance
β-NADPH	β-nitcotinamide adenine dinucleotide phosphate
B-cells	bone marrow derived lymphocytes
BPB	4-bromophenacyl bromide
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
$CaCl_2$	calcium chloride
CIF	Cecropin Immunoresponsive Factor
COX	cyclooxygenase
DIF	dorsal related immune factor
DIG	digoxygenin
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EET	epoxyeicosatrienoic acids
EGTA	ethylenegycoltetraacetic acid
EIA	enzyme immuno assay
ELISA	enzyme-linked immunosorbent assays
ER	endoplasmic reticulum
ETYA	eicosatetraynoic acid
FAD	flavin adenine dinucleotide
FITC	fluorescein isothiocyanate
FMN	flavin mononucleotide
(m)GIM	(modified) Grace's Insect Medium
GS	Galleria saline
H_4B	tetrahydrobiopterin
HETE	hydroxypolenoic fatty acids
HPETE	hydroperoxypolenoic fatty acids
IBMX	3-isobutyl-1-methyl-xanthine
IC_{50}	inhibitory concentration at 50%
Ig	immunoglobulins
IP_3	inositol trisphosphate
L-NAME	N^G-Nitro-L-arginine methyl ester
L-NMMA	N^G-monomethyl-L-arginine citrate
LB	Luria broth
LD_{50}	lethal dose for 50%
LPS	lipopolysaccharide
LT	leukotrienes
NaOH	sodium hydroxide
NADPH	nitcotinamide adenine dinucleotide phosphate
NDGA	nordihydroguaiaretic acid

NF-κB	nucleation factor kappa B
NO	nitric oxide
NOS	nitric oxide synthase
NSAID	nonsteroidal anti-inflammatory drugs
OD	optical density
PBS	phosphate buffered saline
PI	post-injection
PKC	protein kinase C
PLA_2	phospholipase A_2
PG	prostaglandin
PTU	phenylthiourea
PUFA	polyunsaturated fatty acids
LT	leukotrienes
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
SDS	sodium dodecyl sulfate
SNAP	sodium nitroprusside dihydrate
SSC	saline-sodium citrate buffer
T-cells	thymus derived lymphocytes
TLCK	Nα-p-tosyl-*L*-lysinechloro-methyl ketone
TX	thromboxane

Chapter 1

Introduction

Introduction

Comparative immunology is a discipline encompassing the many diverse animal phyla, the members of which face the same common need: to effectively eliminate foreign substances or "non-self" from the internal milieu. The actual mechanisms used by different animals may be as similar as they are diverse; my primary goal in the work presented herein was to elucidate some of the mechanisms by which the insect cellular immune response is modulated. Three putative mediators of particular interest were biogenic amines, eicosanoids and nitric oxide. These were chosen in part due to their similarity to immunomodulators of the vertebrate immune response. Conversely, their putative roles within the invertebrate immunity presented novel opportunities for examining the homology between the two systems.

Research on invertebrate immunology is, in many respects, in its infancy. The intent of this introduction is to provide an adequate context for my research. Therefore, I begin with a historical perspective on the invertebrate immune response, followed by a description of the innate and acquired immune responses of vertebrates and invertebrates. This is followed by an overview of the insect immune system, which I treat as comprising of both humoral and cellular components. Finally, I present background on the presumptive roles of biogenic amines, eicosanoids and nitric oxide and why I believe they play pivotal, and novel roles in the invertebrate immune response.

Historical Perspective

The formal study of cellular immunology began with observations on invertebrates when, in 1884, Elie Metchnikoff embedded a rose thorn in a starfish larva (Chernyak and Tauber, 1988). Due to the translucent nature of this organism, Metchnikoff was able to observe an inflammation response in which an "army" of phagocytic cells migrated towards the thorn, and encapsulated it with cytoplasmic extensions called "plasmodia" (Chernyak and Tauber, 1988). Metchnikoff suggested that since marine invertebrates are capable of mounting such a response, more complex organisms such as vertebrates should posess a similar protective function. Then, as now, invertebrates have frequently proven to be useful models for investigating basic cellular biology. Metchnikoff proposed that such phagocytic cells were the primary elements in natural immunity and critical for acquired immunity (Chernyak and Tauber, 1988).

In 1908, Mechnikoff shared the Nobel Prize in Medicine with Paul Ehrlich, who had proposed the comprehensive theory of antibody formation in 1897 (Silverstein, 1993). Although investigators of the time suggested that both cellular and humoral factors were equally important, the humoral theory of immunity eclipsed the cellular theory. Most investigators at the turn of the century chose to study antibodies which were easier to work with and measure at that time. These investigators considered many of the problems in cellular immunology to be "uninteresting" (Silverstein, 1993).

The majority of advancements made in the field of immunology deal with vertebrate systems. In comparison, there is relatively little known about the immunobiology of invertebrates. Given the wealth of knowledge that has accumulated on the vertebrate immune response, many investigators of invertebrate cellular and humoral defence reactions have looked for homologies and/or analogies to the vertebrates. Primarily as a result of such comparisons, two general responses have been delineated: innate and acquired immunity. Both types of immune reactions have been identified in vertebrates, whereas invertebrates have traditionally been considered to possess only the former (Hoffmann *et al.*, 1996).

Innate and Adaptive Immune Systems

In the course of evolution two mechanisms for responding to non-self have developed: innate (or natural) and adaptive (or acquired) immune systems. The innate immune response is phylogenetically older than the adaptive system and can be distinguished in some form in all multicellular organisms. Adaptive immunity evolved 400 million years ago and is found only in cartilaginous and bony fish, reptiles, birds, and mammals (Thompson, 1995). The primary difference between the two is systems is the means by which they recognize non-self (Fearon and Locksley, 1996).

After the integument, the vertebrate innate response is the first line of defence against invading microorganisms. It consists of a precisely regulated series of events beginning with the recognition of non-self by either cellular receptors or

soluble proteins. Some of the cellular receptors found on the surface of macrophages include: the mannose receptor, which contains C-type lectins with broad carbohydrate specificity; the lipopolysaccharide (LPS) receptor, CD14, which binds LPS (a constituent of the cell wall of gram negative bacteria); and scavenger receptors (type 1 and 2) which bind LPS and yeast cell wall components (Ulevitch and Tobias, 1995). In addition to cellular receptors, non-self is also recognized by soluble factors such as C-reactive proteins, and mannose- and LPS-binding proteins. C-reactive proteins bind microbial polysaccharides and enhance phagocytosis, while the latter two bind microbial cell wall products (Dodds, 1994).

Another major soluble modulator of the innate immune system is complement. Complement can be activated by the classical pathway through binding with specific lectins, or by the alternative pathway involving subtle interactions between enzymatic components and surface structures (Dodds, 1994). The functions of complement include direct killing of foreign microbes by disrupting the invading cells' lipid bilayer, "flagging" foreign substances for recognition by effector cells and release of active fragments which control the regulation of immune complexes and modulation of immune responses (Dodds, 1994).

The innate immune system also plays two important roles in the generation of the adaptive immune response. First, the innate response "fights" the early phases of infection, providing time for the adaptive response to become effective. Second, it is involved in triggering the adaptive response. The two main characteristics of the adaptive immune response are memory and specificity. These characteristics are

achieved via clonal selection and expansion of vertebrate lymphocytes. Vertebrate immunocytes stimulated by specific non-self molecules (antigens) receive an additional signal from host cells which are part of the innate response (Janeway, 1994). This additional signal consists of B7 molecules which are provided by the same cells which are presenting the antigen to lymphocytes (Medzhitov and Janeway, 1997).

According to the theory of clonal expansion, stimulation by a non-self molecule produces thymus-derived lymphocytes (T-cells), antibodies, and lymphocytes (B-cells) (Alberts *et al.*, 1994). When virgin, or previously unstimulated, T or B cells come in contact with a specific antigen they mature and produce clones. Some of the clones produced will initiate an immune response, while others will become memory cells. This confers a form of immunological "memory" on the adaptive immune response due to the selection and amplification of sets of non-self recognizing antibodies. Upon subsequent exposure to the same antigen, memory cells respond more rapidly than virgin cells to produce more activated and memory cells.

Specificity is key to the adaptive immune response: an antigen will only activate lymphocytes or clones that are already committed to respond to it specifically. There are thought to be millions of different lymphocyte clones in the vertebrate immune system. This diversity of clones with specific B- and T cells are encoded by multiple sets of genes that rearrange themselves to produce specific receptors and antibodies (Alberts *et al.*, 1994).

Insects, similar to vertebrates, have developed specific biological defences to protect themselves from invading pathogens and parasites. The means by which they do so are arguably less complex than those of vertebrates, although they are still efficacious. The initial line of defence in insects is also the integument and gut, which each provide a physical barrier to pathogenic microorganisms. However, insects are also capable of rapidly synthesizing antibacterial peptides and proteins when these physical barriers are breached. In contrast, they are not capable of mounting a specific immune response *per se* due to their lack of immunologically-specific lymphocytes or immunoglobulins (Igs). Some insects produce proteins which have Ig-like domains, including amalgam, fasciclin II, and twitchin, although these proteins play roles primarily in the development of the insect nervous system (Lanz-Mendoza *et al.*, 1996). In that insects lack an adaptive immune response, their immune systems more closely resemble the innate immune mechanisms of vertebrates. In the following section, I review the salient features of immunity in insects.

Insect Immune Response

The insect immune response, like the innate system of vertebrates, has evolved means for recognizing characteristic molecular patterns of microbial polysaccharides (Janeway, 1994). The insect immune system is capable of responding to certain microbial constituents such as lipopolysaccharides and peptidoglycans which are unique to the outer membranes of bacteria, and β-1,3-

mannans and β-1,3-glucans from fungal cell walls (Janeway, 1994). However, the mechanisms underlying the recognition of such non-self molecules are poorly understood.

Conceptually, the innate immune response in insects consists of three interconnected phases. The first involves a proteolytic cascade induced by wounding or by recognition of non-self. The end result is a localised clotting of hemolymph and is referred to as the prophenoloxidase cascade. This cascade is believed to play important roles in the defence mechanism and recognition of foreign particles in insects (Ashida and Dohke 1980). The second phase is a rapid and transient synthesis of a battery of antibacterial peptides, produced mainly by the fat body. The third phase is the cellular defence reaction, which consists of hemocytic (blood cell) mediated reactions which include phagocytosis, nodulation and encapsulation.

Humoral Defence Mechanisms

In the absence of antigen-specific antibodies the invertebrate humoral response presents an impressive array of endogenous proteins which serve two broad functions: isolating foreign pathogens via deposition of the tyrosine derivative, melanin and killing bacteria by means of lysozymes and antibacterial peptides.

The melanization cascade, which may be considered analogous (but not homologous) to the vertebrate extrinsic clotting cascade, plays key roles in the insect innate immune response through wound healing and pathogen sequestration. The

proenzyme prophenoloxidase, which is present as an inactive precursor in insect hemolymph and/or in hemocytes, is proteolytically activated to form phenoloxidase, which in turn hydroxylates phenols and oxidizes *o*-phenols. Quinones formed from the tyrosine derivative result in the production of melanin, which is a "sticky" black gelatinous matter capable of physically binding foreign particles (Ratcliffe and Rowley, 1979).

The other humoral components of the insect immune system include inducible antimicrobial peptides and polypeptides. To date, the list of antimicrobial molecules that have been characterised is rapidly approaching one hundred (Hoffmann *et al.*, 1996). These antibacterial peptides/polypeptides can be grouped according to their gene families : cecropins, attacins/ sarcotoxins, deptericins, and defensins.

In 1981, Boman and colleagues isolated the first antibacterial peptide in bacteria-challenged pupae of the moth *Hyalophora cecropia* (Steiner *et al.*, 1981). Since then, these peptides (termed cecropins) have been identified in a number of lepidopteran and dipteran species. Additionally, a cecropin homologue has been found in pig intestine (Hetru *et al.*, 1994). These 4kDa peptides have a broad spectrum of activity against gram-negative and gram-positive bacteria. It is proposed that cecropins kill bacteria by forming voltage-dependent ion channels in the cell membrane (Durell *et al.*, 1992).

The attacins/sarcotoxin II are predominantly found in lepidopteran and dipteran species. These bacteria-induced 20-28 kDa proteins have a relatively

narrow spectrum of activity and are effective only against gram-negative bacteria, in which they prevent the synthesis of the outer membrane proteins. Deptericins are similar to attacins and are effective against a limited number of gram-negative bacteria. These 9 kDa antibacterial peptides have so far been found only in members of the Diptera (Hetru *et al.*, 1994).

Two 4 kDa peptides, the insect defensins, were initially isolated from bacteria-challenged larvae of the flesh fly *Sarcophaga peregina*, and were originally named sapecins. These defensins are active against only gram-positive bacteria and have been identified in the Diptera, Coleoptera, Trichoptera, Hymenoptera, Hemiptera and Odonata. Insect defensins target the bacterial membrane by disrupting the permeability barrier, resulting in a loss of cytoplasmic potassium (Cociancich *et al.*, 1993). A homologous cationic peptide also called defensin has been identified in vertebrate macrophages (Du Pasquier, 1993).

Lysozymes are antibacterial molecules present in both vertebrate and invertebrate phyla. They are found constitutively in insects but can be induced upon immunization with bacteria. This enzyme hydrolyses β-1,4 linkages between N-acetylglucosamine and N-acetylmuramic acid in the peptidoglycan of bacterial cell walls. Insect lysozymes are approximately 14 kDa and have been cloned and characterised in *H. cecropia, Bombyx mori, Drosophila melanogaster and S. peregrina.* The sequence of these lysozymes show the greatest homology with chicken egg white lysozymes (Gillespie *et al.*, 1997)

Sun *et al.* (1990) discovered another humoral factor in the hemolymph from bacteria-induced larvae. This molecule, named hemolin, is the only invertebrate member of the Ig superfamily found in insects to be up-regulated and released upon infection. Hemolin contains four C2-type immunoglobulin domains which are characteristic of vertebrate cell adhesion molecules (Ladendorff and Kanost, 1991). Hemolin inhibits the aggregation of hemocytes, a fact which suggests it may regulate the adhesive properties of hemocytes during an immune response (Kanost *et al.*, 1994).

Cellular Defence Reactions

The primary effector of the cellular immune response in insects is the hemocyte, which is analogous to the immunocyte found in vertebrates. Similar to mammalian macrophages, hemocytes engage in active ameboid movements which facilitate chemotatic movements and are presumed to play key roles in wound healing, phagocytosis, nodule formation and encapsulation. However, numerous functional and morphological differences exist between macrophages and hemocytes. The oxidative burst, typical of vertebrate macrophage activation, has yet to be demonstrated in phagocytic hemocytes. Anderson *et al* (1988) determined that *Blaberus craniifer* hemocytes were capable of bacterial endocytosis, but did not produce oxidative metabolites. This still remains a point of contention. Ahmad *et al.* (1991) detected superoxide dismutase in the hemocytes of the cabbage looper, *Trichoplusia ni*, which implied that superoxide anions are produced in insect

hemocytes. Additionally, Nappi *et al.* (1995) suggested that quinones formed in the melanization cascade may be involved in the production of reactive oxygen species such as superoxide anions and hydroxyl radicals, and that the cytotoxic effects may contribute to the killing of pathogens. Finally, unpublished work in our lab has revealed that insect hemocytes produce a weak respiratory burst when stimulated with phorbol esters.

In contrast, changes in hemocyte morphology, behaviour and population during development and immunological challenge have been extensively documented and have been the subject of many reviews (Wigglesworth, 1959; Jones, 1962; Salt, 1968; Ratcliffe and Rowley 1979; Lackie, 1988). There has been considerable confusion in the literature over the nomenclature and classification of hemocytes. Light and electron microscopy has been used to characterise and classify hemocytes for the last 100 years, as reviewed by Gupta (1979), but until recently there has been vague concordance between investigators. In the last few years, monoclonal antibodies have been used to identify and analyse hemocyte function (Chain *et al.*, 1992; Mullet *et al.*, 1993; Willott *et al.*, 1995; Strand and Johnsson, 1996). The following is a brief summary of the currently accepted hemocyte types, based on the sources listed above.

Of the five main classes of circulating hemocytes in insects, prohemocytes are the smallest, approximately 6-14 μm in length. These cells are found in the hemolymph of larval insects and are considered to be stem cells from which other blood cell types develop. Plasmatocytes are phagocytic, agranular and contain

lysosomal enzymes, and are 3.3-5 μm in width to 3.3-40 μm in length. These hemocytes are round or spindle-shaped in suspension; however, when activated by wounding, plasmatocytes become thigmotatic, pleiomorphic and motile. In contrast, granulocytes are round or oval cells in suspension and are variable in size (10-45 μm long and 4-32 μm wide). Their most distinguishing features are phase-bright granules, which are released during an immune response to produce a granular coagulum. Spherule cells contain spherical phase-bright inclusions and range in size from 9-25 μm long and 5-10 μm wide. The immunological and/or developmental role of these blood cells is still unknown. Finally, oenocytoids are the largest of the insect hemocytes (>16-54 μm), and are infrequent in the circulating population. It has been suggested that these cells may contain prophenoloxidase (Gupta, 1979).

The plasmatocyte is, in most insects, the primary effector of phagocytosis. This process is functionally similar to that of vertebrate immunocytes, and includes the stages of attachment, recognition, signal transduction, pseudopodium formation, ingestion and assembly of phagosomes (Gillespie *et al.*, 1997). In contrast to the vertebrate phagocyte, the molecular biology of phagocytosis by insect hemocytes has not been well studied, although it has been assumed that the fundamental mechanisms underlying ingestion, such as actin-myosin interactions, are similar. Scapagliati and Mazzini (1994) demonstrated that phagocytosis could be blocked in hemocytes of the stick insect, *Bacillus rossius*, by treating the cells with cytochalasin B. The molecular basis of recognition, signal transduction, microbial killing and digestion in insect hemocytes still remain largely unknown.

When challenged by large numbers of circulating bacteria, most insects employ a bacterial trapping mechanism called nodulation. Ratcliffe and Walters (1983) demonstrated in *G. mellonella* larvae that low doses ($< 10^3/\mu l$) of pathogenic and non-pathogenic bacteria were removed by phagocytosis. At higher doses, nodule formation was found to be a quantitatively more efficient mechanism of eliminating bacteria from the hemocoel than phagocytosis. This process is initiated by the random contact of granulocytes with bacteria. Granulocytes release their contents, producing a localized coagulum which entraps a large number of bacteria and non-reactive hemocytes (Ratcliffe and Rowley, 1979). The mixture of bacteria, coagulum and necrotic granular hemocytes become melanized while surrounding plasmatocytes flatten and form a capsule-like structure that adheres to the surfaces of tissues within the hemocoel (Ratcliffe and Gagan, 1976).

Encapsulation is functionally similar to nodule formation, but serves primarily to "wall off" foreign invaders, such as metazoan parasites, which are too large to be phagocytosed or trapped within a nodule. Encapsulation involves the formation of a hemocytic multicellular sheath that becomes melanized, thereby killing the enclosed parasite (Lackie, 1988).

Modulators of the Insect Immune Response

In the last two decades, significant research has been directed at elucidating the factors which modulate the insect immune response. While the major morphological and behavioural components of the insect immune response have

been described, only recently have investigators begun to identify the molecular modifiers of hemocyte activity. There is ample justification for such efforts. From an evolutionary perspective, the issue of homology vs. analogy between the invertebrate and vertebrate immune systems needs to be explored. One approach to this continuous debate is to assess the similarity of immunoregulatory mechanisms of these groups. Furthermore, divergent and/or novel regulatory pathways may be uncovered and of themselves present new insights into aspects of basic cellular physiology. Finally, an understanding of the modulators of the insect immune response may directly yield novel approaches to safe, effective biological control agents.

As stated to earlier, my research has focused on three putative immunomodulators: biogenic amines, eicosanoids, and nitric oxide. In the proceeding sections, I review the current literature, and discuss how these factors converge and diverge with/from vertebrate models.

Biogenic Amines

The three major biogenic amines found in insect hemolymph are octopamine, dopamine and 5-hydroxytryptamine (5-HT). These appear to serve both neural and immunoregulatory functions in insects (Bailey *et al.*, 1984; Downer and Martin, 1987). In particular, octopamine and 5-HT have both been shown to regulate aspects of hemocyte activity in insects. Baines *et al.* (1992) reported that both octopamine and 5-HT elevate phagocytosis *in vitro* and nodule formation *in vivo* in

the cockroach, *Periplaneta americana*. Moreover, both amines increase the survival rate of cockroaches challenged with a LD_{50} dose of *Staphylococcus aureus* (Baines and Downer, 1992). In the lepidopteran *Galleria mellonella*, octopamine accelerates clearance of bacteria from hemolymph, apparently independently of the prophenoloxidase cascade (Dunphy and Downer 1994).

An octopamine-sensitive adenylate cyclase has been identified in cockroach hemocytes (Orr *et al.*, 1985). Baines and Downer (1992) demonstrated that 5-HT and octopamine stimulated increases in cyclic adenosine monophosphate (cAMP) in fragmented hemocyte membranes, with maximal effects at 10 nM and 10 μM, respectively. In comparison, vertebrate macrophages and neutrophils treated with the neurohormones adrenaline and noradrenaline also respond with elevated levels of cAMP, which corresponds with increased pseudopodial extensions (Lew, 1989; Mueller and Sklar, 1989). Rink and Hallam (1984) demonstrated 5-HT-induced increases in cAMP and inositol trisphosphate (IP_3) which correlated with changes in the shape of platelets.

The action of 5-HT is mediated by increasing the intracellular concentrations of cAMP in the salivary glands of the blowfly *Calliphora erythrocephala* (Berridge, 1970). Dalton (1977) demonstrated that low doses of prostaglandin E_1 diminished the stimulatory effects of 5-HT on these salivary glands, presumably by inhibiting adenyl cyclase. The effects of increased cAMP in insect hemocytes, in contrast, have not previously been shown, although octopamine and 5-HT increased the survival of insects by enhancing phagocytosis and nodule formation (Baines *et al.*,

1992). In this thesis, I provide evidence that octopamine modulates locomotory behaviour, polarity, and cytoskeletal dynamics in hemocytes, all of which are important aspects in phagocytosis and nodule formation.

Eicosanoids

To date, prostaglandins and other eicosanoids have been found in essentially every mammalian tissue and fluid, where they exert both specific and non-specific functions similar to that of some steroids. Eicosanoids are highly potent and their multifaceted activity may be due to their solubility in aqueous as well as lipid media (von Euler, 1988). Prostaglandins, thomboxanes, leukotrienes and lipoxins are involved in many basic cellular and physiological processes and seem to be essential to various pathophysiological responses including inflammation, blood-clotting, asthma and tumor growth (von Euler, 1988).

The hypothesis that eicosanoids mediate cellular immunity in insects was first put forth by Stanley-Samuelson *et al.* (1991). Perturbation of the major eicosanoid biosynthetic pathways with selective pharmacological inhibitors was used to examine this idea. Insect larvae injected with dexamethasone, a phospholipase A_2 inhibitor, cleared significantly fewer bacteria from the hemolymph than in control animals. Furthermore, larvae infected with bacteria and injected with dexamethasone had significantly lower survival rates (Stanley-Samuelson *et al.*, 1991). To add support to the concept that eicosanoids are mediators of the insect cellular immunity, these researchers conducted a "rescue" experiment. Subsequent infections with *S. aureus*

and exogenous arachidonic acid resulted in significantly lower mortality than in larvae pre-treated with dexamethasone (Stanley-Samuelson *et al.*, 1991).

The biological significance of eicosaniods in insects is not well understood. Fatty acid composition of lipid preparations from larval *M. sexta* hemocytes are quite different from that of serum lipids and insect culture medium (Ogg *et al.*, 1991). Ogg *et al.* (1991) demonstrated that hemocytes are capable of incorporating exogenous radioactive arachidonic acid into hemocyte phospholipids, suggesting that the requirements of eicosanoid precursors may be regulated from within the cell.

Since nodule formation is one of the means by which insects eliminate bacteria from the hemocoel, Miller *et al.* (1995) examined the effects of dexamethasone and other eicosanoid biosynthesis inhibitors, specifically showing a decrease in nodule formation related to these inhibitors. The mechanism(s) by which eicosanoids may influence nodule formation were unknown. In the present work, I chose to investigate the role of eicosanoids during three discrete phases of nodule formation: prophenoloxidase activation, cell spreading, and phagocytosis. In doing so, I endeavour to show that eicosanoids, in general, modulate immunocyte activity and are specifically involved as modulators of an important insect immune response.

Nitric Oxide

Nitric oxide (NO) is a short-lived molecular messenger with numerous physiological roles in vertebrates, including modulation of synaptic plasticity, regulation of vascular tone and elimination of pathogens during non-specific immune

response (Moncada *et al.*, 1991). Three major isoforms of nitric oxide synthase (NOS) have been cloned in various mammalian tissues: Ca^{2+}/CaM-dependent isoform found in brain tissue; Ca^{2+}-dependent, a membrane-associated enzyme in vascular endothelial tissue and a Ca^{2+}-independent NOS found in induced macrophages (Stuehr *et al.*, 1991).

Recent biochemical data support the idea that NOS/NO exists in insects. Choi *et al.* (1995) found NOS in the fat body and Malpighian tubules of the silkworm, *Bombyx mori*, and Muller and Bicker (1994) show NO present in the nerve tissue of the locust, *Schistocerca gregaria*. In the Malpighian tubules of *Drosophila melanogaster*, Dow *et al.* (1994) have demonstrated a NOS pathway which regulates fluid secretion. In addition to biochemical evidence, the following insect NOS-like genes have been cloned: dNOS from *D. melanogaster* (Regulski and Tully, 1995), nitrophorins from the salivary glands of *Rhodnius prolixus* (Champagne *et al.*, 1995) and a mosquito NOS (genebank # AF053344).

Due to the importance of NO in the vertebrate immune response and its appearance in mollusc hemocytes (Conte and Ottaviani, 1995), I investigated the presence of NOS in *Galleria mellonella* hemocytes using a combined molecular and biochemical approach. Furthermore, I endeavoured to show that NO has a physiologically role in the insect immune response.

Summary

In this thesis, I have attempted to provide new insights into the roles of three putative immune modulators, octopamine, eicosanoids and NO, in regulation of the cellular immune response in insects. The effect of octopamine on the locomotory behaviour, polarity, and cytoskeleton of hemocytes from the greater wax moth, *Galleria mellonella* is examined in Chapter 2. In chapter 3, I demonstrate that eicosanoids modulate all three phases of nodule formation in hemocytes from *Galleria mellonella*. Biochemical, molecular and physiological evidence for the presence and role of nitric oxide synthase in lipopolysaccharide-induced insect hemocytes is presented in Chapter 4. In the latter, I submit that nitric oxide may confer a weak but discrete form of secondary immune response in insects, and that nitric oxide may play important roles in mediating innate immunity in both insects and vertebrates. Finally, in Chapter 5, I present data that support the hypothesis that eicosanoids and NOS pathways are co-regulated, a further homology between insect and vertebrate immune responses.

General Materials and Methods

Insect Rearing

A colony of greater wax moths, *Galleria mellonella* L. (Insecta: Lepidoptera) were maintained under standard conditions (Diehl-Jones *et al.*, 1996). Larvae were reared on a synthetic diet consisting of mixed cereal pablum (Heinz, North York, ON.) moistened with water/glycerol/sugar (1:2:2, v:v:w). Adult moths were collected and eggs were laid in a growth medium composed of a mixture of ground rolled oats and honey. The colony was maintained at 24°C with a photoperiod of 12 hours.

Isolation of Hemocytes

Hemocytes were obtained from sixth instar larvae (0.27-0.28g). Healthy, prewandering stage larvae were chilled at 4°C for approximately 15 minutes then surface-sterilized with 70% ethanol. At this and all subsequent stages, care was taken to keep insects and hemolymph samples chilled. Larvae were injected via the third abdominal proleg using a 30 gauge needle with 75 µl of modified Grace's Insect Medium (mGIM: 10mM NaCl, 30 mM KCl, 9mM CaCl2, 11mM MgCl2, 11mM MgSO4, 2mM trehalose, 4 mM D-glucose, 160 mM Sucrose, and 11mM PIPES, pH=7.2). Larvae were then bled directly into chilled Ependorf microtubes containing cold mGIM by amputating the third thoracic proleg and gently expressing approximately 50 µl of hemolymph per insect. Hemolymph from larvae were pooled in all experiments, and hemocytes were pelleted at 1500g at 4°C for 4 minutes in an

Eppendorf microcentrifuge, which resulted in optimum yield with minimal lysis of the cells. Hemocyte pellets were washed by removal of mGIM, resuspension and re-centrifugation. The washed hemocytes were either utilized immediately or frozen at -70 °C.

Glassware and Plastics

In all assays, care was taken to minimise exposure to endotoxins. All culture chambers were cleaned in 10N KOH and rinsed in E-toxa Clean (Sigma Chemical Co., St. Louis Mi., U.S.A.). Chambers were autoclaved on a dry cycle. Glassware was soaked in 1% (w/v) E-toxa Clean, rinsed in endotoxin-free water, and autoclaved on a dry cycle. Plascticware was sterilized by autoclaving on a dry cycle.

Reagents

The following reagents were purchased from Sigma Chemical Co. (St. Louis Mi., U.S.A.): sodium chloride, potassium chloride, calcium chloride, magnesium chloride, magnesium sulphate, trehalose, D-glucose, sucrose, sodium hydroxide, potassium hydroxide, PIPES (piperazine-N,N'-bis[2-ethanesulfonic acid]), HEPES (N-[2-hydroxyethyl]-N'-[2-ethanesulfonic acid]), dithiothreitol, EDTA, EGTA, leupeptin, antipain, TLCK (Nα-p-tosyl-$_L$-lysinechloro-methyl ketone), valine, citrulline, arginine, IBMX (3-isobutyl-1-methyl-xanthine), β-NADPH, FAD, Dowex 50WX4-400, poly(HEMA) methacrylate, dexamethasone ((11,B,16)-9-fluoro-

11,17,21-trihydroxy-16-methylpregna-1,4-dione)), quinacrine (6-chloro-9-(14-diethylamino)-1-methylbutyl)amino-2-methoxy-acridine), BPB (4-bromophenacyl bromide), ETYA (5,8,11,14-eicosatetraynoic acid), esculetin (6,7-dihydroxycoumarin), NDGA (nordihydroguaiaretic acid), indomethacin (1-(p-chlorophenycyl)-5-methoxy-2-methylindole-3-acetic acid), arachidonic acid (5,8,11,14-eicosatetaenoic acid) Grace's Insect Medium, glutaraldehyde, paraformaldehyde, sodium cacodylate, laminarin, L-dihydrophenylalanine, lipopolysaccaride (from *Escherichia coli* Serotype 055:B5) and TRIREAGENT. Rhodamine-labelled phalloidin was purchased from Molecular Probes Inc., (Eugene, OR., U.S.A.). Latex microspheres were purchased from Polysciences, Inc. (Warrington, Pa., U.S.A.). L-[2,3,4,5-^{3}H]arginine monohydrochloride was purchased from Amersham (Toronto, Ontario). L-NAME (N^{G}-Nitro-L-arginine Methyl Ester) and L- NMMA (L-N^{G}-monomethyl arginine citrate) were purchased from Cayman Chemical (Ann Arbor, Mi., U.S.A.). Tetrahydro-L-biopterin, dihydrochloride and SNAP (s-nitroso-N-acetylpenicillamine) were purchased from Calbiochem-Novabiochem Co. (San Diego, Ca., U.S.A.).

References

Ahmad, S., D.L. Duval, L.C. Weinhold and R.S. Pardini. 1991. Cabbage looper antioxidant enzyme: tissue specificity. Insect Biochem. 21:563-572

Alberts, B., D. Bray, J. Lewis, M. Raff, K. Roberts and J. Watson. 1994. The immune system. In: *Molecular Biology of the Cell.* 1196-1254. Garland Publishing Inc., New York, NY.

Anderson, R.S., B. Holmes and R.A. Good. 1988. Comparative biochemistry of phagocytizing insect hemocytes. Comp. Biochem. Physiol. 46B:595-602.

Ashida, M. and K. Dohke. 1980. Activation of prophenoloxidase by the activating enzyme of the silkworm, *Bombyx mori*. Insect Biochem. 10:37-47.

Bailey, B. A., R. J. Martin and R. G. H. Downer. 1984. Haemolymph octopamine levels during and following flight in the American Cockroach, *Periplaneta americana.* Can. J. Zool. 62:19-22.

Baines, D., T. DeSantis and R. G. H. Downer. 1992. Octopamine and 5-hydroxytryptamine enhance the phagocytic and nodule formation activities of cockroach (*Periplanteta americana*) haemocytes. J. Insect Physiol. 38:905-914.

Baines, D. and R. G. H. Downer. 1992. 5-Hydroxytryptamine-sensitive adenylate cyclase affects phagocytosis in cockroach hemocytes. Arch. Insect Biochem. Physiol. 21:303-316.

Berridge, M. 1970. The role of 5-hydroxytryptamine and cyclic AMP in the control of fluid secretion by isolated salivary glands. J. Exp. Biol. 53: 1717-186.

Chain, B. M., K. Leyshon-Soland and M.T. Siva-Jothy.1992. Haemocyte heterogeneity in the cockroach *Peiplaneta americana* analyzed using monclonal antibodies. J. Cell Sci. 103:1261-1267.

Champagne, D. E., R.H. Nussenzveig and J.M.C. Ribeiro 1995. Purification, partial characterization, and cloning of nitric oxide-carrying heme proteins (nitrophorins) from the salivary glands of the blood sucking insect *Rhodnius prolixus*. J. Biol. Chem 270:8691-8695.

Chernyak, L. and A. I. Tauber. 1988. The birth of immunology: Metchnikoff, the embryologist. Cell. Immunol. 117:218-233.

Choi, S. K., H. K. Choi, K. Kadono-Okuda, K.Taniai, Y, Kato, M. Yamamoto, S. Chowdhury, J. Xu, A. Miyanoshita, N.C. Debnath, A. Asaoka and M. Yamakawa. 1995. Occurance of novel types of nitric oxide synthase in the silkworm, *Bomybx mori*. Biochem. Biophys. Res. Comm. 207: 452-459.

Cociancich, S., A, Ghazi, C. Hetru, J. A. Hoffmann and L. Letellier. 1993. Insect defensins, an inducible antibacterial peptide, forms voltage-dependent channels in *Micrococcus luteus*. J. Chem. Biol. 263: 19239-19245.

Conte, A. and E. Ottaviani. 1995. Nitric oxide synthase activity in molluscan hemocytes. FEBS Lett. 365:120-124.

Dalton, T. 1977. Threshold and receptor reserve in the action of 5-hydroxytryptamine on the salivary glands of *Calliphora erythrocepala*. J. Insect. Physiol. 23: 625-631.

Diehl-Jones, W. L.; Mandato, C. A.; Whent, G.; Downer, R. G. H. Monoaminergic regulation of hemocyte activity. J. Insect Physiol. 42: 13-19.

Dodds, A. W. 1994. Molecular and phylogenetic aspects of the complement system. In: *Phylogenetic Perspectives in Immunity: The Insect Host Defence*. (J.A. Hoffmann, C.A. Janeway and S. Natori, eds) 143-156. R.G. Landes Co., Austin, TX.

Dow, J. T., S. H. P. Maddrell, S. A. Davis, N. J. V. Skaer and K. Kaiser. 1994. A novel role for the nitric oxide-cGMP signaling pathway: the control of epithelial function in *Drosophila*. Am. J. Physiol. 226:R1716-R1719.

Downer, R.G.H. and R.J. Martin. 1987. N-acetylation of octopamine a potential target for insecticide development. In: *The Search for Novel Insecticides: Toxicants Affecting GABA, Octopamine, and Other Neuroreceptors in Invertebrates. ACS Symp.* (M. Green, R. M. Hollingworth and P. A. Hedlin, eds.) 202-210. ACS Press, New York, NY.

Du Pasquier, L. 1993. The development of an immune system. In: *Fundamental Immunlogy, 3rd ed.* (W. E. Paul, ed.) 199-234. Raven Press Ltd., New York, NY.

Dunphy, G. B. and . R. G. H. Downer. 1994. Octopamine, a modulator of the haemocytic nodulation response non-immune *Galleria mellonella* larvae. J. Insect Physiol. 40:267-272.

Durell, S. R., G. Raghunathan and R. Guy. 1992. Modeling ion channel structure of cecropins. Biophys. J. 63:1623-1631.

Fearon, D. T. and R. M. Locksley. 1996. The instructive role of innate immunity in the acquired immune response. Science. 272:50-54.

Gillespie, J. P., M. R. Kanost and T. Trenczek. 1997. Biological mediators of insect immunity. In: *Annual Review of Entomology*. (T. Mittler, F. Radovsky, and V. Resh, eds.) 42: 611-644. Annual Reviews Inc., Palo Alto, CA.

Gupta, A. P. 1979. Hemocyte types: their structure, synonymies, interrelationships, and taxonomic significance. In: *Insect Hemocytes: Development, Forms, Functions and Techniques*. (A. P. Gupta, ed.) 85-128. Cambridge University Press, New York, NY.

Hetru, C., P. Bulet, S. Cociancich, J-L, Dimarcq, D. Hoffmann and J.A. Hoffmann 1994. Antibacterial peptides/polypeptides in the insect host defense: a comparison with vertebrate antibacterial peptides/polypetides. In: *Phylogenetic Perspectives in Immunity*. (J.A. Hoffmann, C.A. Janeway and S. Natori, eds) 143-156. R.G. Landes Co., Austin, TX.

Hoffmann, J. A., J-M. Reichhart and C. Hertu. 1996. Innate immunity in higher insects. Curr. Opin. Immun. 8:8-13.

Janeway, C.A. 1994. The role of microbial pattern recognition in self: non-self discrimination in innate and adaptive immunity. In: *Phylogenetic Perspectives in Immunity*. (J.A. Hoffmann, C.A. Janeway and S. Natori, eds) 115-122. R.G. Landes Co., Austin, TX.

Jones, J. C. 1962. Current concepts concerning insect hemocytes. Amer. Zool. 2:209-246.

Kanost, M. R., M. K. Zepp, N. E. Ladendorff and L. A. Andersson. 1994. Isolation and characterization of a hemocyte aggregation inhibitor from hemolymph of *Manduca sexta* larvae. Arch. Insect Biochem. 27:123-126.

Lackie, A. M. 1988. Haemocyte behavior. In: *Advances in Insect Physiology*. (P. D. Evans and. V. B. Wigglesworth, eds) 21:85-178. Academic Press Ltd., San Diego, CA.

Ladendorff, N. E. and. M. R. Kanost. 1991. Bacteria-induced protein P4 (hemolin) from *Manduca sexta*: a member of the immunoglobulin superfamily which can inhibit hemocyte aggregation. Arch. Insect Biochem. Physiol. 18:285-300.

Lanz-Mendoza, H., R. Bettencourt, M. Fabbri and I. Faye. 1996. Regulation of the insect immune response: the effect of hemolin on cellular immune mechanisms. Cell. Immun. 169:47-54.

Lew, D. P. 1989. Receptor signalling and intracellular calcium in neutrophil activation. Eur. J. Clin. Invest. 19:338-346.

Medzhitov, R. and C. A. Janeway. 1997. Innate immunity: the virtues of a nonclonal system of recognition. Cell 91:295-298.

Miller, J. S., Nguyen, T., and Stanley-Samuelson, D. W. 1995. Eicosanoids mediate insect nodule response to bacteria infection. Proc. Natl. Acad. Sci. 91: 12418-12422.

Moncada, S., R. M. J. Palmer and E. A. Higgs. 1991. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharma. Rev. 43:109-142.

Mueller, H. and L. A. Sklar. 1989. Coupling of antagonistic signalling pathways in modulation of neutrophil function. J. Cell. Biochem. 40:287-294.

Muller, U. and G. Bicker. 1994. Calcium-activated release of nitric oxide and cellular distribution of nitric oxide-synthesizing neurons in the nervous system of the locust. J. Neurosci. 14:7521-7528.

Mullet, H., N. A. Ratcliffe and A. F. Rowley. 1993. The generation and characterisation of anti-insect blood cell monoclonal antibodies. J. Cell Sci. 105:93-100.

Nappi, A. J., E. Vass, F. Frey and Y. Carton. 1995. Superoxide anion generation in *Drosophila* during melanotic encapsulation of parasites. Eur. J. Cell Biol. 68: 450-456.

Ogg, C. L., Howard, R. W. and Stanley-Samuelson, D. W. 1991. Fatty acid composition and incorporation of arachidonic acid into phospholipids of hemocytes from the Tobacco Hornworm *Manduca sex*. Insect Biochem. 21:109-114.

Orr, G. L., J. W. D. Gole and R. G. H. Downer. 1985. Characterization of an octopamine-sensitive adenylate cyclase in haemocyte membrane fragments of the American cockroach *Periplenta americana* L. Insect Biochem. 15:695-701.

Ratcliffe, N. A. and A. F. Rowley. 1979. Role of hemocytes in defense against biological agents. In: *Insect Hemocytes: Development, Forms, Functions and Techniques*. (A. P. Gupta, ed.) 331-414. Cambridge University Press, New York, NY.

Ratcliffe, N. A. and S. J. Gagan. 1976. Cellular defense reactions of insect hemocytes *in vivo*: nodule formation and development in *Galleria mellonella* and *Pieris brassicae* larvae. J. Invert. Path. 28:373-382.

Ratcliffe, N. A. and J. B. Walters. 1983. Studies on the *in vivo* cellular reactions of insects: clearance of pathogenic and non-pathogenic bacteria in *Galleria mellonella* larvae. J. Insect Physiol. 29:407-415.

Regulski, M. and T. Tully. 1995. Molecular and biochemical characterization of dNOS: a *Drosophila* Ca^{2+} / calmodulin-dependent nitric oxide synthase. Proc. Natl. Acad. Sci. 92:9072-9076.

Rink, T. J. and T. J. Hallam. 1984. What turns on platlets? T.I.B.S. 9:215-219.

Salt, G. 1968. The resistance of insect parasitoids to the defence reactions of their hosts. Biol. Rev. 43:200-232.

Scapagliati, G. and M. Mazzini. 1994. *In vivo* and *in vitro* phagocytosis by hemocytes of the stick insect *Bacillus rossius*. Boll. Zool. 61:115-120.

Silverstein, A. M. 1993. The history of immunology. In: *Fundamental Immunology, 3rd ed.* (W. E. Paul, ed.) 21-42. Raven Press Ltd., New York, NY.

Stanley-Samuelson, D. W., E. Jensen, K. W. Nickerson, K. Tiebel, C. L. Ogg, and R. W. Howard. 1991. Insect immune response to bacterial infection is mediated by eicosanoids. Proc. Natl. Acad. Sci. 88:1064-1068.

Strand, M. R. and J. A. Johnsson. 1996. Characterization of monoclonal antibodies to hemocytes of *Pseudoplusia includens*. J. Insect Phys. 42:21-31.

Steiner, H., D. Hultmark, A. Engstrom, H. Bennich and H. G. Boman. 1981. Sequence and specificity of two antibacterial proteins involved in insect immunity. Nature. 292:246-248.

Stuehr, D. J., N. S. Kwon, C. F. Nathan, O. W. Griffith, P. L. Feldman and J. Wiseman. 1991. N omega-hydroxy-L-arginine is an intermediate in the biosynthesis of nitric oxide from L-arginine. J. Biol. Chem. 266:6259-6263.

Sun, S-C., I. Lindstrom, H. Boman, I. Faye and O. Schmidt. 1990. Hemolin: an insect-immune protein belonging to the immunoglobulin superfamily. Science. 250:1729-1732.

Thompson, C. B. 1995. New insights into V(D)J recombination and its role in evolution of the immune system. Immunity 3: 531-539.

Ulevitch, R. J. and P. S. Tobias. 1995. Receptor-dependent mechanism of cell stimulation by bacterial endotoxins. Annu.Rev. Immun. 13: 437-457.

von Euler, U. S. 1988. Biology of prostanoids. In: *Prostaglandins: Biology and Chemistry of Prostaglandins and Related Eicosanoids*. (P. B. Curtis-Prior, ed.) 1-7. Churchill Livingstone, London, England.

Wigglesworth, V. B. 1959. Insect blood cells. Annu. Rev. Entomol. 4:1-16.

Willott, E., C. Lowenberger, B. M. Christensen and M. R. Kanost. 1995. Monoclonal antibodies against *Manduca sexta* hemocytes bind *Aedes aegypti* hemocytes: characterization of six monoclonal antibodies that bind hemocytes from both species. Dev. Comp. Immunol. 19:451-461.

Chapter 2

Monoaminergic Regulation of Hemocyte Activity

as published in: Journal of Insect Physiology 42:13-19.

Abstract

Biogenic amines have previously been shown to enhance both phagocytosis and nodule formation in insects. In this chapter, the effects of octopamine on the locomotory behaviour, polarity, and cytoskeleton of hemocytes from the greater wax moth, *Galleria mellonella* are examined. Octopamine (5×10^{-6}M) induced an initial, transient increase in locomotory rate of exomigrating plasmatocytes *in vitro*, and this effect was abolished by phentolamine, a general β-adrenergic antagonist. Within 5 minutes exposure to octopamine, hemocytes exhibited increased protrusive activity. After 15 minutes exposure, a higher proportion of plasmatocytes assumed lamellar and unipolarized shapes associated with movement, and granulocytes produced extensive, bulbous-tipped filopodia. Morphological changes were reflected in alterations of the hemocyte cytoskeleton and were consistent with changes in the relative concentration of F-actin. These data suggest that octopamine modulates hemocyte activity via reorganization of the actin cytoskeleton.

Introduction

Hemocytes form the cellular component of the invertebrate immune response. There are several morphologically and functionally distinct types of hemocytes. At least two of these, plasmatocytes and granular cells, are involved in phagocytosis and nodule formation in insects (Pech *et al.*, 1994; Anggraeni and Ratcliffe, 1991; Ratcliffe, 1986). A variety of humoral factors have been implicated in modulating hemocyte activity in insects; the hemocyte aggregation inhibitor protein (Kanost *et al.*, 1994), hemolin (Ladendorff and Kanost, 1991), lysozyme (Dunphy and Webster, 1991), and a neuropeptide, metenkephalin (Stephano *et al.*, 1991).

There is strong evidence that biogenic amines regulate hemocyte activity in arthropods. Batelle and Kravitz (1978) showed that octopamine activates non-self responses in the lobster, *Homarus americanus*. More is known about the role of amines in insects. Baines *et al.* (1992) reported that both octopamine and 5-hydroxytryptamine (5-HT) elevate phagocytosis *in vitro* and nodule formation *in vivo* in the cockroach, *Periplaneta americana*. Moreover, both amines increase the survival rate of cockroaches challenged with a LD_{50} dose of *Staphylococcus aureus* (Baines and Downer, 1992). In the lepidopteran *Galleria mellonella*, octopamine accelerates clearance of bacteria from hemolymph, apparently independent of the prophenoloxidase cascade (Dunphy and Downer, 1994).

At least two mechanisms have been suggested to explain the cellular processes by which biogenic amines enhance phagocytosis. Baines *et al.* (1992)

proposed that octopamine and 5-HT target specific cell types. Granulocytes, in particular, are known to release factors which initiate hemocyte clumping (Lackie, 1988). This may be responsible for the *in vivo* hemocyte clumping that is observed in octopamine-treated cockroaches (Baines *et al.*, 1992). By extension, octopamine itself may function as a hemokine, opsonizing bacteria by virtue of its positively-charged amino group (Dunphy and Downer, 1994). A possibility is now suggested: that octopamine modulates cell shape change and motility. It is well established that locomotory activity and changes in cell structure are initiated by receptor-ligand binding, followed by transient increases in both intracellular Ca^{+2} and in the synthesis/hydrolysis of phosphoinositides (Jamney, 1994; Stossel, 1993). In this chapter data is presented on the effects of octopamine on the following related parameters: hemocyte locomotory behaviour, F- and G-actin concentrations, and F-actin distribution in certain hemocytes. Finally, I speculate on the role of octopamine in a two-tiered activation pathway of the insect immune system.

Materials and Methods

Video Time-Lapse Motility Assay

Hemocytes were collected (as described in the General Material and Methods) and resuspended in Grace's insect medium in the presence or absence of various test agents. Cells (approx. 1 x 10^6/ml) were then plated onto clean glass slides and assembled into Inoue chambers. These consisted of a sterile, saline-saturated filter paper dam (20 mm x 20 mm outer diameter, 5mm wide) surrounding 20 µl of resuspended cells, and covered with a 1.5 gauge coverslip. The chamber formed was sealed with tackiwax (Boekek Industries, Philadelphia, PA.) providing an optically transparent chamber which both permitted gas exchange and prevented desiccation. Cells were then viewed on a Leitz Labovert microscope equipped with Hoffman Modulation Contrast optics and a Sony CCD camera.

Video signals were captured on a 386 computer using the Northern Exposure Image Analysis and Archival system (ImagExperts, Mississauga, ON). Video frames were captured every 5 minutes and stored as bitmap files. Cell tracks were then created by averaging resultant bitmap files into one of three 8-bit frame buffers: the frame at time = 0 minutes was stored in the green buffer, subsequent frames were digitally averaged into the red buffer, and the final frame was stored in the blue frame buffer. A 24-bit image was compiled from all three colour frames, such that the starting position of hemocytes were shown in green, hemocyte tracks in red, and the final locations were shown in blue. In this manner, video callipers could be used to accurately measure distances covered during each 15 minute

interval. Only cells exomigrating from clumps of 2-15 hemocytes were measured to insure consistency, and data were not included from cells that travelled less than 5 mm. Statistical significance was determined by one-way ANOVA.

Fluorescence Assay for F-actin

To quantify F-actin in hemocytes, a modification of the method of Howard and Oresajo (1985) was used. Approximately 0.5 to 1.0 x 10^6 hemocytes in Grace's insect medium were allowed to adhere to glass coverslips for 5, 10, or 15 minutes. Adhesion was arrested by the addition of an equal volume of 5% (w/v) formalin. After a 15 minute fixation period, nonadherent cells were washed off with phosphate buffered saline (PBS) and stained with 50 mg/ml rhodamine-labelled phalloidin (Molecular Probes Inc., Eugene, OR.) for 20 minutes at room temperature. Unbound fluorochrome was removed with 3 washes of PBS and bound rhodamine-labelled phalloidin extracted in 1 ml of absolute methanol for 3 hours at room temperature. Relative fluorescence of the extracts was measured in a single emission spectrofluorometer (Photon Technologies International, Inc, South Brunswick, NJ) at an excitation wavelength of 550nm and emission wavelength of 580nm. Rhodamine-labelled phalloidin did not bind to bare coverslips, and negligible amounts remained bound to cells after extraction.

Fluorescence staining for F-actin

Hemocytes were collected, fixed and washed as described for the fluorescence F-actin assay. They were then permeabilized with -20°C acetone for 1 minute, and washed 3 times in PBS. Cells were stained in 20 mg/ml rhodamine-labelled phalloidin for 20 minutes, washed 3 times with PBS, and mounted in 75% glycerol/PBS (v/v). A Zeiss epifluorescence axiovert microscope equipped with neofluor lenses was used for microscopic examination of F-actin. Photomicrographs were taken with T-Max 400 film and printed on polycontrast RC paper under a number 3 filter.

DNase Inhibition Assay for G-Actin

Hemocyte G-actin concentrations were measured according to Ginis *et al.* (1992). Briefly, this assay is based on the ability of G-actin to inhibit DNase activity (Blikstad *et al.*, 1978). Hemocytes were adhered to glass coverslips for 10 minutes and fixed with an equal volume of 5% formalin (w/v). Nonadherent cells were washed off with 3 rinses of PBS and adherent cells were lysed as per Ginis *et al.* (1992) in a 0.2% Triton X-100 DNA solution, with 80 ug/ml lambda dsDNA used instead of calf thymus DNA. Ginis *et al.* (1992) have shown that G-actin fixed in 3.7% fomalin (w/v) is as active as untreated actin in the DNase assay.

Samples were centrifuged at 200g for 5 minutes, and the supernatants (75-100 µl) mixed with 5-10 µl of a DNase solution (Ginis *et al.*, 1992). The rate of DNA hydrolysis was measured spectophotometrically by changes in optical density

at 260nm. Controls consisted of the lysis buffer substituted for cell extracts. The amount of G-actin in the sample was calculated according to Ginis *et al.* (1992), using the Michaelis-Menten equations for non-competitive inhibitors. Control and experimental groups were normalized for total cellular protein with the BioRad protein assay kit.

Results

Plasmatocyte Motility In Vitro

Octopamine (5×10^{-6}M) induced a two-fold increase in plasmatocyte locomotory rate within the first 15 minutes post-treatment (Fig. 2.1). This was statistically significant ($p<0.05$; N=30 cells/interval) and was followed by a slight but not significant increase in velocity over the control between 15 and 30 minutes post-treatment. The decrease in velocity during 30-45 minutes post-treatment was also not significantly different from the control. Concomitant treatment with octopamine and 1×10^{-3}M phentolamine, a specific antagonist of octopamine, effectively blocked an initial increase in locomotory rate, and resulted thereafter in rates comparable to the control. Phentolamine alone (1×10^{-3} M) did not affect locomotory rates (data not shown). In contrast, neither 1×10^{-6}M nor 1×10^{-8}M 5-HT stimulated plasmatocyte motility, resulting instead in locomotory rates comparable to the control (Fig. 2.2).

To determine the effective concentration range for octopamine, cell preparations were treated with 5×10^{-10}M, 5×10^{-8}M, and 5×10^{-4}M octopamine. Both 5×10^{-8}M and 5×10^{-6}M octopamine concentrations enhanced locomotory rate above that of the control ($p<0.05$). The maximal effect occurred at 5×10^{-6}M octopamine. The lowest (5×10^{-10}M) and highest (5×10^{-4}M) octopamine concentrations did not increase locomotory rate (Fig. 2.3). These data were comparable to the effective range of octopamine concentrations which were

Fig. 2.1: Effect of octopamine (5×10^{-6}M) and phentolamine (1×10^{-3}M) on hemocyte motility. Octopamine significantly increases the distance travelled within the first 15minutes *in vitro* compared to the control; this effect is abolished with β-adrenergic antagonist phentolamine (mean ± S.E.).

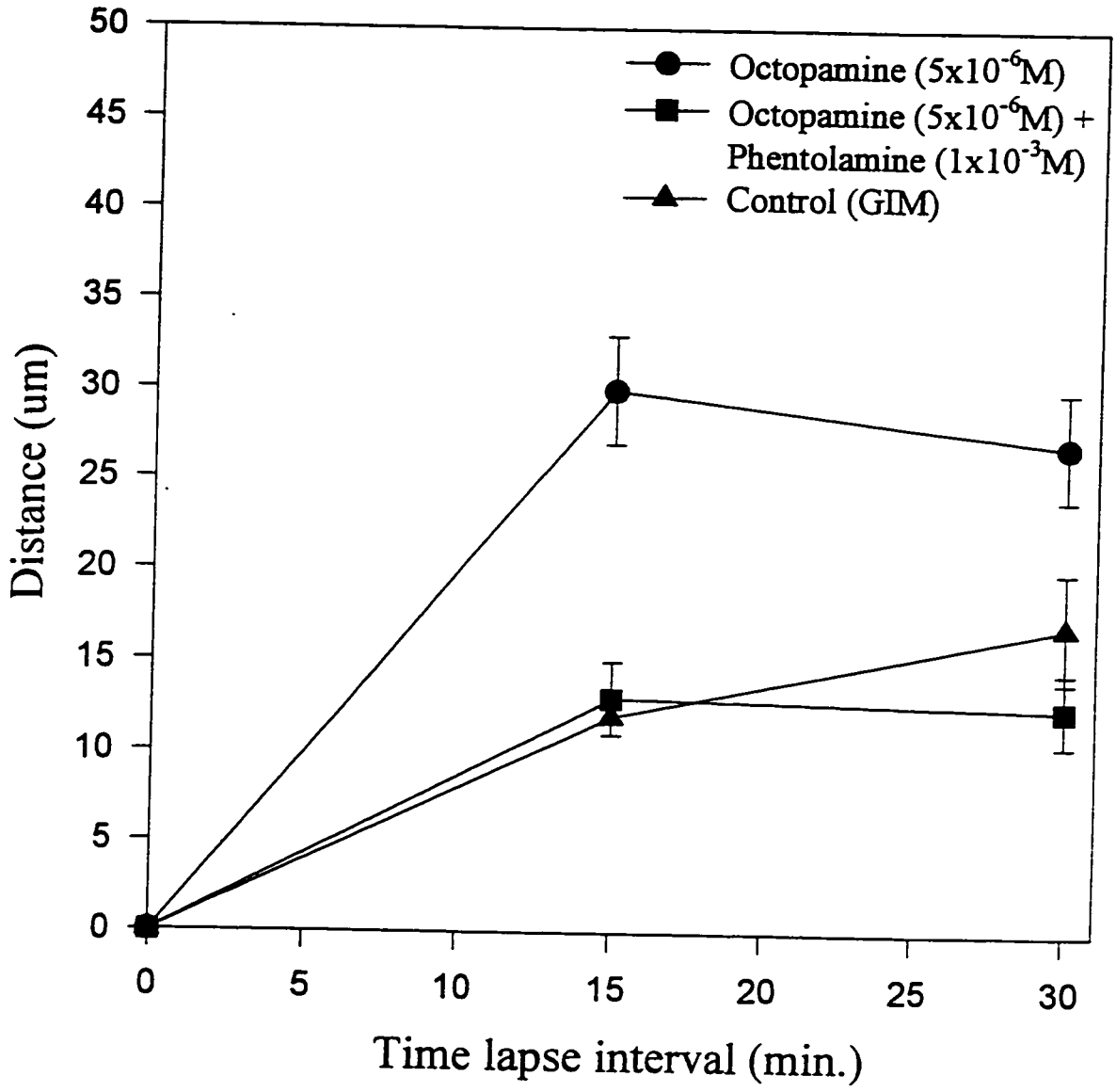

Octopamine (5x10-6M)
Octopamine (5x10-6M) + Phentolamine (1x10-3M)
Control (GIM)
Distance (um)
Time lapse interval (min.)
0
5
10
15
20
25
30
35
40
45
50

Fig. 2.2: Effect of 5-hydroxytryptamine (1×10^{-8} and 1×10^{-6}M) on hemocyte motility. 5-Hydroxytryptamine had no effect on the hemocyte motility *in vivo* (mean ± S.E.).

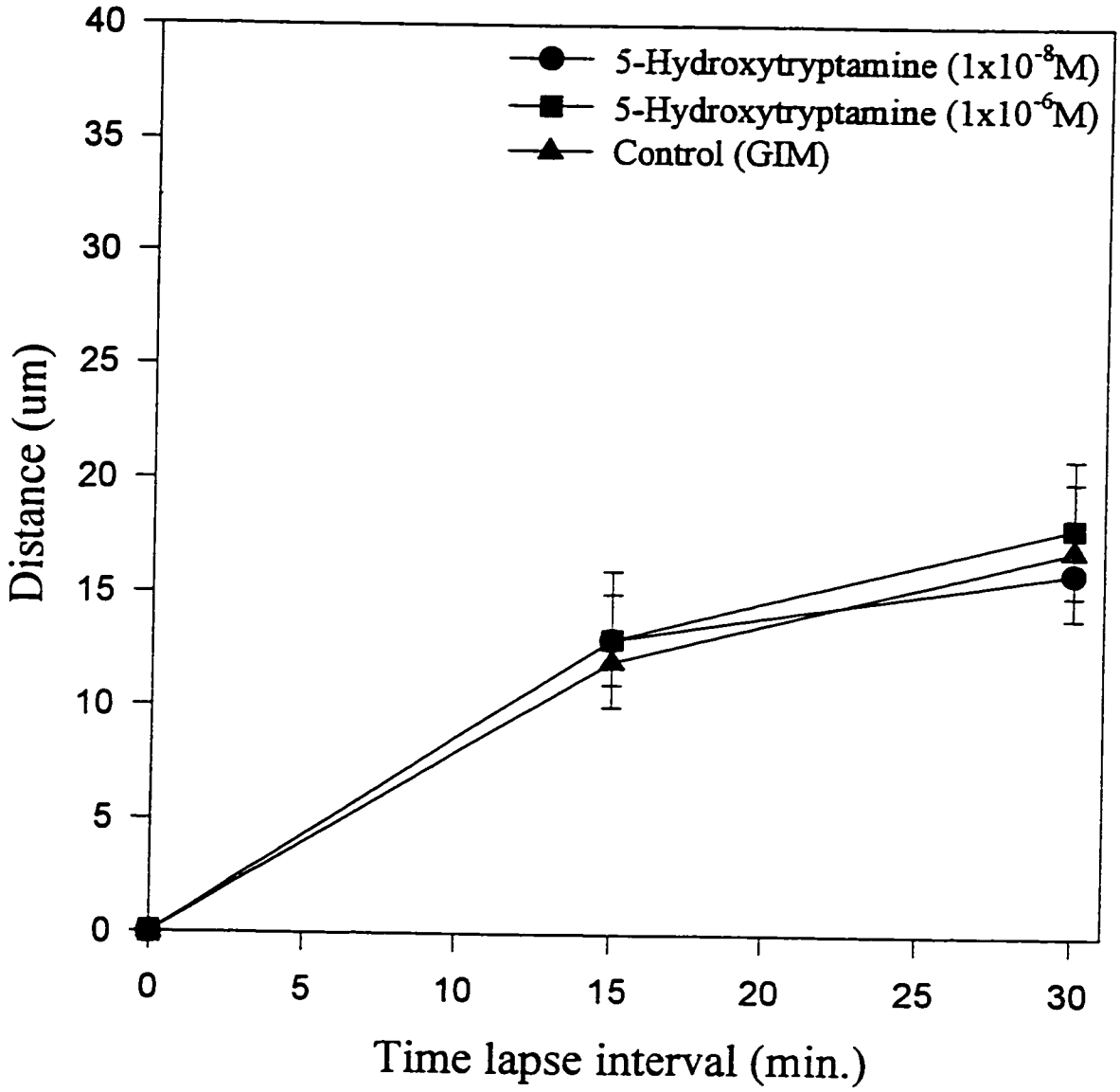
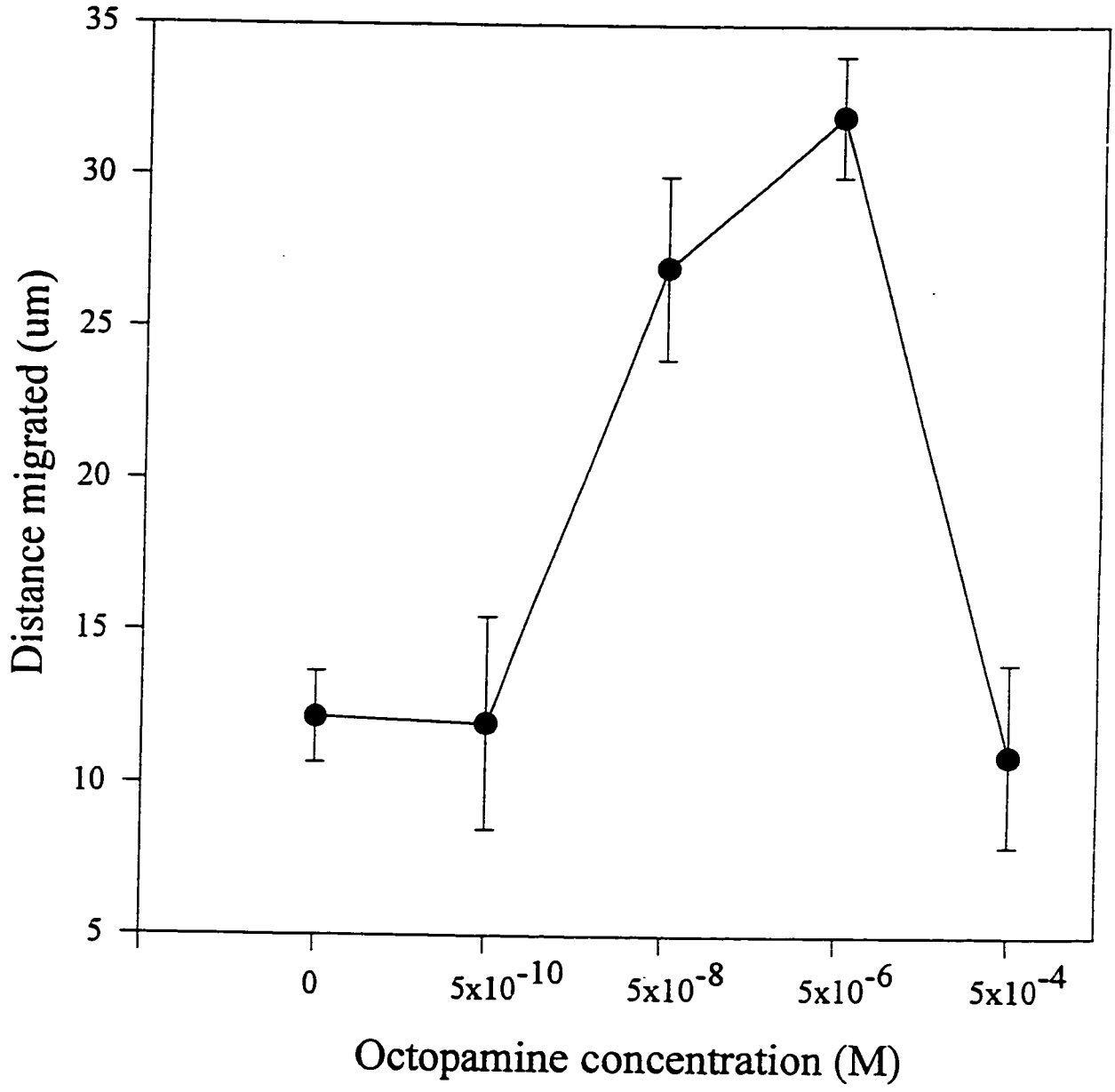

5-Hydroxytryptamine (1x10-8M)
5-Hydroxytryptamine (1x10-6M)
Control (GIM)
Distance (um)
Time lapse interval (min.)
0
5
10
15
20
25
30
35
40

Fig. 2.3: Threshold of hemocyte response to octopamine. Measurement of locomotory rates during the first 15min *in vitro* reveal octopamine is effective at 5×10^{-8} and at 5×10^{-6}M (mean ± S.E.).


35
30
25
20
15
10
5
Distance migrated (um)
0
5x10-10
5x10-8
5x10-6
5x10-4
Octopamine concentration (M)

previously found to enhance phagocytosis by cockroach hemocytes (Baines *et al.*, 1992).

Morphology of Hemocytes In Vitro

A typical video time-lapse sequence of stimulated and non-stimulated hemocytes is presented in Figure 2.4. Within 10 minutes post treatment with 5×10^{-6} M octopamine, broad lamellar extensions are obvious on several plasmatocytes (Fig. 2.4a; time in culture = 0 minutes). Filopodial processes are not yet evident on granular cells. However, after 5 minutes in culture, filopodia are evident in non-motile granular cells, and most plasmatocytes elongate and begin to assume unipolar shapes characteristic of exomigrating hemocytes (Fig. 2.4b). In the video frame taken 5 minutes later, plasmatocytes are clearly dispersing from the original clump (Fig. 2.4c), and after 15 minutes in culture, the original cluster of hemocytes has almost completely dispersed, and granular cells assume a flattened morphology (Fig. 2.4d).

In comparison with the previous sequence, unstimulated hemocytes clearly do not disperse as quickly, and plasmatocytes differentiate more slowly (Fig. 2.4e-h). At time = 5 minutes (Fig. 2.4f), lamellar processes are discernible, but dispersion of hemocytes from cell clumps is limited. After 10 minutes (Fig. 2.4g), the first discernible exomigration is evident, whereas at the comparable time interval in the octopamine-treated cells (Fig. 2.4c) the original clump has largely dispersed. By 15 min (Fig. 2.4h), hemocytes in the control are not completely dispersed, although more plasmatocytes assume polarized shapes and granulocytes remain rounded.

Fig. 2.4a-h: Video time lapse sequence showing the behaviour of hemocytes exposed to 5 x 10^{-6} M octopamine (a-d) and the control (e-h). Frames (a) and (e) were taken at time = 0 minutes; successive frames are at 5minute intervals. Plasmatocytes spread and exomigrate earlier in the octopamine treatment. Pictures were taken directly from a sVGA video monitor with a 58mm lens using Pan F film using a 1/4sec exposure. Arrow in frame (a) indicates a plasmatocyte extending a lamellar process. Mag = 80X.

Differences in granulocyte spreading were not quantified due to difficulties in accurately distinguishing between the many intermediate stages of spreading and flattening.

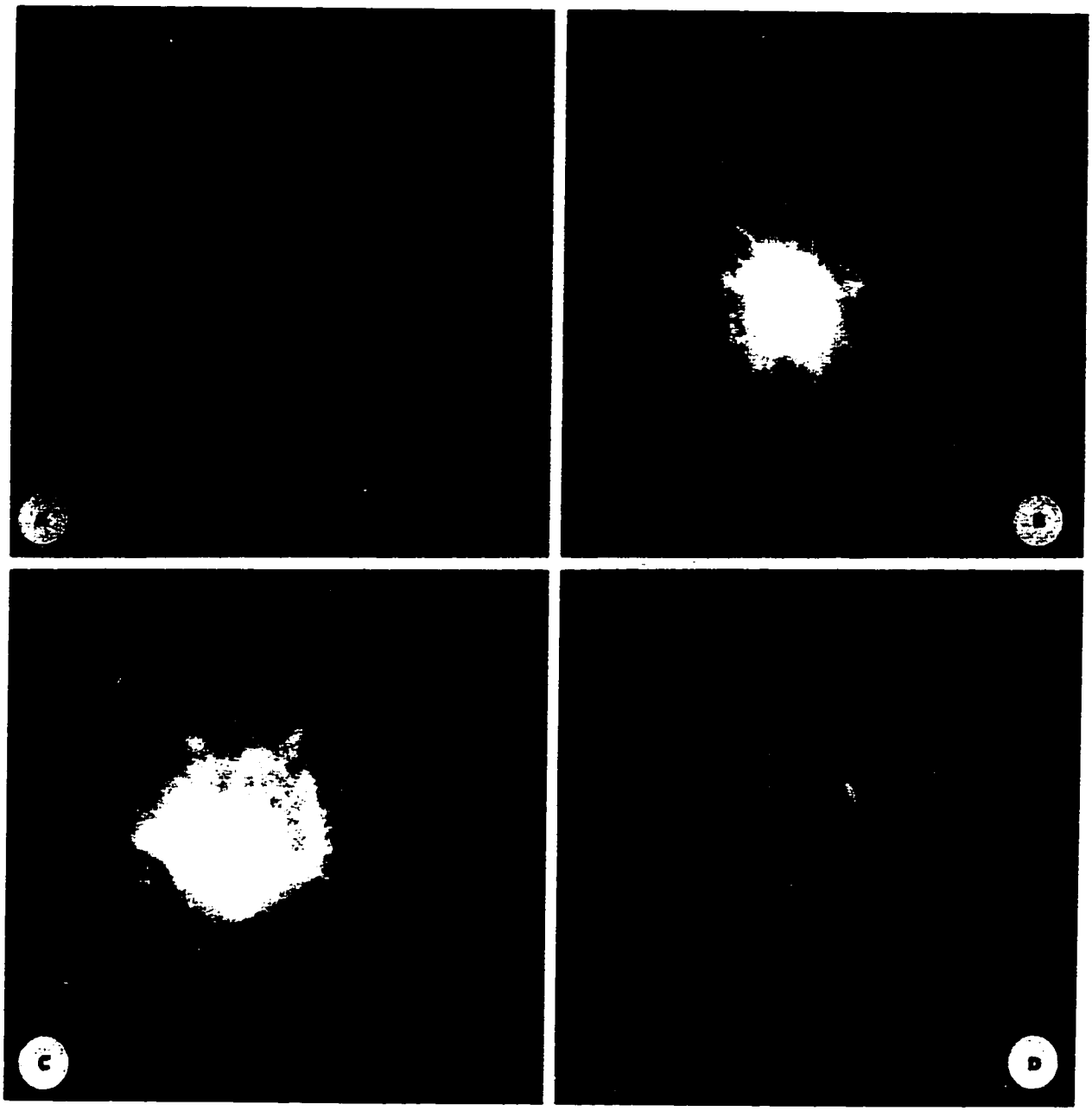
F-actin Staining Pattern

Notable differences in F-actin staining pattern were revealed in comparisons of granular cells. By 10 minutes after plating onto glass, there were striking differences in the F-actin cytoskeleton between control and octopamine-stimulated granulocytes. In Figure 2.5a, an unstimulated granular cell (control) is shown with numerous actin-rich filopodia. However, in Figure 2.5b, an octopamine-stimulated granular cell exhibits thick, F-actin-filled filopodia which end in bulbous tips. Control granulocytes eventually also attain this morphology, but only after 20 minutes elapsed time in culture. Differences between plasmatocytes in control and treatment groups were less obvious, although actin staining revealed considerably greater proportions of plasmatocytes in intermediate lamellar (Fig. 2.5c) and unipolar forms (Fig. 2.5d) after octopamine treatment. Treatment of hemocytes with octopamine and phentolamine resulted in granulocytes with similar staining patterns to the control (data not shown) and comparable numbers of lamellar and polarized plasmatocytes.

Quantification of F-actin and G-actin

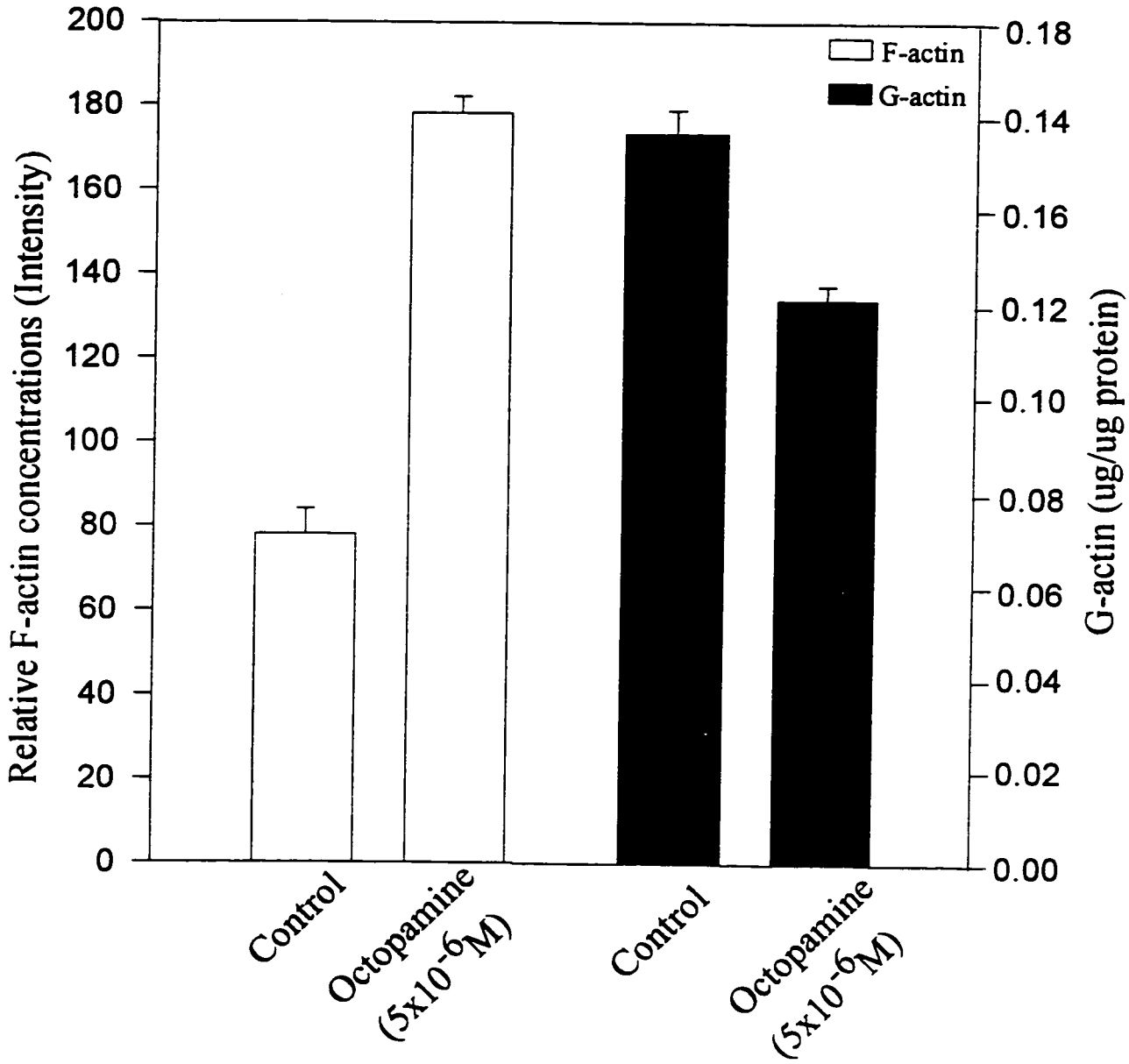
Two different and complementary assays were used to characterize the actin state in heterogeneous (containing granulocytes and plasmatocytes) populations of

Fig. 2.5a-d: F-actin staining pattern of control (a) and octopamine-stimulated hemocytes (b-d) after 10minutes *in vitro*. Mag = 550X.


C
D

hemocytes. The change in relative F-actin concentration was striking in octopamine-treated hemocytes. As shown in Figure 2.6, octopamine stimulated a significant increase ($p<0.05$; $N = 6$ replicates per trial) in F-actin after 15 minutes exposure. This was confirmed by the G-actin assay, which exhibited a smaller but nonetheless significant decrease in G-actin in octopamine-treated (Fig. 2.6).

Figure 2.6: Relative F-actin and G-actin in control and octopamine-stimulated hemocytes ($p<0.05$; N = 6 replicates).


Relative F-actin concentrations (Intensity)
G-actin (ug/ug protein)
F-actin
G-actin
200
180
160
140
120
100
80
60
40
20
0
0.18
0.14
0.16
0.12
0.10
0.08
0.06
0.04
0.02
0.00
Control
Octopamine (5x10-6M)
Control
Octopamine (5x10-6M)

Discussion

The present study demonstrates that octopamine modulates protrusive and locomotory activity of insect hemocytes *in vitro*. Granular cells, which are not mobile *in vitro*, responded to octopamine exposure by producing longer, bulbous-tipped filopodia which had F-actin cores. A practical consequence of octopamine-induced protrusive activity is an increased likelihood of encountering particles *in vivo*, thereby increasing the rate of phagocytosis. Additionally, it has been shown that actin polymerization is necessary for the propulsion of bacteria through the cytoplasm of phagocytic cells (Sanger *et al.*, 1992; Dabiri *et al.*, 1990). Octopamine-induced changes in actin may thus augment phagocytosis via different mechanisms: one involves increased surface area for contacting particles, the other would permit internalization of particles away from the initial site of contact.

Plasmatocytes are phagocytic and are also involved in forming the outer layer around nodules and capsules surrounding metazoan parasites (Ratcliffe and Rowley, 1979). Octopamine-induced increases in plasmatocyte motility may aid these reactions by promoting the spreading and flattening of plasmatocytes around nodules. However, it is not likely that octopamine functions to favour chemotaxis. Amines present in the hemocoel are rapidly inactivated by N-acetyltransferases (Downer and Martin, 1987). This likely explains the observation of Stephano *et al.* (1989) that octopamine does not increase chemotaxis of *Mytilus* hemocytes toward injury sites *in vivo*. Baines *et al.* (1992) predicted that octopamine should only transiently affect hemocytes. This is consistent with our data showing that increases

in locomotory rate occur only within 15 minutes post-exposure to octopamine. While our study was done *in vitro*, it seems plausible that sufficient N-acetyltransferases were still present in the culture medium to acetylate and inactivate octopamine. Therefore, the enhancement of locomotory rates *in vivo* is likely a transient phenomenon which is primarily functional immediately at an injury site or developing nodule.

While the precise mechanisms whereby protrusive activity and locomotion are regulated by octopamine are unknown, there is ample evidence indicating that both Ca^{+2} and phosphoinositides control actin assembly/disassembly in other systems. Mammalian neutrophil motility is regulated by transient increases in intracellular Ca^{+2} (Hendey and Maxfield, 1993; Marks and Maxfield, 1990). The underlying mechanism probably involves Ca^{+2}-activation of the Ca^{+2}/calmodulin-dependent phosphatase, similar to calcineurin, which would initiate substrate-detachment (Hendey and Maxfield, 1993), as well as the severing of subcortical F-actin filaments by free Ca^{+2} (Jamney, 1994). Phosphoinositide signals have roughly the opposite effects to intracellular Ca^{+2} and both signals are likely integrated in inducing cells to spread or crawl (Stossel, 1989). It is likely that octopamine also transduces changes in morphology and behaviour through the actin-based cytoskeleton, in that it stimulates increases in intracellular free Ca^{+2} in hemocytes *in vitro* (Jahagardar *et al.*, 1987) and elevates levels of inositoltrisphosphate (Baines *et al.*,, 1992). Figure 2.7 illustrates the proposed interaction of the signalling

molecules in hemocytes which contribute to the increased motility stimulated by octopamine.

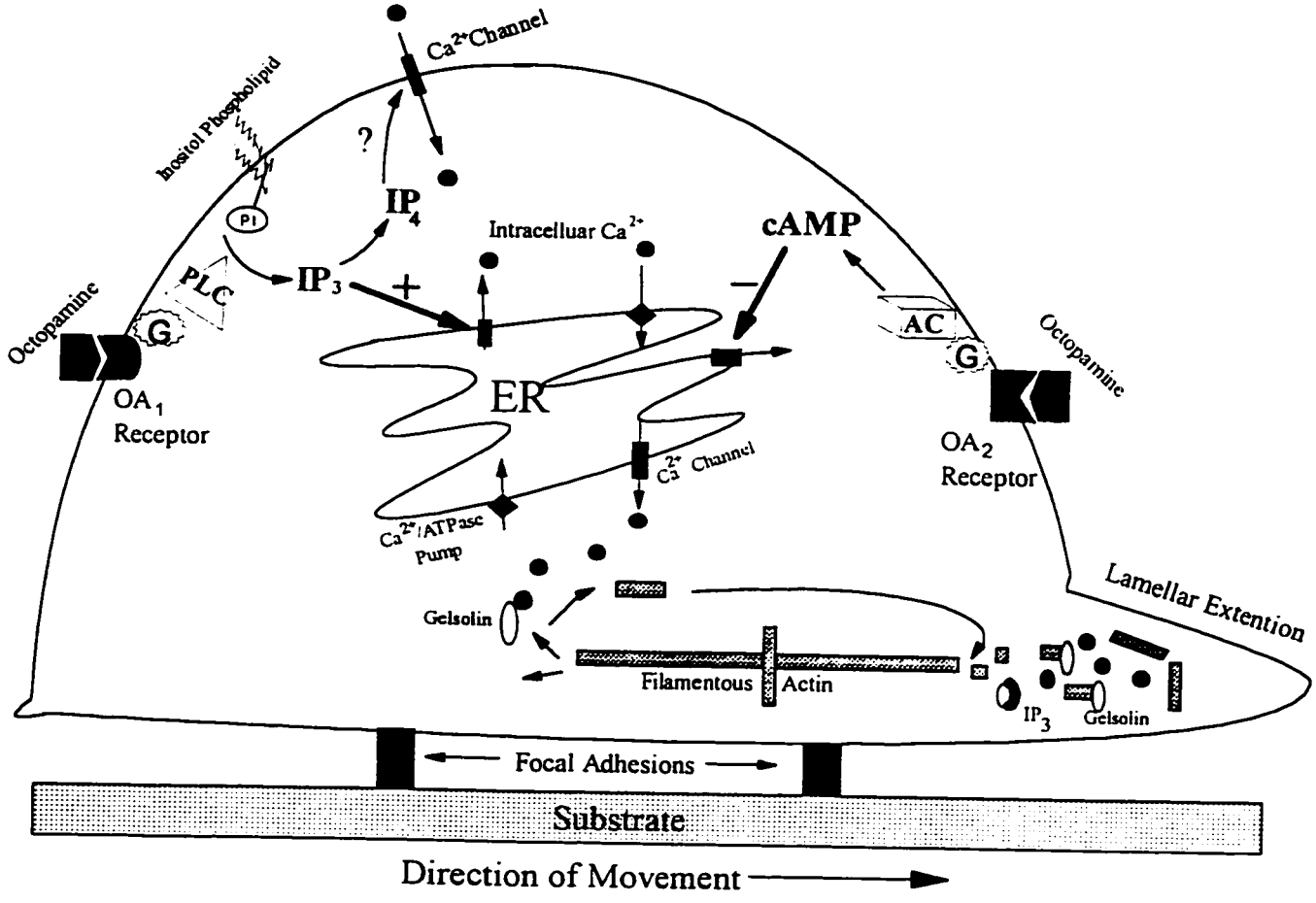
Upon ligand binding to the octopamine$_1$ receptor there is a marked increase of calcium via IP_3 production (Evans, 1981). It is this increase in calcium which I propose is modulating the increase in protrusive activity in hemocytes. This regulation is not based solely on stimulation of the octopamine$_1$ receptor, but rather occurs in concert with the octopamine$_2$ receptor (Fig. 2.7).

Octopamine$_2$ receptors have been linked to adenylate cyclase systems in the peripheral tissue of many insects (Orchard and Lange, 1986). Furthermore, an octopamine-sensitive adenylate cyclase has been reported in hemocytes of the American cockroach (Orr *et al.*, 1985) and the forest tent caterpillar (Gole *et al.*, 1982). In macrophages, increased levels of intracellular cAMP inhibit many defensive functions such as chemotaxis, phagocytosis, release of lysosomal enzyme and superoxide production (Hamachi *et al.*, 1984). The increase in cAMP inhibits Ca^{2+} efflux in stimulated macrophages.

In the model (Fig 2.7), I propose that both octopamine receptors are involved in the regulation of intracellular calcium levels within hemocytes, which ultimately mediates events in cytoskeletal motility. In essence, octopamine$_1$ causes an increase in Ca^{2+} via the IP_3 pathway and octopamine$_2$ causes a decrease in the Ca^{2+} via cAMP. By virtue of their opposing effect on Ca^{2+}, these two receptors could be responsible for the initiation and propagation of transient increases in intracellular

Fig. 2.7: Model of putative octopamine-stimulated signalling pathways in insect hemocytes.

AC Adenylate Cyclase
G G-Protein
PLC Phospholipase C
OA Octopamine
IP_3 Inositol trisphosphate
IP_4 Inositol tetrakisphosphate
PI Phosphatidylinositol



Ca^{2+} which correlate with spreading, psudopodial extension and motility observed in the present study.

I favour a two-step model for the response of hemocytes to non-self. The first phase involves activation of hemocytes. Mammalian neutrophils are normally in a resting state until they are activated by a variety of factors, including amines (Mueller and Sklar, 1989). The initial stimulus need not be octopamine, and could involve some endogenous protein or peptide-like the cell adhesion factor similar to those found in crayfish hemocytes (Johansson and Soderhall, 1989) or platelet activating factor found in mammalian systems (Bengtsson and Grenegard, 1994). The second phase involves changes in hemocyte behaviour and motility, and would be mediated by biogenic amines such as octopamine. The latter phase would be dependent on dynamic changes in the cytoskeleton, including the reversible assembly of actin filaments. Elevation of octopamine by wounding or stress could serve to mobilize hemocytes to respond rapidly to foreign pathogens (Downer and Martin, 1987). Future research needs to be directed at clarifying this role to determine the timing and specificity of the pathways involved. This approach would develop a clearer picture of the insect cellular defense system.

References

Anggraeni, T. and N. A. Ratcliffe. 1991. Studies on cell-cell cooperation during phagocytosis by purified hemocyte populations of the wax moth, *Galleria mellonella*. J. Insect Physiol. 37:453-460.

Baines, D. and R. G. H. Downer. 1992. 5-Hydroxytryptamine-sensitive adenylate cyclase affects phagocytosis in cockroach hemocytes. Arch. Insect. Biochem. Physiol. 21:303-316.

Baines, D., T. DeSantis and R. G. H. Downer. 1992. Octopamine and 5-hydroxytryptamine enhance the phagocytic and nodule formation activities of cockroach (*Periplaneta americana*) haemocytes. J. Insect Physiol. 11:905-914.

Batelle, B. A. and E. A. Kravitz. 1978. Targets of octopamine actin in the lobster: cyclic nucleotide changes and physiological effects in hemolymph, heart and exoskeletal muscle. J. Pharmac Exp.Therap. 205:438-448.

Bengtsson, T. and M. Grenegard. 1994. Platelets amplify chemotactic peptide-induced changes in F-actin and calcium in human neutrophils. Eur. J. Cell Biol. 63:345-349.

Blikstad, I., F.Markey, L. Carlsson, T. Persson and U. Lindberg. 1978. Selective assay of monomeric and filamentous actin in cell extras, using inhibiton of deoxyribonucease I. Cell. 15:935-943.

Dabiri, G., J. Sanger, D. Portnoy and F. Southwick. 1990. *Listeria* monocytogenes moves rapidly through the host cell cytoplasm by inducing directional actin assembly. Proc. Natl. Acad. Sci. 87:6068-6072.

Downer, R. G. H. and R. J. Martin. 1987. N-acetylation of octopamine a potential target for insecticide development. In: *The Search for Novel Insecticides: Toxicants Affecting GABA, Oct, and Other Neuroreceptors in invertebrates*. (M. Green, R. M. Hollingworth and P. A. Hedlin, eds.) 202-210. ACS Symp. ACS Press, New York, NY.

Dunphy, G. B. and R. G. H. Downer. 1994. Octopamine, a modulator of the haemocytic nodulation response of non-immune *Galleria mellonella* larvae. J. Insect Physiol. 40:267-272.

Dunphy, G. B. and J. M. Webster. 1991. Antihemocytic surface components of *Xenorabdus nemtophilus* var. dutki and their modification by serum of nonimmune larvae of *Galleria mellonella*. J. Invert. Pathol. 58:40-51.

Evans, P.D. 1981. Multiple receptor types for octopamine in the locust. J. Physiol. 318: 99-122.

Ginis, I., K. Zaner, J. Wang, N. Pavlotsky and A.I. Tauber. 1992. Comparison of actin changes and calcium metabolism in plastic- and fibronectin-adherent human neutrophils. J. Immunology 149:1388-1394.

Gole, J. W. D., R. G. H. Downer and S. S. Sosi. 1982. Octopamine-sensitive andenylate cyclase in haemocytes of the forest tent caterpillar, Malacosma disstria Hubner (Lepeidoptera: Lasiocampidae). Can. J. Zool. 60: 825-829.

Hamachi, T., M. Hirata and T. Koga. 1984. Effect of cAMP-elevating drugs on Ca^{2+} efflux and actin polymerization in peritoneal macrophages stimulated with N-formyl chemotactic peptide. Biochim. Biophys. Acta. 804:230-236.

Hendy, B. and F. R. Maxfield. 1993. Regulation of neutrophil motility and adhesion by intracellular calcium transients. Blood Cells. 19:143-164.

Howard, T. H. and C. O. Oresajo. 1985. The kinetics of chemotactic peptide-induced change in F-actin content, F-actin distribution, and the shape of neutrophils. J. Cell Biol. 101:1078-1085.

Jahagirdar, A. P., G. Milton, T. Viswanatha and R. G. H. Downer. 1987. Calcium involvement in mediating the action of octopamine and hypertrehalosemic peptides on insect hemocytes. FEBS. 219:83-87.

Jamney, P. A. 1994. Phosphoinositides and calcium as regulators of cellular actin assembly and disassembly. Annu. Rev. Physiol. 56:169-191.

Johansson, M.W. and K. Soderhall. 1989. A cell adhesion factor from crayfish haemocytes has degranulating activity towards crayfish granular cells. Insect Biochem. 19:183-190.

Kanost, M. R., M. K. Zepp, N. E. Ladendorff and L. A. Andersson. 1994. Isolation and characterization of a hemocyte inhibitor from the hemolymph of *Manduca sexta* larvae. Arch. Insect Biochem. Physiol. 27:123-136.

Lackie, A. M. 1988. Hemocyte behaviour. Adv Insect Physiol. 21:85-178.

Ladendorff, N. E. and Kanost, M. R. 1991. Bacteria-induced protein P4 (hemolin) from *Manduca sexta*: a member of the immunoglobulin superfamily which can inhibit hemocyte aggregation. Arch. Insect Biochem. Physiol. 18:285-300.

Marks, P. W. and F. R. Maxfield. 1990. Transient increases in cytosolic calcium appear to be required for the migration of adherent human neutrophils. J. Cell Biol. 110:43-52.

Mueller, H. and L.A. Sklar. 1989. Coupling of antagonistic signalling pathways in modulation of neutrophil function. J. Cell. Biochem. 40:287-294.

Orchard, I and A. B. Lange. 1986. Pharmacological profile of octopamine receptors on the lateral oviducts of the locust, *Locusta migratoria*. J. Insect Physiol. 32:741-745.

Orr, G. L., J. W. D. Gole and R. G. H. Downer. 1985. Characterization of an octopamine-sensitive adenylate cyclase in the membrane fragments of the American cockroach *Periplaneta americana* L. Insect Biochem. 15:695-701.

Pech, L. L., D. Trudeau and M. R. Strand. 1994. Separation and behaviour *in vitro* of hemocytes from the moth, *Pseudoplusia includens*. Cell Tissue Res. 277:159-167.

Ratcliffe, N. A. 1986. Insect cellular immunity and the recognition of foreignness. Symp. Zool. Soc. Lond. 56:21-43.

Ratcliffe N. A. and A. F. Rowley. 1979. Role of hemocytes in defence against biological agents. In: *Insect Hemocytes Development, Forms, Functions and Techniques* (A. P. Gupta, ed.) 331-414. Cambridge University Press, Cambridge, U.K.

Sanger, J., J. Sanger and F. Southwick. 1992. Host cell actin assembly is necessary and likely to provide the propulsive force for intracellular movement of *Listeria monocytogenes*. Infect. Immunol. 60:3609-3619.

Stephano, G. G., M. K. Leung, X. Zhao and B. Scharrer. 1989. Evidence for the involvement of opiod neuropeptides in the adherence and migration of immunocompetent invertebrate hemocytes. Proc. Natl. Acad. Sci. 86:626-630.

Stephano, G. B., M. A. Shipp and B. Scharrer. 1991. A possible immunoregulatory function for [met]-enkephalin-arg^6-phe^7 involving human and invertebrate granulocytes. J. Neuroimmun. 31:97-103.

Stossel, T. 1989. From signal to pseudopod. J. Biol. Chem. 264:18261-19264.

Chapter 3

The Effects of Eicosanoid Biosynthesis Inhibitors on Hemocytic Immune Functions

as published in Journal of Insect Physiology 42: 1-8

Abstract

The invertebrate immune system produces melanotic nodules in response to bacterial infections, which has previously been shown to be mediated by eicosanoids. Nodulation occurs in two phases: the first involves hemocyte degranulation and activation of the prophenoloxidase cascade; the second involves formation of a cellular capsule by chemotaxis and spreading of hemocytes. In this chapter it will be demonstrate that eicosanoids modulate both phases of the nodulation in insects. The phospholipase A_2 inhibitor, dexamethasone, as well as the cyclooxygenase inhibitor, indomethacin, were shown to significantly inhibit phagocytosis *in vitro* and prophenoloxidase activation *in vivo*. The inhibitory effects of dexamethasone were abolished by the addition of exogenous arachidonic acid. Furthermore, the arachidonic acid analogue 5,8,11,14 - eicosatetraynoic acid and indomethacin inhibited hemocyte spreading *in vitro*. The findings suggest that eicosanoid derivatives mediate both phases of the nodulation response and are consistent with previous studies which attribute roles for prostaglandins in other species as modulators of cellular behaviour.

Introduction

The hypothesis suggesting eicosanoids may mediate the insect cellular immunity was first proposed by Stanley-Samuelson *et al.* (1991). Perturbation of the major eicosanoid biosynthetic pathways with selective pharmacological inhibitors was used to test this hypothesis. Eicosanoids are implicated as regulators of bacterial clearance from the insect hemocoel (Stanley-Samuelson *et al.*, 1991).

The word "eicosanoid" was first introduced by Corey *et al.* (1980) and refers to all biological active oxygenated metabolites of certain polyunsaturated fatty acids (PUFA). Eicosanoid biosynthesis is a result of cellular phospholipid membrane alteration, resulting in the release of unesterified precursors (Kuehl and Egan, 1980). Arachidonate and other polyunsaturates are hydrolyzed by the action of the enzyme phospholipase A_2 (PLA_2). In this rate-limiting step of eicosanoid biosynthesis, PLA_2 removes PUFAs from the sn-2 position of the phospholipid membrane (Stanley-Samuelson, 1994). Arachidonate and other polyunsaturates that are released from the lipid membrane are substrates for the three major eicosanoid biosynthesis pathways: the cyclooxygenase pathway, which results in the formation of prostaglandins (PGs) and thomboxanes (TXs); the lipoxygenase pathway, which produces hydroperoxy- and hydroxypolyenoic fatty acids (HPETEs and HETEs), which are further metabolized to leukotrienes (LTs.); and the cytochrome P-450 epoxygenase pathway, which produces epoxyeicosatrienoic acids (EETs) (Yamamoto, 1988).

Pharmacological inhibition of arachidonic acid biosynthesis by PLA_2 antagonists decreases both bacterial clearance and nodule formation, and these effects can be rescued by the addition of arachidonic acid (Stanley-Samuelson *et al.*, 1991; Miller *et al.*, 1994). Inhibition of the formation of down-stream derivatives of arachidonic acid, namely the products of the cyclooxygenase and lipoxygenase pathways, results in inhibition of bacterial clearance and nodule formation. This supports the idea that cyclooxygenase and lipoxygenase products are involved in nodule formation.

Nodule formation is an event which occurs much earlier than the induction of any of the antibacterial factors, indicating that it is one of the most important primary lines of defense against bacterial invasion. Nodulation has been described in *Galleria mellonella* as a biphasic response (Ratcliffe and Rowley, 1979). The first phase is initiated by contact between granular hemocytes and bacteria. This results in degranulation and release of a "sticky coagulum" within 1 minute post-exposure to bacteria (Ratcliffe and Rowley, 1979; Ratcliffe and Gagan, 1976). Concurrently or shortly after degranulation of granular hemocytes, the prophenoloxidase cascade is activated, which ultimately leads to the formation of melanin deposits around bacteria within 5 - 30 minutes post-exposure (Söderhäll and Smith, 1986). Within this early phase, granulocytes and plasmatocytes begin to aggregate. The second phase of nodulation is marked by the beginning of phagocytic activity by plasmatocytes and attachment and spreading of these cells to form an outer sheath encompassing the developing nodule (Ratcliffe and Rowley, 1979).

Miller *et al.* (1994) determined that the PLA_2 inhibitor dexamethasone significantly reduced the aggregation of hemocytes in the tobacco hornworm *Manduca sexta*. However, one possibility as yet unexplored is that eicosanoids may also influence the melanization response. This hypothesis, coupled with the findings that arachidonic acid metabolites influence a variety of cellular activities in mammalian cells, including cell adhesion, spreading and phagocytosis (Chun and Jacobson, 1992; Levine, 1988; Oropeza-Rendon *et al.*, 1980) suggests a possible role of eicosanoids in distinct events during nodule formation. Since nodulation has been thoroughly described in the wax moth, this animal was chosen to investigate the role of eicosanoids during three discrete processes: prophenoloxidase activation, cell spreading, and phagocytosis. Herein I provide evidence that arachidonic acid derivatives are required for all three activities. The results further support the hypothesis that eicosanoids modulate immunocyte activity thereby specifically regulating nodule formation, an important insect immune response.

Materials and Methods

Eicosanoid Biosynthesis Inhibitors

To examine the effects of eicosanoids on nodule formation, a variety of eicosanoid biosynthesis inhibitors were used to interfere with different branches of the eicosanoid pathway (Fig. 3.1). The inhibitors used in these experiments are summarized in Table 3.1.

With the exception of quinacrine which was dissolved in water, all inhibitors and arachidonic acid were prepared in absolute ethanol and frozen in aliquots at -20°C. After each aliquot was thawed, unused portions were discarded. Inhibitors of arachidonic acid hydrolysis include dexamethasone ((11,B,16)-9-fluoro-11,17,21-trihydroxy-16-methylpregna-1,4-dione)), ETYA (5,8,11,14-eicosatetraynoic acid), quinacrine (6-chloro-9-(14-diethylamino)-1-methylbutyl)amino-2-methoxy-acridine), and BPB (4-bromophenacyl bromide). Lipoxygenase inhibitors included esculetin (6,7-dihydroxycoumarin) and NDGA (nordihydroguaiaretic acid). Indomethacin (1-(p-chlorophenycyl)-5-methoxy-2-methylindole-3-acetic acid) was used to inhibit cyclooxygenase activity. The choice of working concentrations was based on a survey of the available literature. Injectant concentrations in the nodulation and the prophenoloxidase assays were either 10 mM, 1 mM or 0.1 mM . The range of inhibitor concentrations in the phagocytosis assay are as indicated in the figure legends.

Fig. 3.1: Eicosanoid biosynthesis pathway. The inhibitor used in these experiments and the steps at which they inhibit the pathway are depicted. This pathway was adapted from Stanley-Samuelson, 1994.

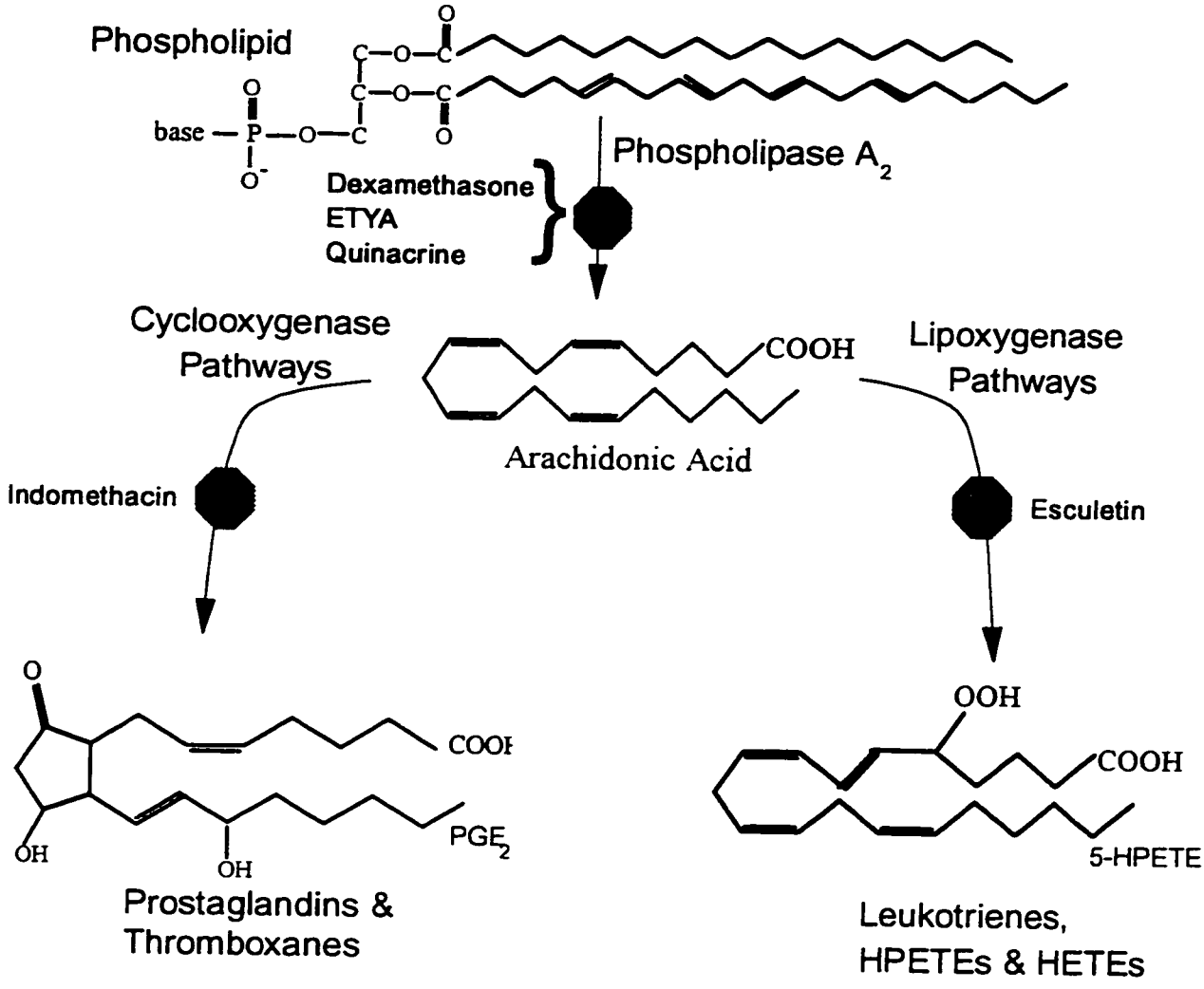

Phospholipid
base
Dexamethasone
ETYA
Quinacrine
Phospholipase A2
Cyclooxygenase Pathways
Lipoxygenase Pathways
COOH
Arachidonic Acid
Indomethacin
Esculetin
OH
OH
PGE2
OOH
5-HPETE
Prostaglandins & Thromboxanes
Leukotrienes, HPETEs & HETEs

Table 3.1: Eicosanoid Biosynthesis Inhibitors and their Modes of Action

Inhibitor	Enzyme	Mode of Action
Dexamethasone (glucocorticoid)	Phospholipase A_2	Upregulates the production of PLA2 inhibitor
Quinacrine (anti-malarial agent)	Phospholipase A_2	Complexes with phospholipids Prevents attack by phospholipase
4-bromophenacyl bromide (BPB)	Phospholipase A_2	Reacts with a histidine residue Binds to the active center of the enzyme preventing substrate binding
5,8,11,14-eicosatetraynoic acid (ETYA)	Phospholipase A_2	Arachidonic Acid analogue Inhibits the uptake of Arachidonic Acid
Indomethacin	Cyclooxygenase	Tightly binds to the active site of the enzyme
Ecsuletin	Lipoxygenase	Unknown, related to its anti-oxidant ability
Nordihydroguaiaretic acid (NDGA)	Lipoxygenase	Unknown, related to its anti-oxidant ability

Toxicity Assay

Trypan blue exclusion was used to assess any obvious toxic effects of either the inhibitors, 0.1% ethanol or arachidonic acid. Hemocytes were collected, washed, and plated at a density of approximately 1 x 10^6 cells/ml in 24-well polystyrene microwell plates and allowed to adhere and spread for 20 minutes. Inhibitors, ethanol or arachidonic acid at the concentrations used in the spreading assay were then introduced and cells were incubated for 60 minutes at room temperature. Cells were then quickly washed 3 times with PBS and incubated in Trypan blue (0.1% w/v in PBS) for 15 minutes. Cells were again quickly washed, fixed, then observed on the Leitz microscope under both brightfield and interference contrast optics. Positive controls consisted of cells incubated in 0.1 % ethanol, whereas cells in the negative controls were killed by exposure to 70% methanol for 15 minutes. Methanol-killed cells all stained with Trypan blue, while viable cells (including granulocytes and plasmatocytes) excluded the dye. One hundred cells were counted in each replicate (3 replicates per treatment), and the number of viable cells expressed by percent viability. The concentration of all inhibitors was 100 μM, with the exception of BPB (25 μM). These relatively extreme concentrations were used to ensure that the lower concentrations used in assays did not reduce hemocyte viability. Significant differences in this and all subsequent assays were determined by paired student's T-test.

Nodulation Assay

To determine if inhibitors of eicosanoid biosynthesis also inhibit nodulation in *G. mellonella*, 1.0 mm carboxylated latex microspheres (Polysciences, Warrington, PA.) were used as nodulation elicitors. Animals were injected with 5 µl of beads (0.001% latex solids in carrier) suspended in 100% ethanol with one of the following: 10 mM dexamethasone, 10 mM indomethacin, 10 mM esculetin, or 10 mM dexamethasone with 10 mM arachidonic acid. The control consisted of animals injected with 5 µl of the bead suspension in 100 % ethanol. Each replicate included fourteen larvae, which were allowed to incubate for one hour post-injection before being fixed by intrahemocoelic injection of approximately 200 µl fix (2% glutaraldehyde, 4% paraformaldehyde in 0.1 M sodium cacodylate). This ensured nodulation was stopped precisely after 1 hour. Larvae were then dissected and examined for the presence of melanotic nodules.

Prophenoloxidase Assay

The prophenoloxidase assay was modified from that of Brookman *et al.* (1989). Chilled larvae were injected with 5 µl of the inhibitors (10mM, 1mM or 0.1mM in ethanol). Controls consisted of larvae injected with 5 µl ethanol. After 30 minutes at room temperature, larvae were injected with 75 µl of chilled *Galleria* saline (GS:186 mM NaCl, 13 mM KCl, 17 mM EDTA, 10 mM HEPES, 1mM $NaHCO_3$; pH 6.8) and bled (see Chapter 2, General Materials and Methods). Hemolymph was collected directly into a chilled 1.5 ml Eppendorf tube.

Approximately 50 µl hemolymph was collected per animal and hemolymph from two animals was pooled in each replicate. Samples were sonicated for 30 seconds on a 50% duty cycle using a Bronson Cell Disrupter, and cellular debris was removed by centrifugation at 10,000 g for 4 minutes. Total serum protein was measured with the Biorad protein assay kit using bovine serum albumen as the standard. Fifty ml of serum was added to Nunc immunomodule microlitre wells containing 50 µl laminarin (1 mg/ml). Following a 30 minute incubation at room temperature, 200 µl of L-dihydrophenylalanine (3 mg/ml) were added and absorbance was measured on an ELISA plate reader (Bio-Tek Instruments model EL-301) at 490 nm. Specific activity was expressed as units per mg total protein per minute, then converted to percent inhibition of the control. The Dunnet test was used to determine statistical significance. Results are presented as percent inhibition compared to the control.

Phagocytosis Assay

Larval hemocytes were collected into 1.5 ml Eppendorf tubes from insects injected with 75 µl cold GS saturated with phenylthiourea (PTU), an inhibitor of the prophenoloxidase cascade. Hemolymph from five insects was pooled and hemocytes pelleted (see General Material and Methods). The pellet was resuspended in Grace's Insect Medium (GIM) + PTU containing either the appropriate inhibitor and 0.1% ethanol at the concentrations listed in Figure 3.5 or in 0.1% ethanol. Two hundred and fifty milliliters of the hemocyte suspension were placed in separate poly(HEMA) methacrylate coated wells in 24 well microwell plates, to which was added an

additional 245 µl GIM containing inhibitor at the working concentration. Following a 15 minute incubation, 5 µl of fluorescein isothiocyanate (FITC)-conjugated 1 mm diameter carboxylated latex beads were added to each well, the cells incubated for an additional 30 minutes with gentle agitation to ensure beads and cells remained in suspension. The assay was terminated by the addition of 1 ml 3.7% formalin in GS, and the fixed hemocytes pelleted at 500g for 4 minutes. Hemocytes were washed to remove adherent beads and the suspensions were diluted 1:1 with glycerol and plated on glass slides. Hemocytes were examined on a Zeiss photomicroscope equipped with phase contrast optics and a 40X phaco objective. An average of 100 cells were counted on each slide, and the phagocytic index scored as percentage of cells containing the phase-bright latex beads. Significance was determined by the Dunnet test.

Spreading Assay

To analyze the effects of inhibitors and arachidonic acid on hemocyte spreading, hemocytes were collected in cold GIM + PTU (see General Materials and Methods). Hemocytes were pelleted then resuspended in GIM + PTU with inhibitors at the concentrations indicated previously. Controls consisted of hemocytes incubated with 0.1% concentrations of carrier solvents, either ethanol or water. After incubation in 1.5 ml microcentrifuge tubes for 20 minutes at 4°C, the cells were plated on clean glass coverslips and incubated in moist chambers. The cells were fixed after 60 minutes with 3.7% formalin and washed 3 times with PBS.

Cells were then overstained in 0.1 % Janus Green B, washed 3 times in PBS then mounted in PBS:glycerol (3 replicates were used in each experiment, except for experiments with dexamethasone (n=7)). These cells were examined under brightfield optics on a Leitz inverted microscope equipped with a 20X objective and a Sony CCD video camera. An image analysis system was used to analyze the resultant video images. This consisted of a Matrox frame-grabbing board in a 486 computer driven by the Northern Exposure Image Analysis system (Empix Imaging Co., Mississauga, ON). The procedure for measuring hemocyte surface area consisted of first using spot densitometry to measure the highest discernible pixel grey level values (0-255) in the cortical cytoplasm of hemocytes in each field. The image was then digitally equalized from 0 to the highest pixel grey level value below the background, and a threshold applied to provide a binary image from which the area around cells could be subtracted. Approximately 100 non-confluent cells were examined per coverslip, and to eliminate subjective bias the observer was given samples which were coded so as not to reveal any particular treatment. Each separate experiment was done with a corresponding control, and the paired student's T-test was used to determine statistical significance.

Results

Toxicity Assays

The results of toxicity assays with various eicosanoid inhibitors and arachidonic acid are presented in Figure 3.2. Two of the inhibitors, NDGA and BPB, significantly reduced hemocyte viability compared to the control ($p<0.05$). Consequently, these inhibitors were omitted in following assays. ETYA also reduced hemocyte viability to a lesser, although significant extent ($p<0.05$). .Indomethacin, dexamethasone, esculetin and arachidonic acid did not significantly reduce hemocyte viability.

Effect of Eicosanoid Inhibitors on Nodulation

Dexamethasone, indomethacin and esculetin all significantly inhibited nodule formation compared to the control ($p<0.01$)(Fig. 3.3). Coinjection of arachidonic acid and dexamethasone rescued the effect of dexamethasone and significantly increased the number of nodules per insect over that of the control. Uninjected larvae typically had no nodules (data not shown).

Effect of Eicosanoid inhibitors on Prophenoloxidase Activation

Dexamethasone, indomethacin and ETYA all significantly inhibited prophenoloxidase activation ($p<0.05$) (Fig. 3.4). Treatment with indomethacin resulted in maximal inhibition (94.4 % +/- 2.5). The effect of dexamethasone was rescued by coinjection with arachidonic acid, resulting in an approximately 11%

Fig. 3.2: Toxicity of eicosanoid biosynthesis inhibitors to hemocytes. Indo = indomethacin; Dex = dexamethasone; AA = arachidonic acid; Esc = esculetin. NDGA and BPB significantly decreased hemocyte viability ($p<0.05$; $n = 3$).

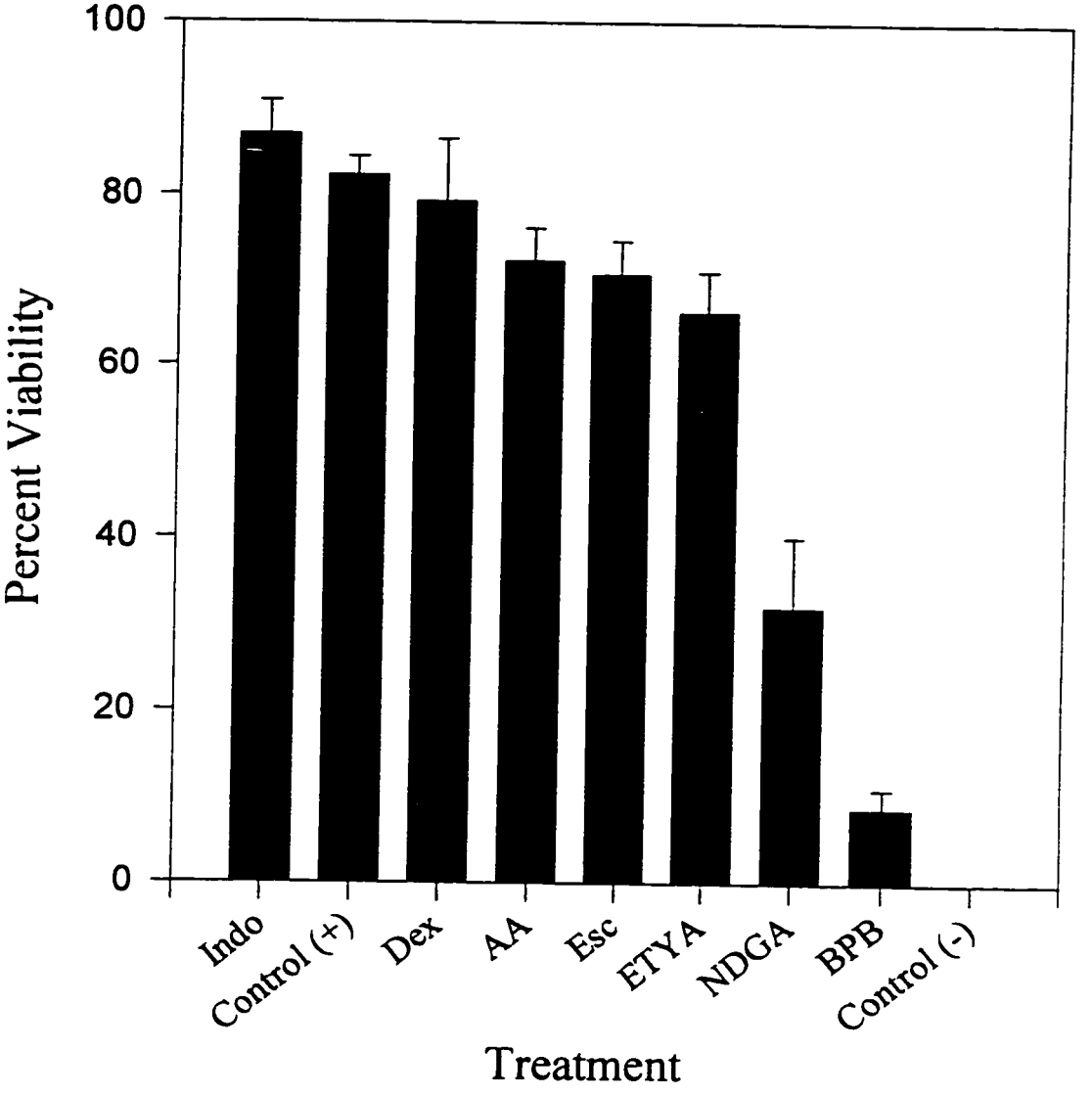

100
80
60
40
20
0
Percent Viability
Indo
Control (+)
Dex
AA
Esc
ETYA
NDGA
BPB
Control (-)
Treatment

Fig. 3.3: Effect of eicosanoid biosynthesis inhibitors on nodule formation in *G. mellonella*. Dex = dexamethasone; Indo = indomethacin; Esc = esculetin. All three inhibitors significantly reduced the number of nodules ($p<0.01$). Arachidonic acid and dexamethasone significantly increased nodule formation compared to the control ($p<0.05$; $n = 14$).

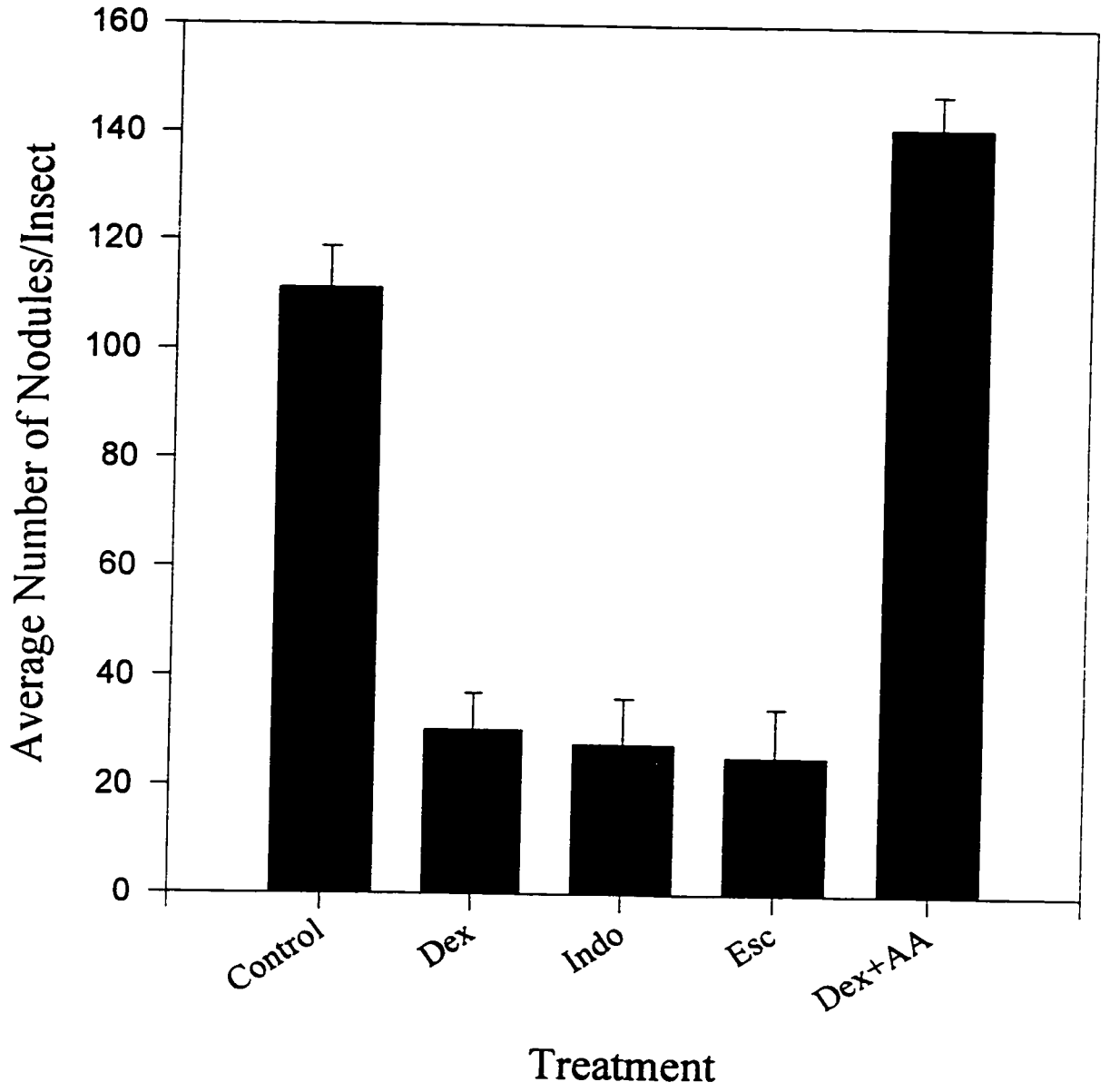
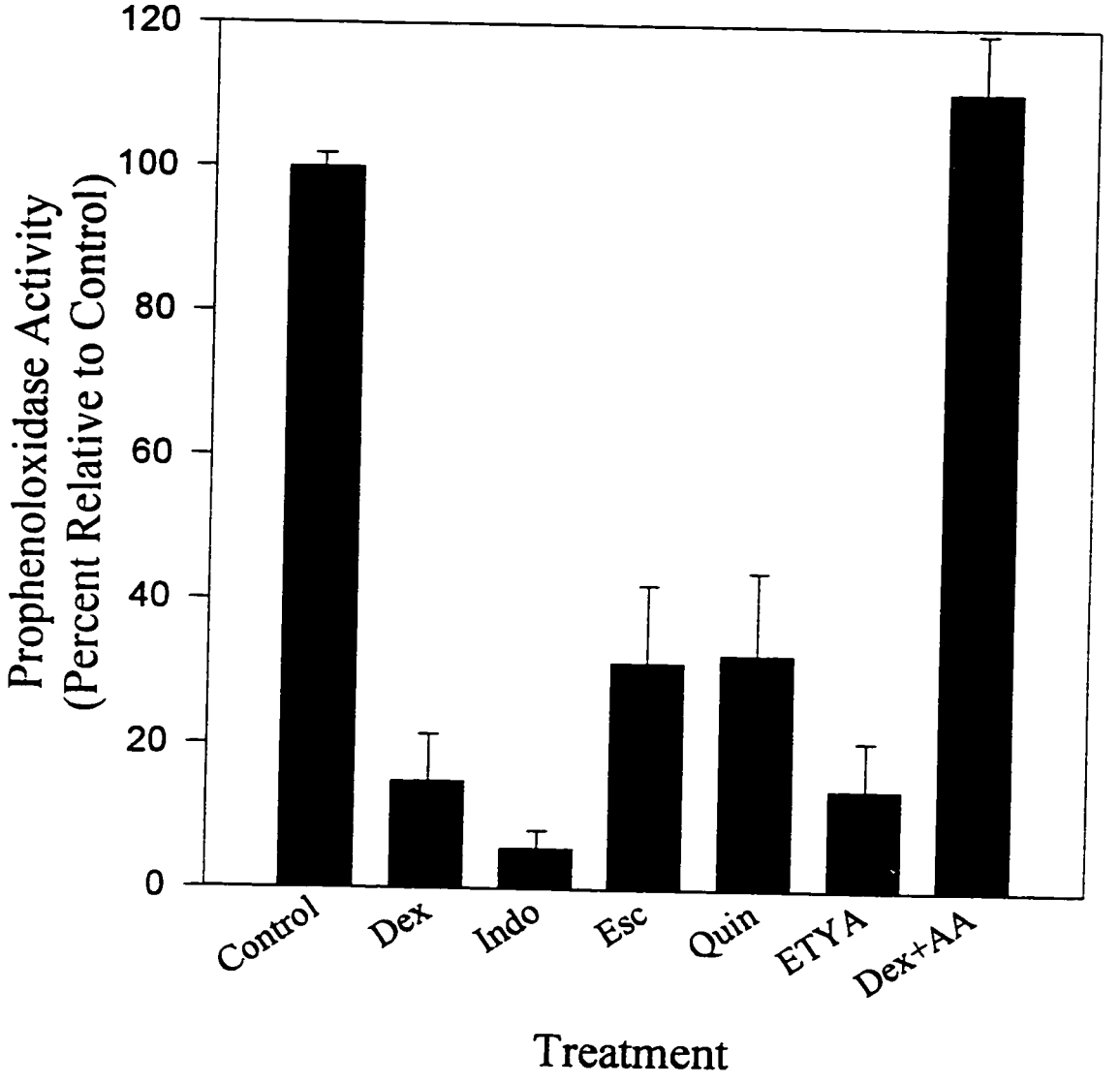

160
140
120
100
80
60
40
20
0
Average Number of Nodules/Insect
Control
Dex
Indo
Esc
Dex+AA
Treatment

Fig. 3.4: Inhibition of prophenoloxidase activity by eicosanoid biosynthesis inhibitors on. Quin = quinacrine; ETYA = eicosatetraynoic acid. All the inhibitors (10mM) significantly decreased prophenoloxidase activity ($p<0.05$). Results are presented as percent inhibition relative to controls (control = 100% activity; n = 6).


120
100
80
60
40
20
0
Prophenoloxidase Activity
(Percent Relative to Control)
Control
Dex
Indo
Esc
Quin
ETYA
Dex+AA
Treatment

increase in prophenoloxidase activity. In dose-response experiments with esculetin, dexamethasone, and indomethacin, the latter two inhibitors caused significant inhibition of prophenoloxidase activation at injectant concentrations as low as 1mM (Fig. 3.5). Esculetin significantly inhibited prophenoloxidase activity only when injected at a concentration of 10 mM ($p<0.05$). All these inhibitors were not effective in preventing prophenoloxidase activity when added to cell lysates and incubated *in vitro* (data not shown).

Effect of Eicosanoid Inhibitors on Phagocytosis

The results of phagocytosis assays with indomethacin, esculetin, and dexamethasone are presented in Figure 3.6. Cells exposed to indomethacin at concentrations as low as 0.1 mM had a significantly lower phagocytic index ($p<0.05$) whereas, at the same concentration neither dexamethasone nor esculetin noticeably inhibited phagocytosis. At 1 mM, esculetin and indomethacin both significantly inhibited phagocytosis, whereas dexamethasone caused significant inhibition only at 50 mM. Coinjection of arachidonic acid and dexamethasone abolished the effect of dexamethasone, yielding a phagocytic index not significantly different from the control (Fig. 3.6, inset).

Effect of Eicosanoid Inhibitors on Hemocyte Spreading In Vitro

Quinacrine and esculetin at a concentration of 100 μM did not significantly inhibit hemocyte spreading after 60 minutes (Fig. 3.7, inset). Dexamethasone,

Fig. 3.5: Dose response of dexamethasone, esculetin and indomethacin in the prophenoloxidase assay. Dexamethasone and indomethacin significantly inhibited prophenoloxidase activity at an injectant concentration of 1 mM; all three inhibitors were effective at 10 mM ($p<0.05$; $n = 3$).

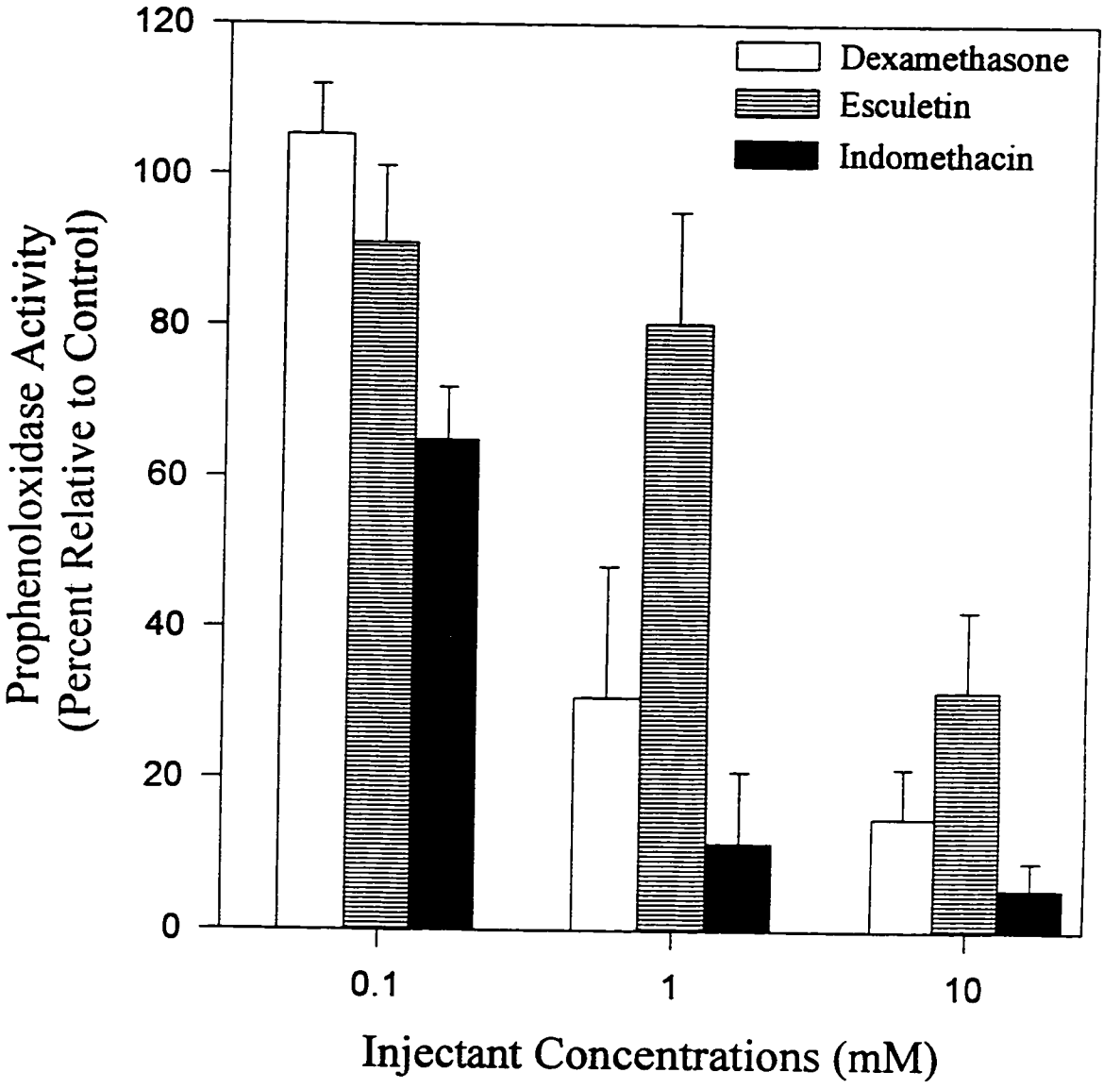

120
100
80
60
40
20
0
Prophenoloxidase Activity
(Percent Relative to Control)
Dexamethasone
Esculetin
Indomethacin
0.1
1
10
Injectant Concentrations (mM)

Fig. 3.6: Inhibitory effect of dexamethasone, esculetin and indomethacin on phagocytosis. Results are presented in terms of the phagocytic index (# phagocytic cells/total # cells) The lowest concentration of indomethacin to significantly inhibit phagocytosis was 0.1 μM, whereas the lowest effective concentrations of esculetin and dexamethasone were 1 μM and 50 μM, respectively ($p<0.05$; $n = 6$). Inset: Arachidonic acid rescue of dexamethasone inhibition. There was no significant difference between the control and the dexamethasone/arachidonic acid treatment ($p<0.05$; $n = 3$).

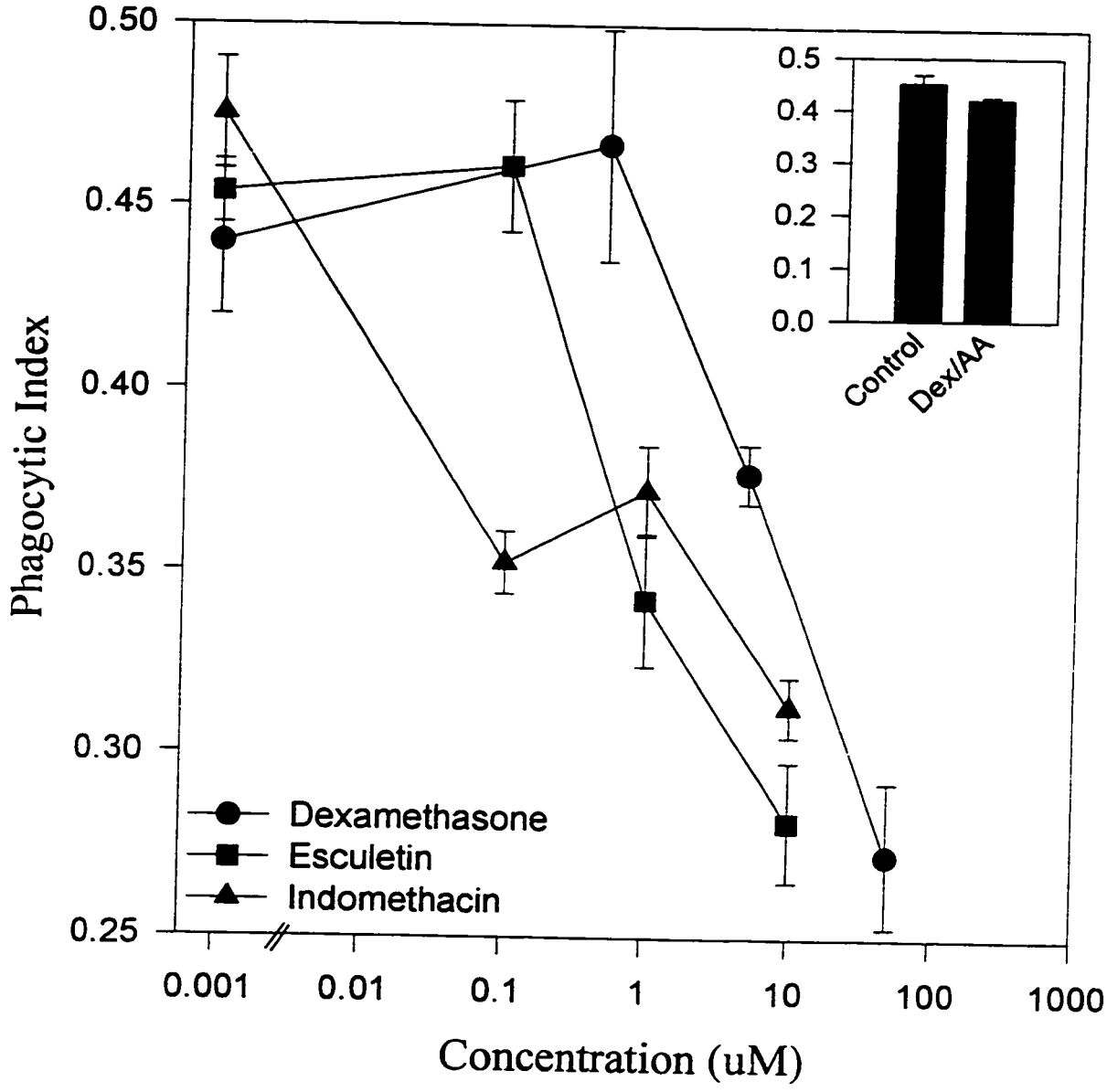
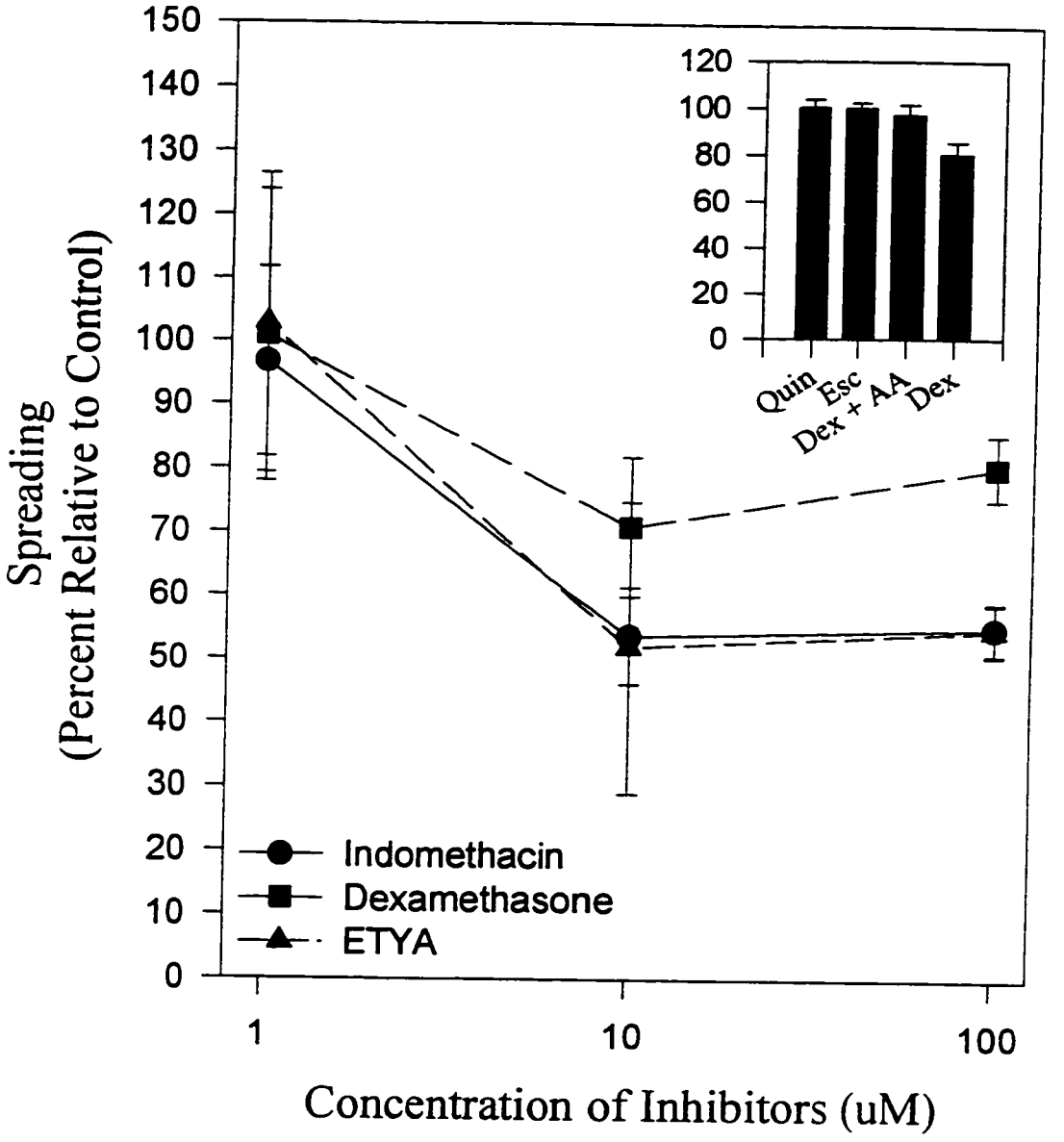

0.50
0.45
0.40
0.35
0.30
0.25
Phagocytic Index
0.001
0.01
0.1
1
10
100
1000
Concentration (uM)
Dexamethasone
Esculetin
Indomethacin
0.5
0.4
0.3
0.2
0.1
0.0
Control
Dex/AA

Fig. 3.7: Effect of eicosanoid biosynthesis inhibitors on hemocyte spreading. Dexamethasone, indomethacin and ETYA all significantly inhibited hemocyte spreading at concentrations of 100 μM. ETYA and indomethacin also inhibited spreading at 10 μM. Inset: The effect of dexamethasone was abolished by coincubation with arachidonic acid ($p<0.05$; $n = 3$).


Spreading
(Percent Relative to Control)
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
1
10
100
Concentration of Inhibitors (uM)
Indomethacin
Dexamethasone
ETYA
120
100
80
60
40
20
0
Quin
Esc
Dex + AA
Dex

indomethacin and ETYA all significantly inhibited cell spreading at concentrations of 100 μM ($p<0.05$); indomethacin and ETYA both inhibited spreading at 10 μM concentrations (Fig. 3.7). Coinjection of dexamethasone and 5 mM arachidonic acid abolished the effects of dexamethasone on hemocyte spreading (Fig. 3.7), although treatments of higher concentrations of arachidonic acid inhibited spreading (Fig. 3.8). The average diameter of control hemocytes after 60 minutes *in vitro* was 131.7 +/- 5.2 mm^2. By comparison, the average surface area of hemocytes exposed to indomethacin was 65.5 +/- 4.6 mm^2. In Figure 3.9 (A-D) video images are presented of control, dexamethasone-, ETYA- and indomethacin-treated hemocytes after 60 minutes *in vitro*. Compared to the control hemocytes (Fig. 3.9A), inhibitor-treated cells remain circular with scant cytoplasm visible (Fig. 3.9, B-D).

Fig. 3.8: Inhibitory effects of arachidonic acid on hemocyte spreading *in vitro*. Arachidonic acid significantly decreased hemocyte spreading at 100 mM ($p<0.05$; $n = 4$).

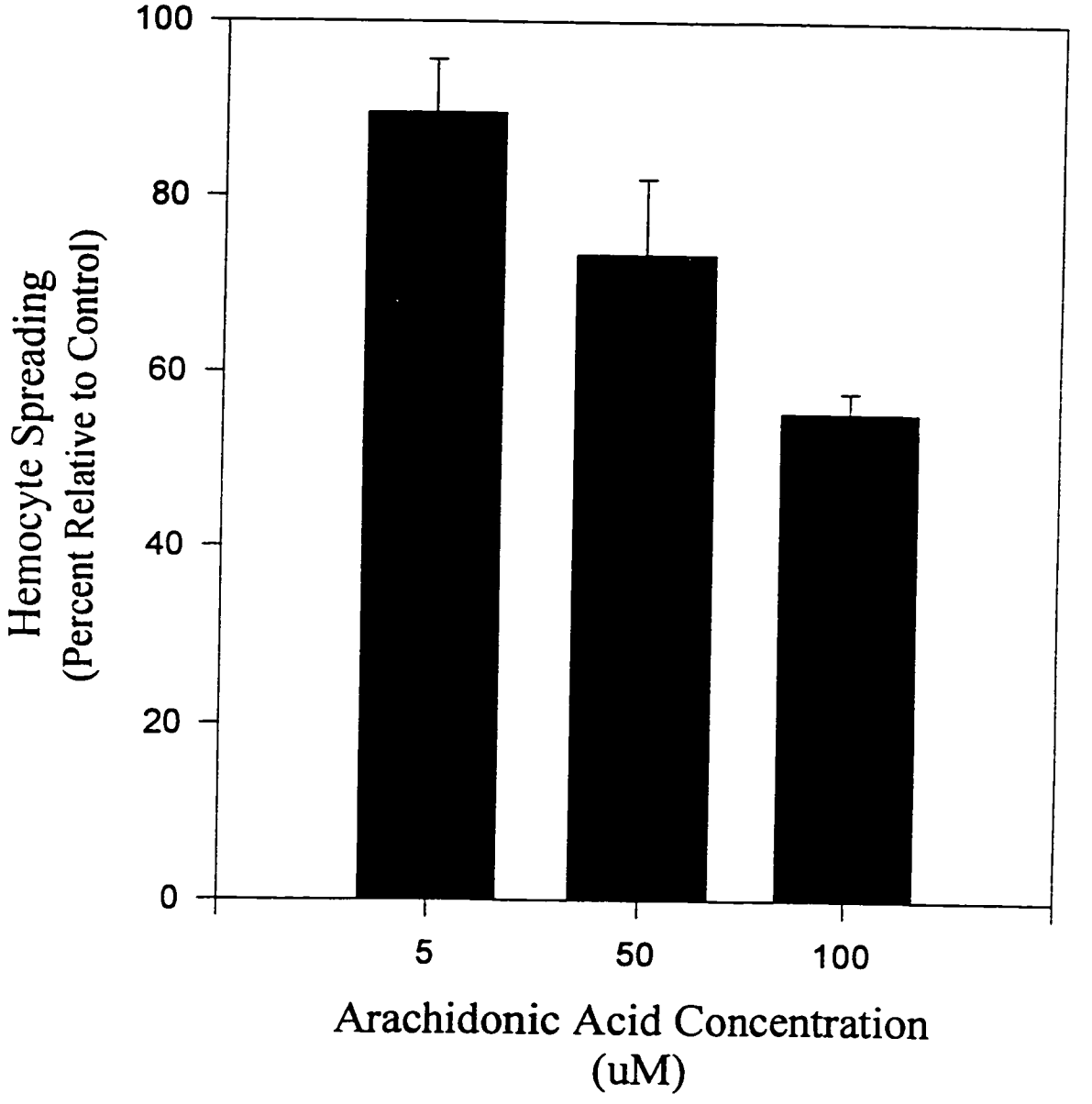

100
80
60
40
20
0
Hemocyte Spreading
(Percent Relative to Control)
5
50
100
Arachidonic Acid Concentration
(uM)

Fig. 3.9 A-D: Video still images of control (A), dexamethasone- (B), ETYA- (C) and indomethacin- (D) treated hemocytes. Scale bar = μm.


A
B
C
D

Discussion

These data support the hypothesis that eicosanoids mediate the immune response in insects. The novel findings in this study include: (1) eicosanoids are likely involved in three discrete processes that occur during nodulation, including prophenoloxidase activation, hemocyte spreading, and phagocytosis; (2) exogenous arachidonic acid inhibits hemocyte spreading *in vitro*; and (3) two commonly-used inhibitors of eicosanoid activity, NDGA and BPB, are toxic to insect hemocytes at pharmacological concentrations.

One of the initial phases of nodule formation involves activation of prophenoloxidase cascade by B-1,3 glucans, fungal spores, lipopolysaccharide and peptidoglycan (Brookman *et al.*, 1989; Dularay and Lackie, 1985; Ashida, 1981). This cascade has been linked to non-self recognition (Ratcliffe *et al.*, 1984) and in the melanization response that occurs during nodule formation, encapsulation, and wound healing (Brookman *et al.*, 1989). In that arachidonic acid metabolites appear to be required for prophenoloxidase activation, this is a novel function for eicosanoids in the regulation of a central pathway in the insect immune response. However, it is not yet possible to ascribe dominant role(s) for cyclooxygenase products versus lipoxygenase products. One possibility is that prostanoids may be involved in the activation of protein kinase C (PKC)(Axelrod *et al.*, 1988). This is due to the fact that prostanoids, in particular, have been proven to activate G_s proteins that stimulate adenylyl cyclase (Axelrod *et al.*, 1988). PKC activates a variety of serine proteases, which are known to be required for activation of the

phenoloxidase proenzyme (Kanost, 1989; Ashida and Sasak, 1994). Another hypothesis is that eicosanoids are required for the activation of cAMP, which may be involved in some as yet unknown component of prophenoloxidase activation. Both hypotheses could be tested by manipulating PKC and cAMP in hemocytes.

The effects of eicosanoid inhibitors on cell spreading and phagocytosis may be distinct from those on prophenoloxidase activation. Phenylthiourea is a potent inhibitor of prophenoloxidase activation (Barrett and Andersen, 1981) and its inclusion in media used in these assays precludes melanization from having collateral effects on either phagocytosis or spreading. Furthermore, there is ample evidence that eicosanoids influence cellular activity. Cyclooxygenase products affect cell spreading, shape change and phagocytosis of mammalian macrophages while lipoxygenase products affect adhesion and chemotaxis in neutrophils (Levine, 1988), and the spreading of HeLa cells requires a lipoxygenase metabolite (Chun and Jacobson, 1992). Oropeza-Rendon *et al.* (1980) present somewhat contradictory findings in reporting that prostaglandin E_1 inhibits phagocytosis and cell shape change in mammalian bone marrow macrophages. Nevertheless, a relationship between eicosanoids, shape change and phagocytosis is generally accepted. The precise mechanism(s) by which eicosanoids modulate hemocyte activity is unknown, although there are several possible models. These include eicosanoid-stimulated increases in cAMP and intracellular Ca^{2+} ,which could promote cytoskeletal rearrangement and cytoplasmic extensions, or eicosanoid modulation of surface receptor clustering (Chun and Jacobson, 1992).

Exogenous arachidonic acid inhibition of cell spreading is an unexpected finding and there are several avenues of investigation with respect to possible mechanisms. One possibility is that arachidonic acid may modulate ion channels in hemocytes. It is well established that arachidonic acid and other polyunsaturated fatty acids affect virtually all known ion channels (Meves, 1994). These actions can be either inhibitory or excitatory and can occur at concentrations as low as 1 -10 mM (Meves, 1994). In one specific example, arachidonic acid stimulates H^+ conductance, NADPH oxidase and hyperpolarization in mouse peritoneal macrophages (Kapas *et al.*, 1994). It is conceivable that, aside from metabolic activation and membrane potential changes, arachidonic acid-stimulated cytoplasmic alkalinization could induce global changes in hemocyte behaviour. Future research needs to be directed at examining the effects of exogenous arachidonic acid as well as specific eicosanoid inhibitors on intracellular pH in hemocytes, and the effects of both extracellular and intracellular pH on hemocyte morphology and behaviour. The effect(s) of arachidonic acid on the intracellular concentrations of other ions, such as Ca^{2+}, should also to be addressed. Before these avenues can be explored, however, further work is required to ascertain that these effects are not due to the oxygenation of arachidonic acid into biologically active compounds.

It is important to note that BPB and NDGA are toxic to hemocytes, and that their effects on cell spreading are likely to be unrelated to any putative function as eicosanoid inhibitors. NDGA in particular has been reported to have nonspecific side effects in other cells (Huang *et al.*, 1992; Keyser and Alger, 1990) and, the

context of the use of these inhibitors with invertebrate model systems, needs to be considered carefully. Both the toxicity and specificity of these inhibitors in invertebrates are points which must be recognized as crucial in interpreting these results.

An essential point to address in this work is the use of pharmacological inhibitors of eicosanoid synthesis and their potential lack of specificity. It could be argued that the inhibitors did not specifically suppress eicosanoid biosynthesis and that their observed actions were due to nonspecific effects. The following is offered as evidence that the inhibitors used probably influenced hemocyte activity via disruption of eicosanoid biosynthesis. First, the effects of dexamethasone on cell spreading, phagocytosis and prophenoloxidase were all abolished by exogenous arachidonic acid. Second, indomethacin and ETYA both reduced hemocyte spreading at concentrations as low as 10 μM. To test for the possibility that indomethacin might non-specifically affect Ca^{2+} transfer across the cell membrane, intracellular Ca^{2+} concentration was measured in hemocytes exposed to 10 μM indomethacin. Indomethacin did not perturb the Ca^{2+} homeostasis at this concentration (data not shown). This concentration is comparable or lower than the effective concentrations reported for these inhibitors in studies on mammalian cells (Carabaza *et al.*, 1993; Nakano et al., 1989; Levine, 1989). Third, the effects of these inhibitors are similar between widely divergent animal species including mammals, fish and invertebrates (von Euler, 1988; Rowley *et al.*, 1995; Stanley-Samuelson, 1991). Furthermore, the biosynthesis of arachidonic acid or

prostaglandin, arachidonic acid uptake, and the presence of arachidonic acid or prostaglandins have been reported in many different insect species and tissues (Radallah *et al.*, 1995; Bowman *et al.*, 1995; Stanley-Samuelson and Ogg, 1995; Jurenka *et al.*, 1988; Wakayama *et al.*, 1986). Also, the incorporation of polyunsaturated fatty acids into phospholipids of insect hemocytes has been reported (Gadelhak and Stanley-Samuelson, 1994). Forth, Miller and Stanley-Samuelson (1996) examined the pharmacology of indomethacin in larvae of the tobacco hornworm and found indomethacin to be taken up by immune tissue to be unmetabolized for physiological relevant periods of time.

This study supports the hypothesis that eicosanoids are important regulators of the immune response in vertebrates and invertebrates. Certainly, experiments wherein exogenous cycloxygenase and lipoxygenase products are used to abolish the effects of eicosanoid biosynthesis inhibitors will be useful and will ultimately clarify the roles of specific arachidonic acid derivatives in insect cellular immunity.

References

Ashida M. 1981. A cane sugar factor suppressing activation of prophenoloxidase in haemolymph of the silkworm *Bombyx mori*. Insect Biochem. 6:37-47.

Ashida, M. and T. Sasak. 1994. A target protease activity of serpins in insect hemolymph. Insect Biochem. Molec Biol. 24:1037-1041.

Axelrod V., R.M. Burch and C. L. Jelsema. 1988. Receptor-mediated activation of phospholipase A_2 GTP-binding proteins: arachidonic acid and its metabolites as second messengers. Trends Neurosci. 11:117-123.

Barrett F. M. and S. O. Andersen. 1981. Phenoloxidase in larval cuticle of the blowfly, *Callephora vinina*. Insect Biochem. 11:17-23.

Bowman, A. S., J.W. Dillwith, R.D. Madden and J.R. Sauer. 1995. Uptake, incorporation and redistribution of arachidonic acid in the isolated salivary gland of the lone star tick. Insect Biochem. Molec. Biol. 25:441-447.

Brookman J. A., N. A. Ratcliffe and A.E. Rowley. 1989. Studies on the activation of prophenoloxidase system by bacterial cell wall components. Insect Biochem. 19:47-57.

Carabaza A., F. Cabre, F. Garcia, M. Gomez, J. Sanchez , D. Mauleon and G. Carganico. 1993. Inhibition of phospholipase A_2 purified from human herniated disc. Biochem. Pharmacol. 45:783-786.

Chun J.-S. and B. S. Jacobson. 1992. Spreading of HeLa cells on collagen substratum requires a second messenger formed by the lipoxygenase metabolism of arachidonic acid released by collagen receptor clustering. Mol. Biol. Cell. 3:481-492.

Corey, E. J., J. O. Albright, A. E. Barton, and S. Hashimoto. 1980. Chemical and enzymic syntheses of 5-HPETE, a key biological precursor of slow-reacting substance of anaphylaxix (SRS) and 5-HETE. J. Am. Chem. Soc. 102:1435-1436.

Dularay B. and A.M. Lackie. 1985. Haemocytic encapsulation and prophenoloxidase activating pathway in the locust *Shistocerca gregaria* forsk. Insect Biochem. 14: 827-834.

Gadelhak, G. G. and D.W. Stanley-Samuelson. 1994. Incorporation of polyunsaturated fatty acids into phospholipids of hemocytes from the tobacco hornworm, *Manduca sexta*. Insect Biochem. Molec. Biol. 24:775-785.

Huang, J. M. -C., H. Xian and M. Balaner. 1992. Long chain fatty acids activate calcium channels in ventricle myocytes. Proc. Natl. Acad. Sci. 89:6452-6456.

Jurenka, R. A., D. W. Stanley-Samuelson, W. Loher and G. J. Blomquist. 1988. *De novo* biosynthesis of arachidonic acid and 11,14-eicosatrienoic acid in the cricket *Teleopryllus commodus*. Biochim. Biophys. Acta. 963:21-27.

Kanost M. R. 1989. Serine protease inhibitors from the serpin gene family in *Manduca sexta* and *Drosophila melanogaster*. In: *Molecular Insect Science* (H. H. Hagedorn, J. G. Hildebrand, M. G. Kindwell and J. H. Law, eds.) 139-146. Plenum Press, New York, NY.

Kapas A., R. Romanek and S. Grinstein. 1994. Arachidonic acid stimulates the plasma membrane H^+ conductance of macrophages. J. Biol. Chem. 269: 4736-4745.

Keyser, D. O. and B. E. Alger. 1990. Arachidonic acid modulates hippocampal calcium currents via protein kinase C and oxygen radicals. Neuron. 5:545-553.

Kuehl, F. A. and R. W. Egan. 1980. Prostaglandins, arachidonic acid and inflamation. Science. 210:978-984.

Levine L. 1988. Arachidonate metabolism in immunologic systems. Karger, Basel.

Meves H. 1994. Modulation of ion channels by arachidonic acid. Prog. Neurobiol. 43:175-186.

Miller J. S. and D. W. Stanley-Samuelson. 1996. The pharmacology of indomethacin, a prostaglandin biosynthesis inhibitor, in larvae of the tobacco hornworm, *Manduca sexta*. J. Insect Physiol. 42:893-901.

Miller J. S., T. Nguyen T. and D. W. Stanley-Samuelson 1994. Eicosanoids mediate insect nodulation response to bacterial infection. Proc. Natl. Acad. Sci. 91:12418-12422.

Nakano T., K. Hanasaki and H. Arita. 1989. Possible involvement of cytoskeleton in collagen-stimulated activation of phospholipases in human platelets. J. Biol. Chem. 264:5400-5406.

Oropeza-Rendon R. L., H. C. Bauer and H. Fisher. 1980. Effect of prostaglandin E_1 on the levels of cAMP in bone marrow macrophages inhibition of phagocytosis and cell shape changes. J. Immunopharm. 2:133-147.

Radallah, D., M. Nogoro and B. Fournier. 1995. Arachidonic acid and prostaglandin E2 stimulate phospholipase C activity in the rectum of the African locust *Locusta migratoria migratoriodes*. J. Insect Physiol. 41:7-16.

Ratcliffe, N.A. and S.J. Gagan. 1976. Cellular defense reactions of insect hemocytes *in vivo*: nodule formation and development in *Galleria mellonella* and *Pieris brassicae* larvae. J. Invert. Path. 28:373-382.

Ratcliffe N. A. and A. F. Rowley. 1979. Role of hemocytes in defence against biological agents. In: *Insect Hemocytes Development, Forms, Functions and Techniques* (A. P. Gupta, ed.) 331-414. Cambridge University Press, Cambridge, U.K.

Ratcliffe N. A., C. Leonard and A. F. Rowley. 1984. Prophenoloxidase activation: non-self recognition and cell co-operation in insect immunity. Science. 226: 557-559.

Rowley, A. F., J. Knight and J.W. Holland. 1995. Eicosanoids and their role in immune regulation in fish. Abstract in: Modulators of immune response.

Söderhäll K. and V. J. Smith. 1986. Prophenoloxidase-activating cascade as a recognition and defence system in arthropods. In: *Hemocytic and Humoral Immunity in Arthropods* (A. P. Gupta, ed.) 251-286. John Wiley and Sons, New York, NY.

Stanley-Samuelson D. W. 1991. Comparative eicosanoid physiology in invertebrate animals. Am. J. Physiol. 260:R849-R853.

Stanley-Samuelson D. W. 1994. Prostaglandins and related eicosanoids in insects. Adv. Insect Physiol. 24:115-212.

Stanley-Samuelson D. W., E. Jensen, K. W. Nickerson, K. Tiebel, C.L. Ogg and R. W. Howard. 1991. Insect immune response to bacterial infection is mediated by eicosanoids. Proc. Natl. Acad. Sci. 88:1064-1068.

Stanley-Samuelson, D. W. and C. L. Ogg. 1995. Prostaglandin biosynthesis by fat body from the tobacco hornworm, *Manduca sexta*. Insect Biochem. Molec. Biol. 24:481-491.

von Euler, U. S. 1988. Biology of prostanoids. In: *Prostaglandins: Biology and Chemistry of Prostaglandins and Related Eicosanoids*. (P. B. Curtis-Prior, ed.) 1-7. Churchill Livingstone, London, England.

Wakayama, E. J., J. W. Dillwith and G. J. Blomquist. 1986. Characterization of prostaglandin synthesis in the housefly. *Musca domestic* (L). Insect Biochem. 16:903-909.

Yamamoto, S. 1988. Characterization of enzymes in prostanoid synthesis. In: *Prostaglandins: Biology and Chemistry of Prostaglandins and Related Eicosanoids*. (P. B. Curtis-Prior, ed.) 37-51. Churchill Livingstone, London, England.

Chapter 4

Identification and Characterization of a Novel Nitric Oxide Synthase from Insect Hemocytes

Abstract

Nitric oxide production is a key element in the innate (non-specific) immunity of vertebrates, and is involved in the elimination of bacteria and the stimulation of immunocytes. There are few data on the molecular biology and physiology of nitric oxide synthase (NOS) in insect blood cells. In this paper, biochemical, molecular and physiological evidence for the presence and role of nitric oxide synthesis induced by lipopolysaccharide- insect hemocytes is presented. NOS activity was demonstrated by enzymatic conversion of the NOS substrate ^{3}[H]-arginine to ^{3}[H]-citrulline. The NOS activity was shown to be calcium independent and inhibited by the mammalian NOS inhibitors, N^{G}-Nitro-L-arginine methyl ester (L-NAME) and N^{G}-monomethyl arginine citrate (L-NMMA). The transcript for NOS was isolated mainly in hemocytes and was upregulated by 4 hours post-injection of larval insects with LPS. Exposure of hemocytes to LPS resulted in increases in cGMP, a putative second messenger of NO. It has also been demonstrated that NO confers an increased resistance to bacterial infection. Furthermore, the hemocyte NOS cDNA displayed strong homologies to other NOS genes. From these data, it is apparent that insect hemocytes produce both NOS and NO, and that the latter plays an important role in mediating insect immunity.

Introduction

Within the last decade, an increasing body of knowledge has accumulated on the molecular modulators and effectors of insect immunity. Insects possess an innate immune system that has many of the same components as are found in vertebrates, including antibacterial molecules, lectins and the RGD adhesion sequence. In terms of a specific or acquired immune response, however, there appears to be very limited homology between insects and vertebrates. While vertebrates possess antigen-specific immunoglobulins (Ig) which yield an enhanced secondary immune response to pathogens, insect immune modulators with Ig-like domains, such as hemolin, function quite differently (Kanost *et al.*, 1994).

Nitric oxide (NO), in addition to having a broad range of functions in vertebrate physiology, has recently been recognized as an immunomodulator of the innate vertebrate immune response. Other functions of NO include serving as a signalling molecule that regulate synaptic plasticity, modulates blood pressure and eliminates pathogens. As a regulator of the immune response, NO has been implicated as the molecule which confers the cytostatic activity produced by activated macrophages (Hibbs *et al.*, 1987).

There are three major isoforms of nitric oxide synthase (NOS) which have been cloned in various mammalian tissues: a Ca^{2+}/Calmodulin-dependent isoform found in brain tissue, neuronal NOS (type I or nNOS); a Ca^{2+}-dependent, membrane associated enzyme discovered in vascular endothelial tissue (type III or eNOS); and a Ca^{2+}-independent NOS found in induced macrophages (type II or iNOS).

Recent biochemical evidence supports the idea that mollusc hemocytes possess some forms of NOS (Conte and Ottaviani, 1995). Biochemical evidence for the presence of NO in insects includes the finding of NOS/NO in fat body and Malpighian tubules of the silkworm, *Bombyx mori* (Choi *et al.*, 1995), in nerve tissue of the locust, *Schistocerca gregaria* (Muller, 1994), in the salivary glands of *Rhodnius prolixus* (Ribeiro *et al.*, 1993) and in the Malpighian tubules of *Drosophila melanogaster* (Dow *et al.*, 1994). In addition to such biochemical markers, insect NOS-like genes have been cloned, including dNOS from *D. melanogaster*, which shows a high degree of homology with mammalian neuronal NOS (Regulski and Tully, 1995), nitrophorins from the salivary glands of *Rhodnius prolixus* (Champagne *et al.*, 1995) and the mosquito NOS (genebank #AF053344).

Although Choi *et al.* (1995) were unable to biochemically detect either NOS or NO in insect hemocytes, the importance of NO in the vertebrate immune response, its production and function in other insect tissues, and its presence in mollusc hemocytes compelled a re-examination of Choi's findings. Therefore, a combined molecular and biochemical approach was chosen to resolve the debate as to the presence of NOS and/or NO in insect hemocytes. Herein is presented the first definitive evidence for the existence and role(s) of NOS and NO in LPS-induced insect hemocytes. Furthermore, I demonstrate that NO has functional significance within the insect immune response.

Material and Methods

Larval infection

Galleria mellonella larvae were injected with various concentrations of *E.coli* LPS (Serotype 055:B5) in modified Grace's Insect Medium (mGIM) via the third abdominal proleg using a 30 gauge needle attached to a 100 µl Hamilton syringe. Concentrations were calculated assuming an average hemolymph volume of 200 µl. Controls consisted of animals which had been injected with mGIM. At various times post injection hemocytes were collected (General Materials and Methods).

Determination of NOS Activity

Larvae were injected with 10 µl doses of increasing concentrations of LPS (40 replicates/dose). Hemocytes were collected and pelleted as described in the General Materials and Methods. Pellets were homogenized in a 5 ml ground glass tissue homogenizer (Kontes, Vineland, NJ.) for 20 -30 seconds in 200 µl of ice-cold mGIM containing 1mM ethylenediaminetetraacetic acid (EDTA), 1mM dithiothreitol (DTT), 10 µg/ml leupeptin, 10 µg/ml antipain and 10 µg/ml Nα-p-tosyl-*L*-lysinechloro-methyl ketone (TLCK). Hemocyte homogenates were centrifuged at 16 000 g for 30 minutes at 4°C. The supernatant was assayed for NOS activity by measuring the conversion of radiolabelled arginine to citrulline (Bredt and Snyder, 1989). This method involved adding the supernatant to a standard reaction buffer containing 50mM HEPES (pH=7.2), 0.5mM EDTA, 60mM valine, 1mM citrulline,

200μM arginine, 1.4 mM calcium chloride ($CaCl_2$), 1mM β-nitcotinamide adenine dinucleotide phosphate (β-NADPH), 50μM tetrahydrobiopterin (H_4B), 10μM flavin adenine dinucleotide (FAD), and L-[2,3,4,5-^{3}H]arginine monohydrochloride (~ 100 000cpm). After incubation for 30 minutes at 30°C ^{3}H-arginine was separated from ^{3}H-citrulline by eluting the reaction mixture over 2 ml columns of cation exchange resin (DOWEX 50WX4-400, Sigma Chemical Co., St. Louis, Mo.). Prior to adding the reaction mixture, the Na^+ form of the resin was prepared by washing the columns 4 times with 1M NaOH and then washing the columns with double distilled water until the pH of the eluent was 7.2. The ^{3}H-citrulline produced in this enzymatic reaction was eluted from the columns with 2 ml of water and quantified by counting the beta emissions in a Beckman liquid scintillation counter. Enzymatic activity is reported as pmol of ^{3}H-citrulline produced per minute of the reaction per mg of protein in the hemocyte homogenate. Protein in the cell extract was measured with the Biorad protein assay kit (Bradford assay) using bovine serum albumin as the standard.

Mammalian NOS inhibitors were used to further characterize this NOS-like enzyme in LPS-induced hemocytes. The NOS inhibitors N^G-nitro-D-arginine methyl ester hydrochloride (L-NAME) and Ng-monomethyl-L-arginine monoacetate salt (L-NMMA) are soluble analogues of arginine, and thus are competitive inhibitors of NOS. The biological activity (IC_{50}) of these inhibitors are 500 nM/ 1mM (eNOS/ iNOS) and 700 nM/ 3.9 μM (eNOS/ iNOS), respectively (Calbiochem, San Diago,

CA). Both of the inhibitors, at a final concentration of 1 mM, which is above the IC_{50} concentration, were added to the standard reaction mixture.

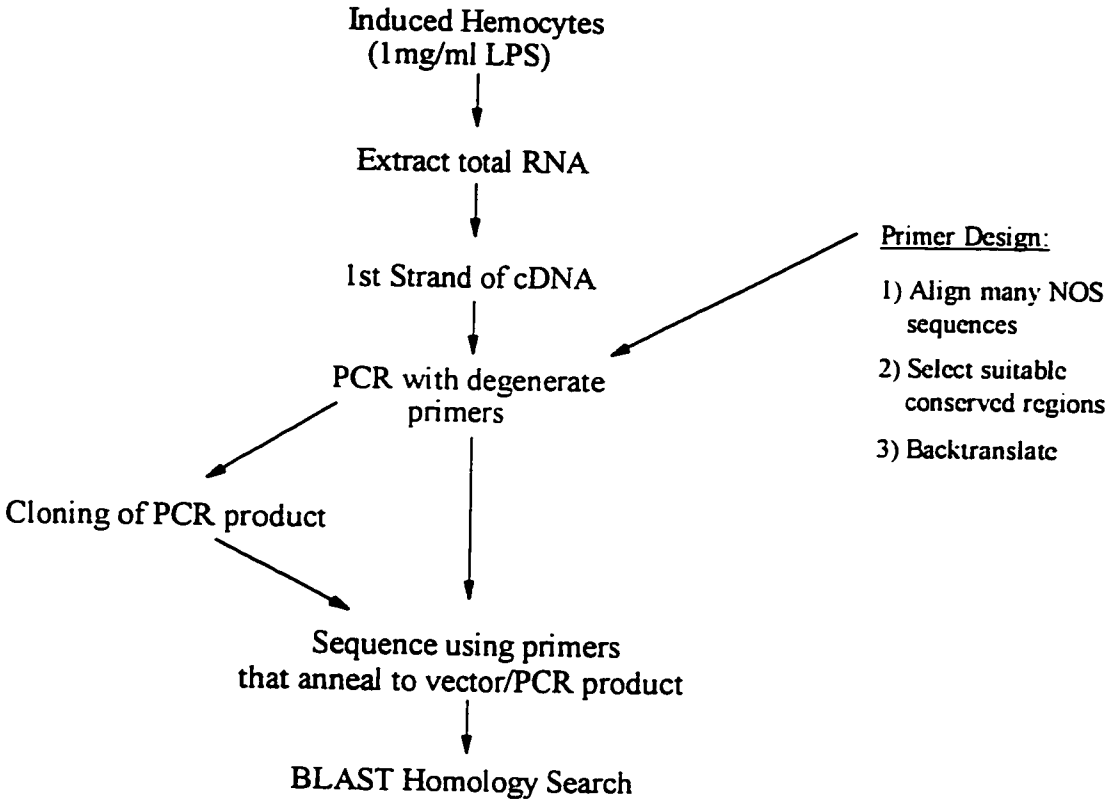
To determine whether NOS activity found in insect hemocytes was Ca^{2+}-dependent, hemocytes were collected from 40 larvae that had been injected with 50 µg/ml of LPS and incubated for 8 hours. $CaCl_2$ was omitted from the reaction mixture and 5 mM EGTA (an effective calcium chelator) was added to the NOS activity assay. Statistical significance for this assay and all subsequent assays were determined by paired Student's T-test ($p<0.05$).

Probe Design

NOS sequences from *Drosophila* (genebank #D25117), mouse (genebank #U26686) and human (genebank #P29475) were aligned and suitable conserved regions were selected and backtranslated to design degenerative primers for reverse transcriptase polymerase chain reaction (RT-PCR) (see Fig. 4.1). The primers were constructed to span the putative heme binding domain (NOS 3, see Appendix B for primer sequences) to the putative calmodulin domain (NOS 4). Total RNA was extracted from LPS-induced *G. mellonella* larvae hemocytes using the single step method reported by Chomczynski and Sacchi (1987). The method involved homogenizing hemocyte pellets in a microcentrifuge tube containing 1ml (per 50-100mg of tissue) of an acid guanidium thiocynate/phenol mixture (TRI REAGENT, Sigma). Two hundred microlitres of chloroform were added to the homgenized mixture, which was then vigorously shaken for 15 seconds and allowed to stand at

Fig 4.1: Strategy for cloning the nitric oxide synthase gene from *Galleria mellonella* hemocytes. NOS protein sequences from *Drosophila* (genebank # D25117), mouse (#X59949) and human (#P29475) were aligned and degenerate primers were designed based on conserved region. The primers were used in reverse transcriptase polymerase chain reactions with cDNA constructed from the RNA collected from LPS-induced hemocytes as the template. The resulting products were either cloned in a vector which was then sequenced or directly sequenced.

RT-PCR Strategy for Cloning NOS from *Galleria mellonella*



room temperature for 10 minutes. The mixture was centrifuged at 12 000 *g* for 15 minutes at 4°C thus separating the mixture into three discrete phases. The upper aqueous phase containing RNA was transferred to a new micro centrifuge tube containing 0.5 ml of isopropanol, then mixed and allowed to stand at room temperature for 10 minutes. The RNA was pelleted by centrifuging the samples at 12 000 *g* for 10 minutes at 4°C. After removing the supernatant, the RNA pellet was washed by vortexing the pellet with 75% ethanol. All RNA preparations had a 260/280 ratio of ≥ 1.8. The total hemocyte RNA was used to synthesize cDNA.

cDNA synthesis was performed using a 1st Strand cDNA Synthesis Kit (Boehringer Mannheim). One µg of total RNA and 50 pmoles of Oligo$(dT)_{15}$ primer were incubated for 10 minutes at 65°C in a thin-walled PCR tube, and the following reagents were added on ice: 4 µl of 5X *Expand* reverse transcriptase buffer, 2 µl of 100 mM DTT, 2 µl of 10 mM dATP, dTTP, dGTP, dCTP, 0.5 µl of RNase inhibitor (40 units/µl) and 1µl of *Expand* reverse transcriptase (30 units/µl) in a total volume of 20 µl RNase-free water. The PCR tube was incubated for 60 minutes at 42°C to synthesize the cDNA, after which the reverse transcriptase was heat inactivated at 95°C for 2 minutes. The first-strand cDNA was amplified in a PCR experiment using the degenerate primers NOS 3 and NOS 4. The PCR reaction mixture included 5µl of cDNA (approximately 0.5 µg), 10 µl of 10X PCR buffer (100mM TRIS-HCl, pH 8.3, 500 mM KCl and 15 mM $MgCl_2$), 2µl of dNTPs (10 mM each),

1 µl of each primer and 2.5 µl of Taq DNA polymerase (1 unit/µl; Boehringer Mannheim). Amplification was performed in the Amplitron II thermocycler (Thermolyne) under a heated lid with the following temperature profile: a "hotstart" at 95°C for 3 minutes (Taq polymerase was added at this time to the hot samples), followed by 30 cycles of denaturing at 94°C for 30 seconds, annealing at 57°C for 30 seconds and extension at 72°C for 2 minutes. This was followed by an elongated extension step of 72°C for 10 minutes.

RT-PCR of the cDNA produced an 800 bp product which was cloned into the plasmid vector pUC 18 (Clone N1). The PCR product was cloned using the Di/Trinucleotide Sticky End Cloning Protocol which relies on the generation of complimentary di- or trinucleotide sticky ends on the linearized vector and PCR-amplified DNA (Dietmaier and Fabry, 1995). The vector was linearized by digestion with the restriction enzymes *Eco* RI and *Hind* III, followed by the addition of dATPs with Klenow polymerase (a 5'-3'-polymerase which filled in the 3' end of the vector with dATPs). The PCR product was prepared by trimming with T4 DNA polymerase (a 3'-5' exonuclease) in the presence of dTTP which made a complimentary end to the vector. Following purification of the vector and PCR product using the NaI/glassmilk methods (Geneclean), the vector and PCR products were added to a ligation mixture containing 10X concentrated ligase buffer (660 mM TRIS-HCl, 50 mM $MgCl_2$, 10 mM dithioerythritol, 10 mM ATP, pH 7.5) and T4 DNA ligase (1 unit/µl; Boehringer Mannheim) and incubated at 15°C overnight.

The ligation mixtures were used to transform competent *E.coli* (strain HB101). Competent cells were made following the protocol by Sambrook *et al.* (1989). Approximately 100 µl of an overnight culture of HB101 was incubated in Luria-Bertani medium (LB: 10g bacto-tryptone, 5 g of bacto-yeast extract and 10 g of NaCl in 950 ml of sterile distilled water, pH 7.0, sterilized by autoclaving) at 37°C with shaking for 4 hours or until the optical density (O.D.) reached 600. The cells were centrifuged at 2000 *g* for 10 minutes at 4°C in a SA600 rotor and then resuspended in 2.5 ml of 50 mM $CaCl_2$ and incubated on ice for 60 minutes. Following this step they were centrifuged and resuspended on ice in 0.5 ml of 50 mM $CaCl_2$.

One hundred microliters of the transformed cells were combined with approximately 50 ng of the ligated DNA in a transformation mixture containing 20 µl of 0.5 M TRIS-HCl, pH 7.4, 200 µl 50 mM $CaCl_2$, 5 µl of $MgCl_2$, 775µl of sterile doubled distilled water. The bacteria/DNA mixture was then incubated on ice for 45 minutes, 42°C for 90 seconds then placed on ice for 2 minutes. Pre-warmed LB broth (350 µl) was added to the mixture and incubated at 37°C for 45 minutes. One hundred µl of the DNA transformation mixture was used to evenly coat agar plates (LB, 20 mM $MgSO_4$, 15 g/l bacto-agar and 75 µg/ml ampicillin). The plates were inverted and incubated at 37°C overnight.

Single colonies that formed were removed from the plates and used to inoculate 5 ml of LB broth with 75µg/ml of ampicillin and incubated overnight at

37°C with shaking. The plasmid DNA from these colonies was isolated using a miniprep kit (Qiagen), which involved centrifuging the cells at 12 000*g* for 5 minutes at 4°C and resuspending the cells in 250 µl of buffer P1 (RNase solution). Two hundred and fifty microliters of P2 (NaOH/ SDS) were then added and the mixture was inverted 5 times and allowed to stand for 5 minutes. Three hundred and fifty microliters of buffer N3 (a chaotropic salt) were added and immediately gently inverted 5 times. The mixture was centrifuged at 12 000*g* for 5 minutes at 4°C and the supernatant pipetted into QIAprep columns. The prep columns were centrifuged for 30 seconds, the flow-through discarded and 0.75 ml of PE (ethanol wash) was added to the columns, which were then centrifuged for an additional 30 seconds. The ethanol wash that contained RNA, cellular proteins and salts was discarded and 60 µl of water was used to elute the plasmid DNA bound to the prep columns.

The 800bp RT-PCR product was Digoxigenin-labelled (DIG, Boehringer Mannheim) and used as a probe in subsequent northern blots. A standard random primed DNA labelling reaction was performed to incorporate Digoxigenin-11-dUTP. One µg of the RT-PCR product was added to 15 µl of water in a thin-walled PCR tube and denatured in boiling water for 10 minutes, then immediately chilled on ice. Two µl of 10X hexanucleotide mixture (1.56 mg/mg of random hexanucleotides, 500 mM Tris-HCl, 100mM $MgCl_2$, 1 mM dithioerythritol and 2 mg/ml BSA; pH 7.2), 2 µl of 10X dNTPs labelling mixture (1 mM dATP, 1 mM dCTP, 1 mM dGTP, 0.65 mM dTTP and 0.35 mM alkali-labile DIG-dUTP; pH 6.5) and 1 µl of

Klenow enzyme (labelling grade, 2 units/µl) were added to the tube. The contents of the reaction tube were mixed by inversion and incubated at 37°C overnight. The reaction was stopped by the addition of 2 µl of 0.5 M EDTA.

Northern Blot Analysis

Total cellular RNA was isolated (as described above) from hemocyte pellets and 20µg samples were separated on 1% agarose/formaldehyde gels for 3 hours at 55 volts. RNA was then transferred by capillary action to nylon membranes (Hybond-N+, Amersham) and immoblized by UV-cross-linking (Sambrook *et al.*, 1989). To assess RNA quality, the membranes were stained with methylene blue (0.02% methylene blue in 0.3 M NaOAc; pH 5.5) and photographed (Polaroid 667 film). The membranes were prehybridized in High SDS buffer (7% sodium dodecyl sulfate (SDS), 50% formamide, 5x saline-sodium citrate buffer (SSC: 75mM sodium citrate and 750 mM NaCl), 2% blocking reagent (a proteolytic digest of gelatine; Boehringer Mannheim), 50 mM sodium phosphate (pH 7.0) and 0.1% N-lauroylsarcosine) for 2 hours at 42 °C and hybridized with the DIG-labelled probes in High SDS Buffer at 42 °C for 48 hours. Membranes were washed twice in 2x SSC/0.1% SDS at room temperature for 5 minutes and once in 0.5 SSC/0.1% SDS at 55°C for 15 minutes.

The hybrized probes were detected using anti-digoxigenin antibodies conjugated to alkaline phosphate, which were then exposed to the chemiluminescent substrate 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5 chloro)tricyclo[3.3.1.1^{3,7}]decan}

phenyl phosphate (CSDP, Boehringer Mannheim). Detection was performed by washing membranes twice in 2X SSC/ 0.1% SDS for 5 minutes at room temperature, followed by a third wash in 0.5 SSC/ 0.1%SDS. The transfer membrane, or blot, was then equilibrated for 1 minute in a maleic acid buffer (0.1 M maleic acid and 0.15 M NaCl; pH 7.5) containing 0.3% Tween 20 and non-specific binding reduced by incubation for 30 minutes in blocking solution (10X blocking reagent (Boehringer Mannheim) in maleic acid buffer, 1:10) at room temperature. The blocked membrane was incubated with an anti-DIG antibody (1/10000 in blocking solution) at room temperature for 30 minutes. The blot was then washed twice in maleic acid buffer for 15 minutes at room temperature and equilibrated in detection buffer (0.1 M Tris and 0.1 M NaCl; pH 9.5). A mylar "sandwich" was formed by placing the blot on a mylar sheet of slightly larger dimensions and adding 0.3 ml of the chemiluminescent substrate (1:10 in detection buffer), followed by an additional mylar sheet. Bubbles between the sheets were removed by gently wiping a damp tissue over the top sheet. After 5 minutes the blot was extracted, the excess substrate removed and the blot sealed with tape between two similar sheets of mylar. Following 15 minutes incubation at 37°C, the membrane was exposed to standard X-ray film (X-OMAT, Kodak). The film was exposed to the blot for 45 minutes at room temperature then developed using standard film developing procedure: film was placed in 1:1 Kodak D-19 developer for 2 minutes, followed by immersion in a 5% solution of acetic acid (stopbath) for 30 seconds, fixed for 5 minutes in Kodak rapid fixer then washed 5 times for 5 minutes in distilled water.

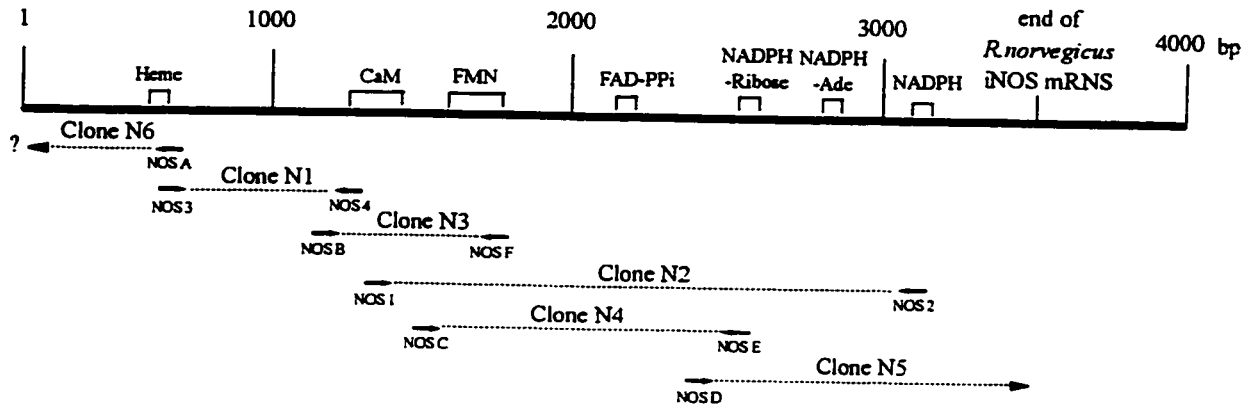
Sequence Analysis

Similar to the design used for constructing the initial northern blot probe, degenerate primers were designed to encompass the entire gene (see Fig. 4.2; Appendix B). In addition to clone N1, five other clones/ PCR products were generated by the RT-PCR strategy described above. In Figure 4.2, I present a schematic representation of a putative NOS cDNA, with the relative position of the cofactors binding sites (distances based on mouse macrophage NOS gb #U26686). The PCR product resulting from the amplification with primers NOS 1 and NOS 2 was cloned into pUC 18 (Clone N2). Both clones N1 and N2 were sequenced using M13/pUC 18 forward and reverse primers (Mobix, Hamilton, ON). The remaining PCR fragments (N3, N4, N5 and N6) were amplified with their appropriate sets of primers (see Fig. 4.2). The amplified PCR fragments were "gene cleaned" as previously described and concentrated to 50ng/µl. These were sequenced using the upstream primer (0.8 pmole/µl) as a sequencing primer (Mobix, Hamilton, Ontario). Each sequence was examined for homology with all the sequences found in *Genebank.*

Larval Survival

A NO donor and a NOS inhibitor were used to determine the effects of NO on the survival of larvae challenged with the Gram negative bacteria *Serratia marcescens.* Bacteria were grown at room temperature in LB broth to midlogarithmic phase (O.D.≈ 600). Larvae were injected with 10 µl of 50 µg/ml

Fig. 4.2: A representative NOS gene with co-factor binding domains. The approximate nucleotide distances are based upon the *Rattus norvegicus* iNOS cDNA (gb|U67309). Below the gene, the placement of the 6 clones are indicated with their appropriate primer pairs. Each clone is listed along with the size of the PCR product, the primer used in the sequencing reaction and the number of base pairs resulting from sequencing.



Clone	PCR Product (bases)	Primer	Sequence Results (# of bases)
N1a	800	M13/pUC reverse	760
N1b	800	M13/pUC forward	933
N2a	2050	M13/pUC reverse	939
N2b	2050	M13/pUC forward	961
N3	725	NOS B	553
N4	1700	NOS C	1023
N5	1000	NOS D	949
N6	1100/750	NOS A	???

LPS, LPS+ L-NAME or modified Grace's Insect Medium (mGIM) (3 replicates of 20 larvae each). Following an 8 hour incubation at room temperature, larvae were injected with 10 colony forming units (cfu) *of S. marcescens.* Survivors in each group were counted at 0, 9, 12, 15, 18 and 24 hours post-infection. To study the direct effects of NO on bacteria-induced mortality, larvae were injected with either 10 µl nitric oxide donor, sodium nitroprusside dihydrate (SNAP: 1mM), or mGIM and immediately infected with 10 cfu *of S.marcescens.*

cGMP Immunoassay

Soluble guanylate cyclase activation is a sensitive indicator of the production of NO and has been linked to immunological functions such as platelet aggregation and adherence and neutrophil chemotactic locomotion (Mayer, 1994; Beauvais *et al.*, 1995). To assay for the presence of cGMP, larvae were injected with 10 µl of either 50 µg/ml LPS, LPS+ L-NAME, or mGIM (30 replicates per treatment). Following an 8 hour incubation hemocytes were collected (see General Materials and Methods) and homgenized in ice cold mGIM containing 1 mM dithiothretiol, 0.5 mM EDTA and 5 mM 3-isobutyl-1-methyl-xanthine(IBMX). IBMX is a phosphodiesterase inhibitor and was added to the homogenization buffer to inhibit the degradation of cGMP. The preparation was centrifuged at 16 000 g for 30 minutes at 4°C.

One hundred microliters of the hemocyte supernatant described above was assayed for cGMP in a correlated enzyme immunoassay kit (EIA; Assay Designs).

In this quantitative competitive immunoassay for cGMP, samples were pipetted into a microtiter plate coated with goat antibody specific to the Fc portion of rabbit IgG. A blue alkaline phosphatase antigen conjugate and the cGMP antibody were then added to the plate which was incubated at room temperature for 2 hours with shaking. The plate was then aspirated and washed 3 times and the colourimetricurmetric substrate (para-nitrophenyl phosphate) was added. cGMP from samples or from standards (0.5-5000 pmol/ml) competed with the antigen conjugate for the cGMP antibody bound to the plate. After 45 minutes at room temperature the colourimetricurmetric reaction was stopped by the addition of NaOH. The absorbance of the colourimetricurmetric substrate was measured on a microplate reader at 405nm, the intensity of which was inversely proportional to the concentration of cGMP in both the samples and the standard. The concentrations are reported in pmol of cGMP per mg of protein.

Results

Enzymatic Activity of NOS

The induction of NOS activity by LPS was confirmed by measuring the formation of ^{3}H-citrulline in *G. mellonella* hemocytes. The relationship between NOS activity and LPS concentration over an 8 hour time course is shown in Figure 4.3. LPS induced a significant increase in NOS activity ($p<0.01$). This increase in NOS activity appears to be dose-dependent, with an LPS concentration of 50 μg/ml producing the highest activity (138± 17 pmol of ^{3}H-citrulline/mg of soluble protein/minute).

At 0, 2 and 4 hours post-injection (P.I.), there were no significant differences in NOS activity between LPS- and mGIM-treated animals, although at 4 hours P.I. both mGIM- and LPS-injected animals exhibited slight, but not statistically significant increases in hemocyte NOS activity compared to time 0. Eight hours P.I., there was a 3-fold increase in NOS activity in the LPS-treated group compared to that of the mGIM-treated group (130±10 pmol/mg/min to 45 ± 2 pmol/mg/min, respectively; Fig. 4.4). Twelve hours P.I., NOS activity in hemocytes from the LPS-injected animals remained significantly elevated above that of the corresponding controls (93.1 pmol/mg/min to 50.67, respectively).

NOS activity was not affected by the absence of Ca^{2+} (Fig. 4.5). LPS-induced hemocytes assayed for NOS activity in the presence of the Ca^{2+} chelator EGTA produced 122 pmol/mg/min of ^{3}H-citrulline, compared to 135 ± 8.3

Fig. 4.3: Effect of increasing LPS dose on nitric oxide synthase activity. Eight hours post-injection with 10 μl of increasing concentrations of LPS (40 larvae/dose) cytosolic protein was collected from hemocytes and assayed for NOS activity. NOS activity increased in a dose-dependent manner, with a concentration of 50 μg/ml injectant inducing the greatest NOS activity. The means and standard errors of triplicate assays are presented. Values represent NOS activity measured in pmol of ^{3}H-citrulline produced per mg of hemocyte supernatant per minute. Values significantly ($p<0.01$) greater than the control (0 μg/ml LPS) are indicated by asterisks.

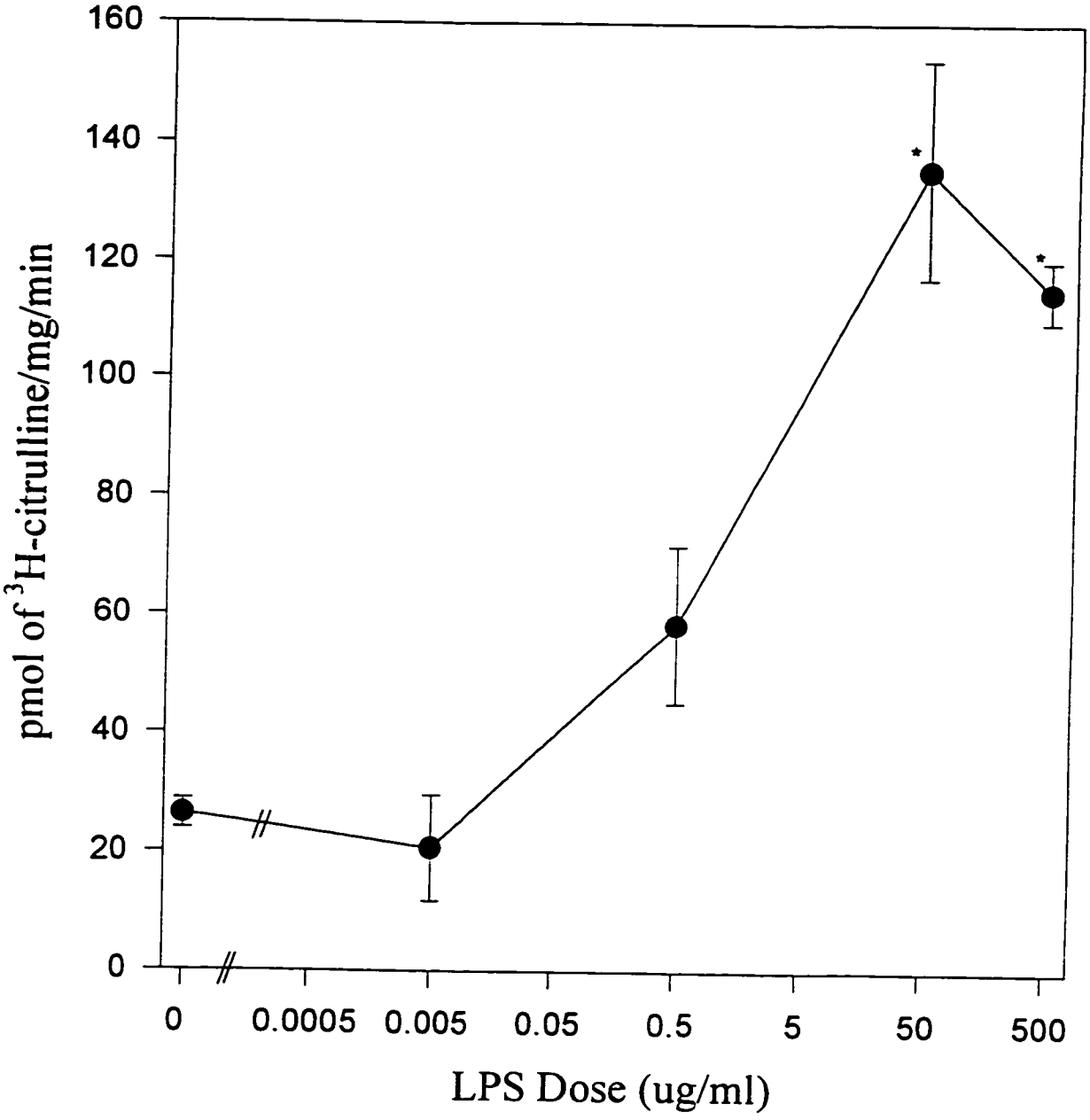

160
140
120
100
80
60
40
20
0
pmol of ^{3}H-citrulline/mg/min
0
0.0005
0.005
0.05
0.5
5
50
500
LPS Dose (ug/ml)
*
*

Fig. 4.4: Time course for LPS-stimulated NOS activity. At times 0, 2, 4, 8 and 12 hours post-injection with 10 μl of mGIM or 50 μg/ml of LPS cytosolic protein was collected from hemocytes (40 larvae/time point). By 8 hours post-injection the NOS activity in LPS-stimulated cells was significantly ($p<0.05$) greater than the mGIM-treated cells. The means and standard errors of triplicate assays are presented. Values represent NOS activity measured in pmol of ^{3}H-citrulline produced per mg of hemocyte supernatant per minute. Values significantly greater than the control (mGIM) are indicated by asterisks.

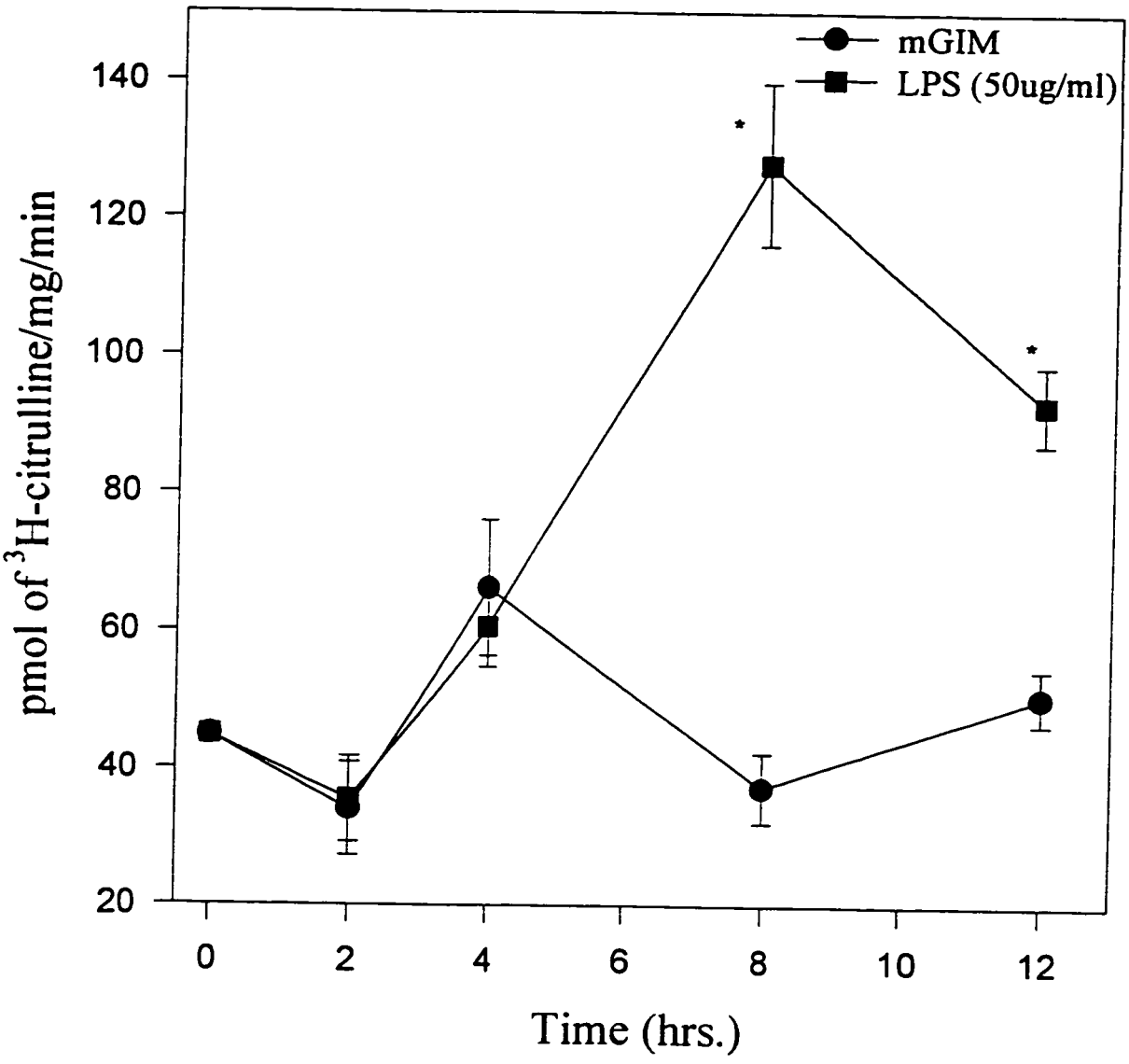
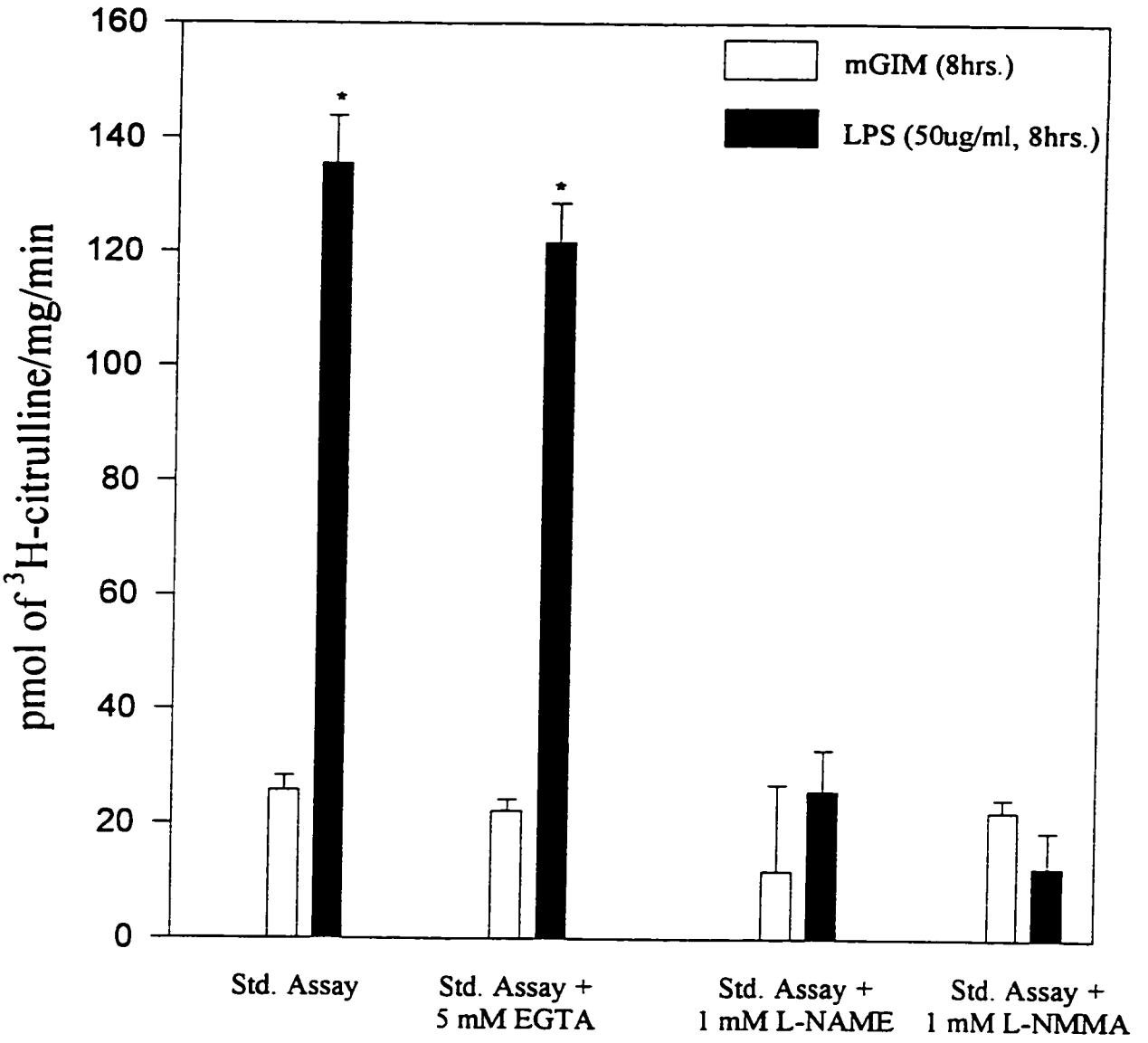

mGIM
LPS (50ug/ml)
pmol of ^{3}H-citrulline/mg/min
140
120
100
80
60
40
20
0
2
4
6
8
10
12
Time (hrs.)
*
*

Fig 4.5: Inhibition of LPS-stimulated NOS synthase activity. Eight hours post-injection with 10 μl of mGIM or 50μg/ml of LPS (40 larvae/treatment) cytosolic protein was collected from hemocytes. The Ca^{2+} chelator EGTA and the NOS inhibitors L-NAME and LNMMA were added to the standard (std.) NOS activity assay. EGTA had no significant effect in reducing NOS activity in LPS-stimulated hemocytes, while both NOS inhibitors abolished the LPS-stimulation of NOS in hemocytes. The bars represent NOS activity measured in pmol of ^{3}H-citrulline produced per mg of hemocyte supernatant per minute (mean ± S.E). The asterisks indicate a significant difference ($p<0.05$) from the control value.


160
140
120
100
80
60
40
20
0
pmol of ^{3}H-citrulline/mg/min
mGIM (8hrs.)
LPS (50ug/ml, 8hrs.)
*
*
Std. Assay
Std. Assay + 5 mM EGTA
Std. Assay + 1 mM L-NAME
Std. Assay + 1 mM L-NMMA

pmol/mg/min for the LPS-treatment. This data indicates that the NOS isoform found in *G.mellonella* hemocytes is Ca^{2+}-independent.

The effects of the mammalian NOS inhibitors L-NAME and L-NMMA on NOS activity are depicted in Figure 4.5. Both inhibitors significantly reduced NOS activity in insect hemocytes ($p>0.01$). The NOS activity of LPS-induced hemocytes decreased five-fold in the presence of either L-NAME or L-NMMA (Fig. 4.5). These inhibitors decreased the NOS activity in LPS-induced hemocytes to 25.7 ± 7.2 pmol/mg/min and 12.5 ± 6.3 pmol/mg/min respectively, compared to the hemocytes treated with LPS alone (135 ± 8.3 pmol/mg/min).

Northern Blot Analysis

A single NOS transcript was detected in hemocytes induced by LPS (Fig. 4.6). A 4.4 kb band was first detected at 2 hours post-injection, and this signal increased in intensity to a maximum at 8 to 12 hours post-injection. No signal was detected in hemocytes at time 0 hours post-injection. To determine if NOS mRNA was present in tissues other than hemocytes, a northern blot was performed on RNA extracted from various mGIM- and LPS-treated tissues (heads, Malpighian tubules, fat body and hemocytes). The DIG-labelled RT-PCR probe hybridized to a single band (4.4 kb) in the LPS-induced hemocyte lane (lane 4); a relatively faint band was also detected in the adjacent lane (lane 3; Fig. 4.7). In all other lanes there was no evidence of NOS mRNA.

Fig. 4.6: Time course of hemocyte NOS mRNA induction following LPS stimulation. Northern blot analysis of NOS mRNA was performed on LPS-stimulated hemocytes collected 0-12 hours post-injection. Membranes were hybridized with a NOS-specific DIG-labelled 800 bp RT-PCR probe and a cDNA probe for cytosolic actin. Lanes are labelled according to the time in which RNA was collected from hemocytes post-injection of 10 μl of 50μg/ml LPS (30 larvae/time point).

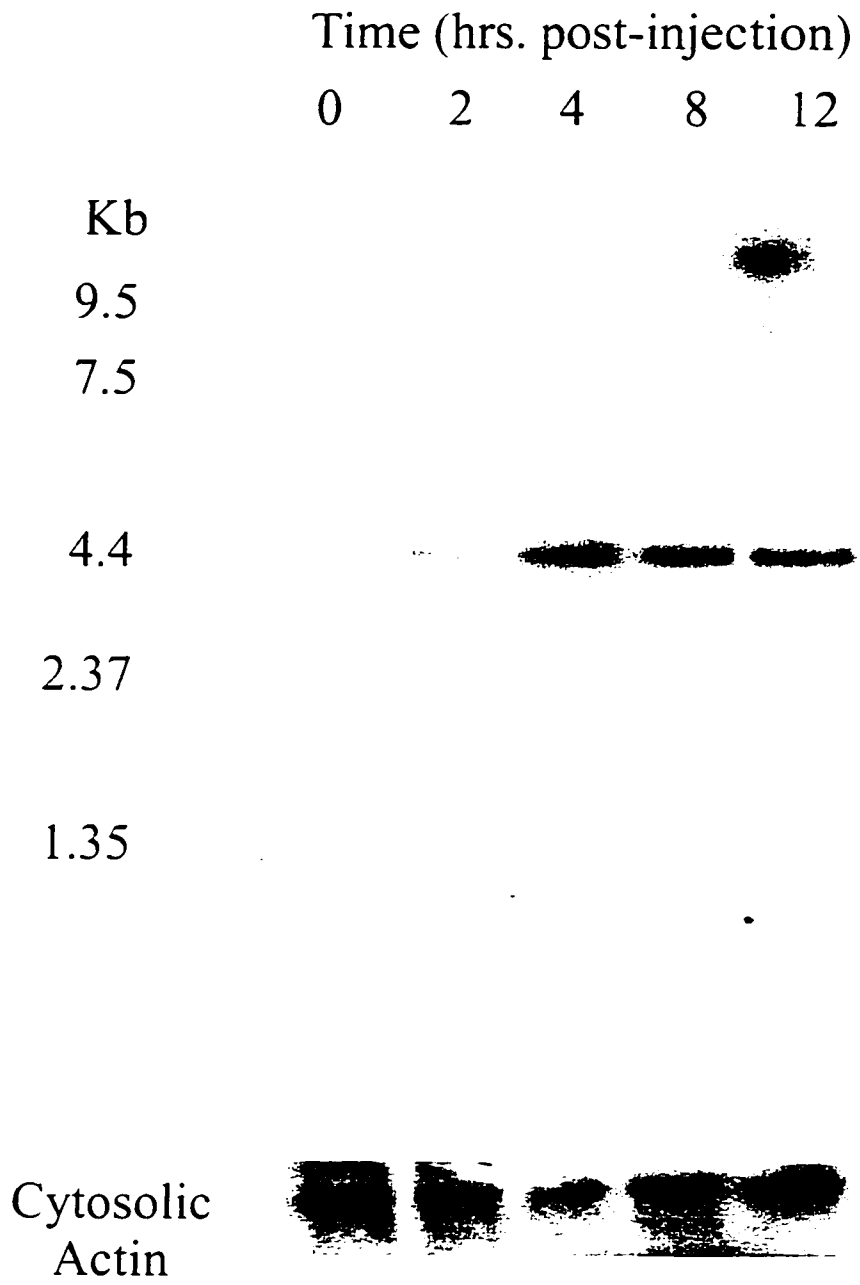
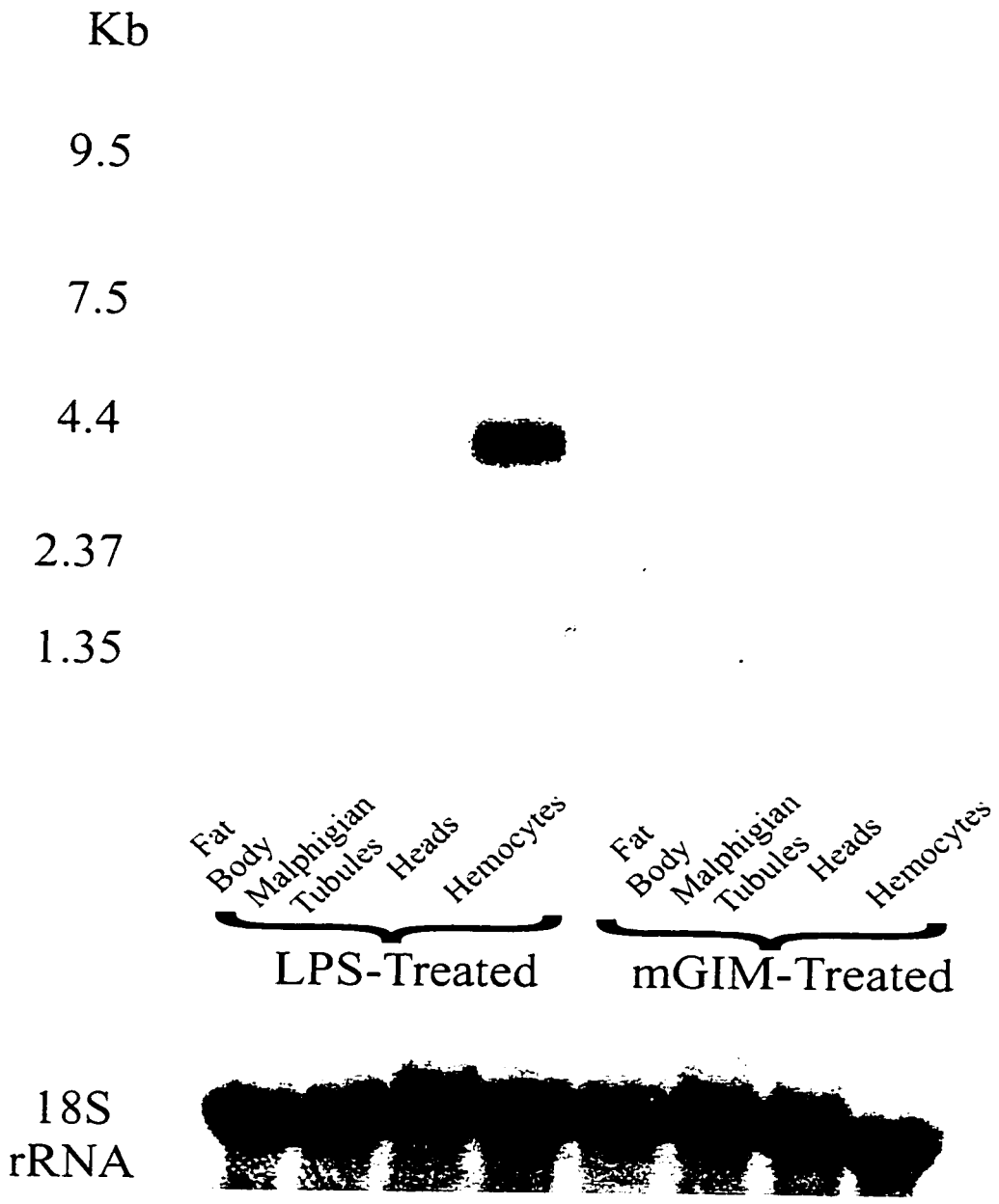

Time (hrs. post-injection)
0
2
4
8
12
Kb
9.5
7.5
4.4
2.37
1.35
Cytosolic
Actin

Fig. 4.7: Tissue-specific expression of NOS mRNA. Northern blot analysis of NOS mRNA was performed on fat body, Malphigian tubules, heads and hemocytes collected from LPS-injected larvae 8 hours post-injection. Membranes were hybridized with a NOS-specific DIG-labelled 800 bp RT-PCR probe. To confirm equal RNA loading the membranes were stained with methylene blue; 18S rRNA methylene blue stained bands are shown below the northern blot.


Kb
9.5
7.5
4.4
2.37
1.35
Fat Body
Malphigian Tubules
Heads
Hemocytes
Fat Body
Malphigian Tubules
Heads
Hemocytes
LPS-Treated
mGIM-Treated
18S rRNA

Sequence Analysis

Partial sequence (≈85%) of the NOS cDNA found in the hemocytes of *G. mellonella* is shown in Appendix A. The nucleotide sequence displays a high degree of homology with the rat iNOS and NOS cloned from *Rhodnius prolixus.* In Figure 4.2, the positions where clones N1-N6 align compared to a putative NOS cDNA is illustrated. In addition to their position, the number of bases reported from direct sequencing is also listed (Table 4.1). Complete results from the direct sequencing for each clone is outlined in Appendix C. The homology of each sequence was determined and representative homologous genes are shown in Table 4.1. Most of the clones display a high degree of homology with previously-published NOS genes and align at the appropriate location of the specified gene. A comparison of the clones N1-N5, the species percent homology and the nucleotide matching sequence are shown in Table 4.1.

Effect of LPS-Induced NO Production and Larval Survival

Eight hours P.I. with either mGIM or LPS larvae were infected with 10 cfu of the pathogenic bacteria *S. marcescens.* Larvae pre-injected with LPS had significantly higher survival rates compared to larvae pre-injected with mGIM (26.22 ± 4.3 % and 18.59 ± 5.7 %; $p>0.05$) at 12 and 15 hours post-infection (Fig. 4.8). In contrast, the treatment group pre-injected with LPS + L-NAME, had significantly lower survival rates (-33.15 ± 5.7%, -25.54 ± 4.9%, and -25.49 ± 2.4%; $p>0.05$) at 9, 12 and 15 hours post-infection when compared to larvae pre-injected with mGIM.

Table 4.1: NOS Clones and their Species Homologies

Clone	Species	Percent Homology	Sequence Location	Genebank #
N1a	*Anophelos stephensi*	67%	684-1297	AF053344
	Rattus norvegicus	66%	1354-1956	U67309
	R. norvegicus	66%	1354-1956	X59949
N1b	*R. norvegicus*	66%	1700-2376	X59949
	Oryctolagus cuniculus	65%	1434-2108	91584
	Gallus gallus	65%	937-1607	U34045
N2a	*D. melanogaster*	68%	2157-2397	D25117
	Oryctolagus cuniculus	66%	2122-2543	91584
	Human	65%	2247-2486	31466
N2b	*Rhodnius prolixus*	66%	1700-2376	U59389
	A.stephensi	59%	3062-3157	AF053344
	Cavia porcellus	56%	2932-3157	AF027180
N3	*Drosophila melanogaster*	69%	2598-3135	D25117
	R. norvegicus	61%	1999-2536	U67309
	Human	61%	2598-3135	U17327
N4	*R. prolixus*	52%	2770-2901	U59389
N5	*R. prolixus*	61%	3078-3485	U59389
	Gallus gallus	60%	3448-3610	U34045
	Human	56%	3345-3525	M95296

The effects of NO on larval survival were examined by co-injecting 10 µl of the NO donor SNAP (1mM) with 10 cfu of *S. marcescens* into larvae pre-treated with mGIM (Fig. 4.8). Survival rates of the larvae in the SNAP-treated group increased 35.14 ± 4.1%, 49.69 ± 5.1 % and 44.49± 3.5 % at 12, 15 and 18 hours post-infection respectively ($p<0.05$) when compared to control larvae (mGIM pre-treatment and 10 cfu of *S. marcescens)* .

Analysis of cGMP Concentration in Hemocytes

Competitive immunoassays for cGMP were performed to determine whether LPS-induced NO stimulated an increase in the cGMP concentration in insect hemocytes. There was a significant increase in cGMP concentration in hemocytes collected from larvae induced with LPS compared to hemocytes from mGIM-treated animals, as shown in Figure 4.9 ($p>0.05$). There was an approximate 5-fold increase in cGMP concentration in LPS-stimulated hemocytes compared to the controls (16.34 ± 2.07 pmol/mg/ml compared to 3.4 ± 1.6 pmol/mg/ml, respectively). The effect of a NO donor on cGMP was examined by treating larvae with 10 µl of SNAP (1mM). As with LPS-treated hemocytes, SNAP treatments induced a significant increase in the concentration of cGMP (9.3 ± 0.8 pmol/mg/ml) in comparison to control hemocytes ($p>0.05$). The LPS-induced increase in cGMP concentration was abolished by co-injecting the larvae with L-NAME. In the presence of the NOS inhibitor the cGMP concentration decreased to 5.7 ± 1.8 pmol/mg/ml, which was not significantly different from control hemocytes (Fig. 4.9).

Fig. 4.8: Effects of LPS-induced NO production and larval survival. Eight hours post-injection with 10 μl of mGIM, LPS (50 μg/ml) or LPS (50 μg/ml) and L-NAME (1mM), larvae were infected with 10 colony forming units of the pathogenic bacteria *S. marcescens* (40 larvae/treatment) and larval survival was monitored for 24 hours. Within 9 to 15 hours post-infection, the LPS/L-NAME treated larvae had significantly ($p<0.05$) lower survival rates than the control larvae (mGIM). LPS-treated larvae had significantly ($p<0.05$) higher survival rates between 12 and 15 hours post-infection compared to control larvae. To determine the direct effects of NO on larval survival, the NO donor SNAP (1mM) was co-injected with the *S. marcescens* in mGIM pre-treated larvae. SNAP-treated larvae had significantly ($p<0.05$) greater survival rates at 12 to 18 hours post-infection compared to control larvae (mGIM without SNAP).

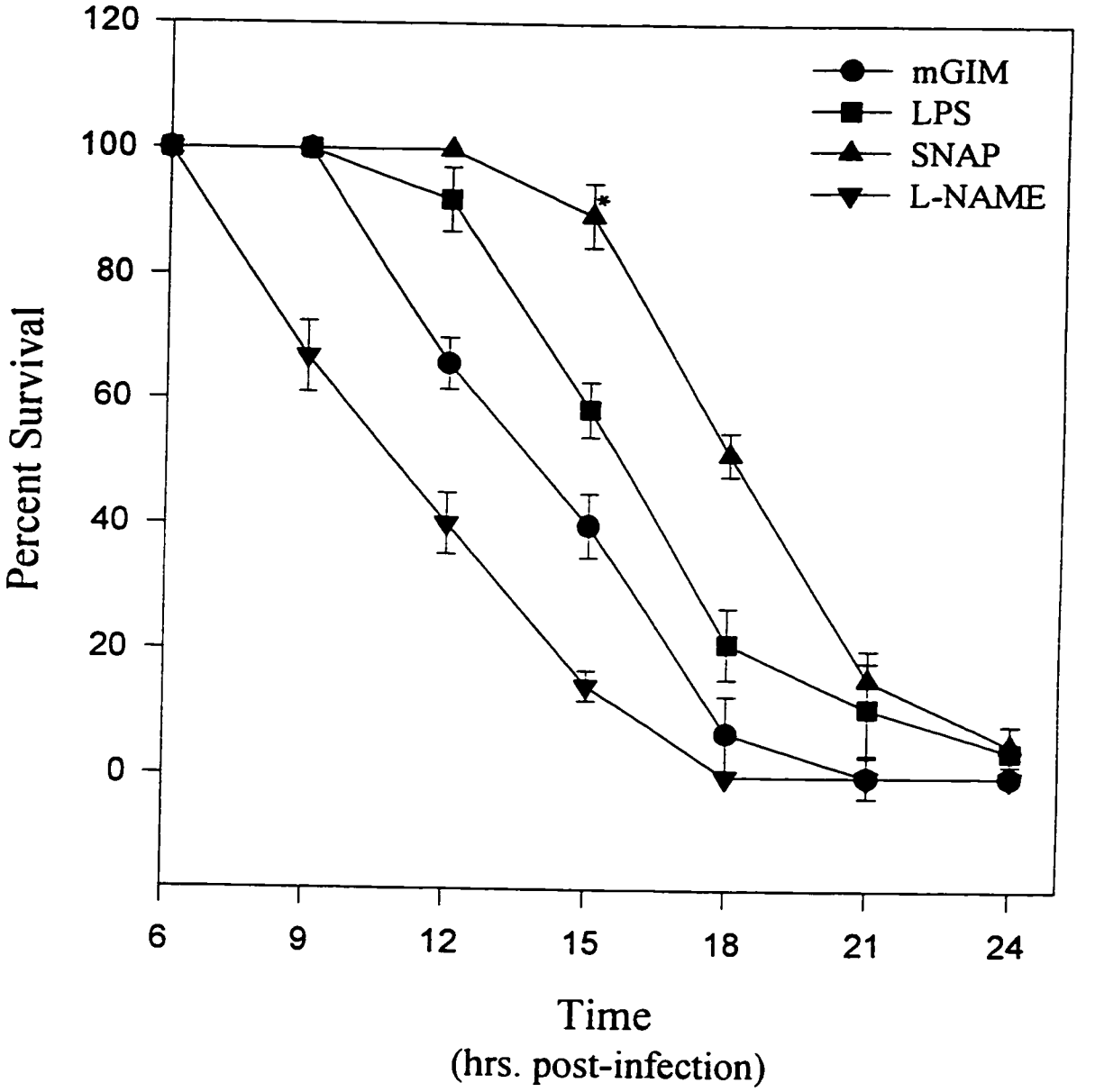
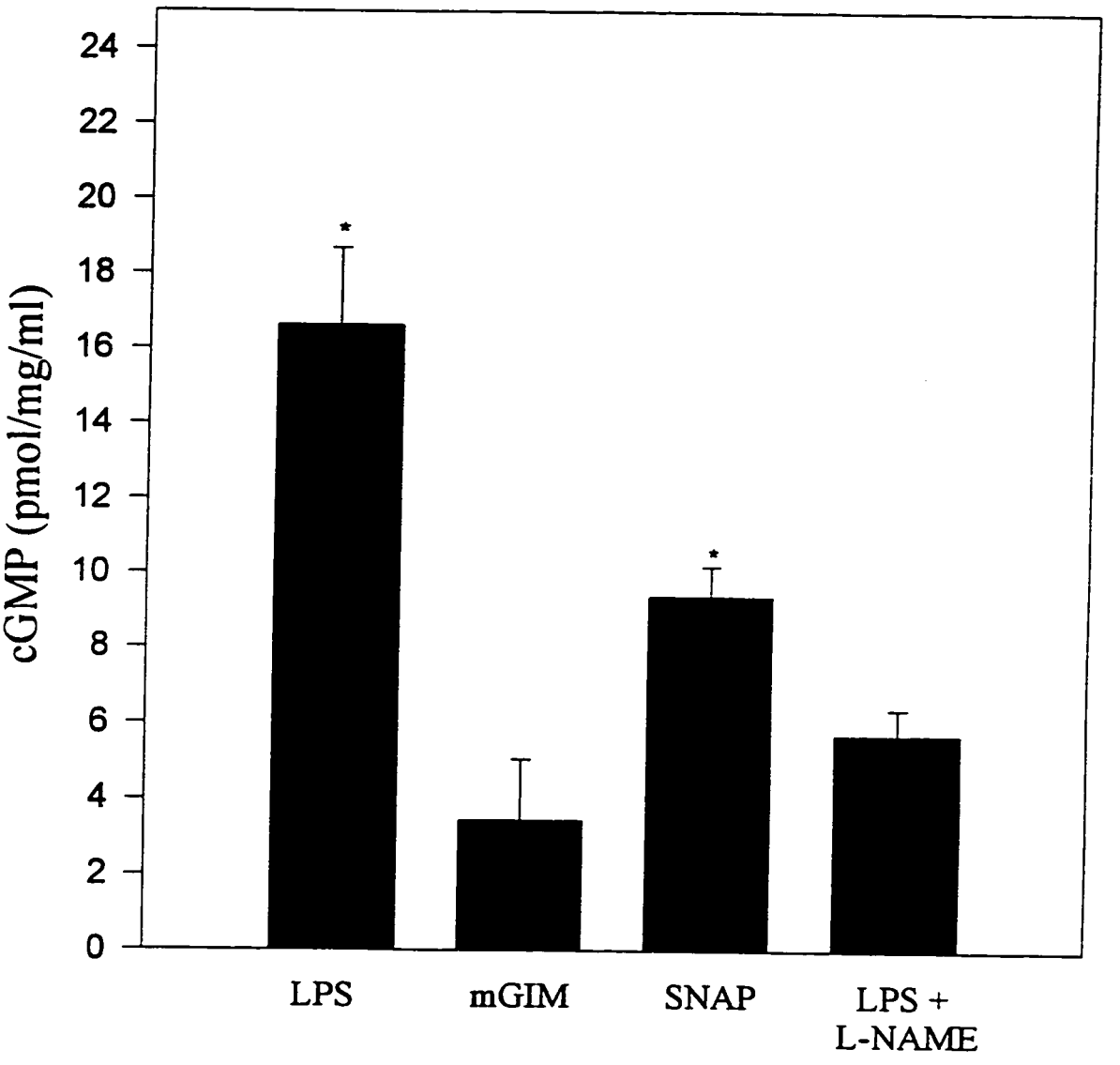

120
100
80
60
40
20
0
Percent Survival
mGIM
LPS
SNAP
L-NAME
*
6
9
12
15
18
21
24
Time
(hrs. post-infection)

Fig. 4.9: The effects of NO on hemocyte cGMP. Eight hours after injecting with mGIM, LPS, SNAP and LPS plus L-NAME (40 larvae/treatment) cytosolic protein was collected from hemocytes. Results from an EIA for cGMP showed a significant increase in cGMP in larvae injected with either LPS or SNAP, compared to control larvae (mGIM). The NOS inhibitor reduced the LPS-stimulated increase in cGMP. The bars represent the concentration of cGMP in pmol per mg of hemocyte supernatant (mean ± S.E). The asterisks indicate significant differences ($p<0.05$) from the control value.


cGMP (pmol/mg/ml)
24
22
20
18
16
14
12
10
8
6
4
2
0
*
*
LPS
mGIM
SNAP
LPS +
L-NAME

Discussion

These results demonstrate for the first time the presence of an inducible form of nitric oxide synthase, which I call gNOS, in insect hemocytes. The NOS activity found in insect hemocytes is comparable to that observed in mammalian immunocytes; similar to mammalian iNOS, gNOS is Ca^{2+}-independent and inhibited by the NOS inhibitors L-NAME and L-NMMA. Northern blot analysis revealed the existence of NOS-like mRNA in the hemocytes of *G. mellonella* larvae which had been injected with LPS. This NOS-like mRNA is found primarily in the hemocytes, although traces of mRNA found in heads may possibly be due to hemocyte contamination or non-specific binding with another NOS-like species (eg. dNOS). Furthermore, it was shown that NO upregulates the production of cGMP in hemocytes, and that nitric oxide has a physiological role in enhancing larval survival during bacterial sepsis. I propose that the insect hemocyte NOS is similar to the iNOS in mammalian macrophages; moreover, it has analogous functions to that found in vertebrate immunocytes.

In mammalian macrophages and neutrophils, NO acts as a major effector molecule for non-specific cell-mediated immunity. NO has been shown to mediate cytotoxic actions against tumours (Hibbs *et al.*, 1987) and parasites (Green *et al.*, 1990). NO has also been implicated in inducing chemotactic locomotion in human neutrophils (Beauvais *et al.*, 1995). Some of these effects have been suggested to be mediated by cGMP, due to the activation of soluble guanylate cyclase by NO.

All NOS identified in the literature catalyse a two-step 5 electron oxidation of L-arginine via an NAPDH-dependent activation of molecular oxygen, which is subsequently incorporated in NO and citrulline (Moncada *et al.*, 1991). In addition to the substrates NADPH and O_2, NOS requires flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), tetrahydrobiopterin and calmodulin as cofactors. The only chemically defined intermediate of NOS is N-hydroxyarginine, which requires the same substrates for the production of NO and citrulline (Marletta, 1993). Conte and Ottaviani (1995) confirmed that the NOS present in mollusc hemocytes require all the cofactors and substrates used by vertebrate NOS. Serendipitously, I also established that substrate β-NADPH and cofactor tetrahydobiopterin are essential for full NOS activity in insect hemocytes (data not shown).

Mechanisms of NOS induction

Other researchers have shown that the three isoforms of NOS are potentially regulated by a similar mechanism, since NO can act as a negative feedback molecule for all isoforms (Marletta, 1993). Additionally, the two constitutive isoforms, nNOS and eNOS, are regulated by Ca^{2+} in a calmodulin-dependent manner. While iNOS is not found constitutively in tissue, it must be produced *de novo* to be present, and its activity is independent of calcium. iNOS is primarily regulated at the level of transcription by lipopolysaccharide, alone or in combination with a variety of cytokines (interleukin-1β, gamma interferon, and tumour necrosis factor-α)(Marletta, 1993). LPS stimulated NOS activity in insect hemocytes at a

concentration of 50 μg/ml. This concentration is higher than the concentrations used in mammalian cell culture experiments. In my *in vivo* assay a larger concentration is like required to elicit the same activity due to the rapid clearance of LPS from the hemolymph by incorporation into the lipid transfer protein, lipophorin (Gillespie *et al.*, 1997).

Inhibitory cytokines such as interleukin-4 and interleukin-10, as well as certain growth factors have been reported to interfere with the induction of NOS in certain cell types (Ignarro, 1997). In addition to inhibitory cytokines, tyrosine kinase inhibitors (genistein, herbimycin A and typhostin) and glucocorticoids also prohibit the induction of iNOS (Wu, 1995). Little is known about insect cytokines, and it remains to be shown whether they regulate gNOS activity.

NOS and Transcriptional Regulation

Despite the report by Choi *et al.* (1995) to the contrary other evidence that prompted me to look for an inducible NOS in insect hemocytes was the great homology between the major iNOS transcription factor NF-κB and Dorsal, a transcription factor for immune proteins in insects.

In mammals, the function and regulation of NF- κB were first characterized in cultured cells (Siebenlist *et al.*, 1994). NF- κB encompasses a family of dimer-forming proteins that have homology to the *rel* oncogene (Grilli *et al.*, 1993). The modulation of gene expression by NF- κB/ Rel family is proving to have great significance in intracellular regulation of the vertebrate immune cellular response. A

variety of genes in the vertebrate immune system use the NF- κB transcriptional regulatory pathway including the induction of inflammatory cytokines, B and T cell proliferation, B cell maturation and the acute phase response (Siebenlist *et al.*, 1994).

In unstimulated cells, NF-κB remains in the cytoplasm bound to an inhibitory protein, IκB-α, which tightly binds the NF- κB dimer (p50/p65). This complex inhibits nuclear translocation (Lin *et al.*, 1995). A variety of cytokines and inducers of the immune system cause inactivation of IκB-α, which allows NF- κB to enter the nucleus where it induces gene transcription through cis-acting κB elements (Siebenlist *et al.*, 1994). The original model for the inactivation of IκB-α involved phosphorylation of the inhibitory protein complex. Presently, it is generally accepted that phosphorylation of the IκB-α alone will not release NF-κB, but that the phosphorylation initiates the proteolytic degradation of IκB-α (Lin *et al.*, 1995).

Homologous Transcription Activation in Vertebrates and Invertebrates

The NF- κB mediation of innate immune genes in vertebrates shows functional and structural homologies with the regulatory cascade controlling dorsal-ventral patterning in *Drosophila* (Ip *et al.*, 1991). Analysis of the genes involved in the development of *Drosophila* embryo reveals that they are mediated by the Toll-Dorsal pathway. Toll, an IL-1 receptor analogue, is activated by a specific morphogen; this initiates nuclear translocation of the transcription factor Dorsal.

Similar to the way NF-κB is retained in the cytoplasm by IκB-α, Dorsal cannot enter the nucleus when bound to the inhibitor complex cactus (Ip *et al.*, 1995). When dorsal is released from cactus and translocates to the nucleus, it activates genes which regulate developmental patterning. The great homology in structure and function between the NF-κB/IκB-α and the dorsal/cactus indicates an evolutionary conservation of this ancient signalling pathway.

There are also indications that the Toll/Dorsal signal pathway has a function within the immune system of insects. LPS is a strong inducer of many insect immune molecules such as cecropins, attacins and lysozymes. Sequences similar to the NF- κB binding sites in vertebrates have been identified in the promoter regions of these immune molecules in *D. melanogaster* and *H. cecropia* (Sun *et al.*, 1991). Upstream regions with homology to the NF- κB binding site have been detected in all the cecropin genes (except D), acidic and basic attacins genes and defensin genes (Sun *et al.*, 1991; Engstrom *et al.*, 1993; Kappler *et al.*, 1993). Sun and Faye (1992) demonstrated the presence of a protein, Cecropin Immunoresponsive Factor (CIF), which has a similar structure to mammalian NF- κB and binds NF-κB DNA motifs. CIF appears to be a crucial transcription factor for the expression of certain immune genes in insects.

Based on such evidence, I expect that gNOS in hemocytes is also induced by CIF or some like factor. There are some data to suggest components of this pathway exist in hemocytes. Lemaitre *et al.* (1995) demonstrated that *Drosophila* larvae lacking cactus have a high frequency of melanotic tumors (nodules) in the

absence of infection. However, the fact that mutants which lack both cactus and dorsal still produce nodules in the absence of infection suggests that another signalling pathway leading to the activation of hemocytes must exist.

Role of NOS in Invertebrate Physiology

Nitric oxide synthase has been immunocytochemically demonstrated and characterized in the hemocytes of the snail, *Viviparus ater* (Conte and Ottaviani, 1995). Furthermore, they suggest that bacterial elimination by the *V. ater* hemocytes correlates with increased nitric oxide production (Franchini *et al.*, 1995), a finding congruent with the enhancement of larval survival shown in my study. Although my findings demonstrate a physiological role for NO in the insect immune system, there have been prior examples of physiological action of NO in other insect tissues.

In insects, nitric oxide synthase has been characterized on the molecular level in *Drosophila* dNOS (Regulski and Tully, 1995). dNOS, which is regulated by intracellular Ca^{2+}, is very similar to the constitutively-expressed mammalian nNOS. Regulski and Tully (1995) postulated that dNOS may play a role in neuronal plasticity in *Drosophila*. Nitric oxide has also been demonstrated to act as an antiproliferation agent during *Drosophila* development and controls the balance between cell proliferation and cell differentiation (Kuzin *et al.*, 1996). Kuzin *et al.*(1996) have demonstrated that NOS is expressed at very high levels in developing imaginal discs and that inhibition of NOS in larvae causes hypertrophy of developing organs.

Choi *et al.* (1995) presented evidence for the existence of NOS/NO in the fat body and Malpighian tubules of *Bombxy mori*, and proposed that the NOS gene may be regulated by ecdysone during metamorphosis of the insect. The observation that mammalian eNOS and nNOS are regulated by estrogen during pregnancy led these researchers to postulate that NOS may play a role in morphological and physiological changes during insect development. Alternatively, if NOS is upregulated by ecdysone, it could prove to be an effective way of heightening the insect's immune system at a crucial time in development, when the insect is most vulnerable to pathogen. If such speculations are correct, then it is possible that activation of gNOS in hemocytes may be developmentally cued at various points in the molt cycle.

The biosynthesis of small quantities of NO appears to be important for the maintenance of normal physiological functions in vertebrate cardiovascular and nervous systems. Nitric oxide has also been proven to be involved in invertebrate neurophysiology. Nitric oxide synthase has been histochemically identified in the brains of *Drosophila* and *Apis*, with the most intense staining expressed in neuropils associated with the olfactory pathways (Muller and Bicker, 1994). The association of NOS with the antennal lobe and olfactory bulb appears to be similar in both insects and vertebrates (Bredt and Snyder, 1992; Muller, 1994). In vertebrates, a major signalling pathway in the olfactory system is NO/cGMP, which may mediate lateral interaction between dendrites and the glomeruli of the olfactory bulb (Breer

and Shephard, 1993). This same regulatory pathway may be working in the glomeruli of the antennal lobe in insects (Muller, 1994).

Dow *et al.* (1994) presented evidence that all the elements of the NO pathway are present and control epithelial fluid secretion in the Malpighian tubules of *Drosophila*. These authors proposed that NO-induced cGMP acts as a second messenger affecting cGMP-dependent protein kinases (PKG).

In addition to regulatory functions, Ribeiro *et al.* (1993) describe another use for NO in insects. NO causes vasodilatation of vascular smooth muscle by activating soluble guanylate cyclase, which has a heme-iron-containing protein. The salivary glands of the blood sucking insect *Rhodnius prolixus* are bright red due to the abundance of heme-containing proteins. *R. prolixus* produces a salivary vasodilator with the properties of NO. Furthermore, a mixture of these nitrosylated heme-containing proteins in the salivary glands both binds and releases NO (Ribeiro *et al.*, 1993). Once the insect has injected the nitrosylated heme complex into the blood of its prey, there is a shift in equilibrium towards the dissociation of NO from Fe^{3+} to the Fe^{2+} in the soluble guanylate cyclase in smooth muscle. This achieves local dilation at the site of the bite and may also inhibit platelet aggregation which could interfere with feeding by the insect (Ribeiro *et al.*, 1993).

Mechanisms of NO Toxicity

The biosynthesis of large quantities of NO by the inducible form of NOS in vertebrates seems to be associated with widespread cytotoxicity of NO to the

invading micro-organisms and viral particles and some host cells. The mechanism(s) by which NO exerts antimicrobial and antiviral actions and/or cytotoxicity are subject to controversy. One suggestion has been that NO may act indirectly on critical cellular components of foreign micro-organism, such as protein-bound iron or iron-sulfur clusters, and cause their destruction. This would lead to cytostatic or cytocidal actions (Henry *et al.*, 1993). A second mechanism for the cytotoxicity of NO is the interaction of NO with superoxide ions to generate peroxynitrite. Peroxynitrite is a powerful oxidant and favours the selective attack and modification of key chemical moieties involved in cellular signalling pathways. Peroxynitrite is particularly reactive with zinc-fingers, which are important transcription factors and iron-sulfur centres which are crucial in cellular respiration (Beckman, 1991).

In this chapter, it was reported that larvae which have been stimulated to produce NO or injected with a NO donor exhibit increased survival rates when infected with the pathogenic bacteria *S. marcescens*. This augmentation of the immune response could be viewed as an adaptive process, conferring a larger secondary response. At this time, however, it is not clear if this adaptive response is due to the formation of peroxynitrite or the direct actions of NO.

NO as a Second Messenger

Not only does NO have direct effects on cellular physiology, it also acts as a first order second messenger. Due to its high solubility, NO moves relatively freely throughout the cytosol, where it can bind to, and subsequently activate soluble

guanylate cyclase. NO has an exceptionally high affinity for heme iron and it has been speculated that NO binds to guanylate cyclase through the formation of a nitrosyl-heme complex. This binding subsequently changes the protein conformation and activates guanylate cyclase (Ignarro 1996).

The increase in NO-induced cGMP has been suggested to play a major role in neutrophil chemotaxis by increasing cell polarization (Caterina and Deverotes , 1991). However, cGMP in insect hemocytes does not seem to be involved in chemotaxis or motility. Hemocytes treated with a non-degradable analogue of cGMP, 8-bromo-cGMP, exhibited no significant changes in motilty (data not shown). The function of NO-mediated elevation of cGMP therefore remains to be resolved, and this should prove to be a fruitful area of future research.

In summary, biochemical, molecular and physiological evidence presented in this chapter all strongly suggest the presence of NOS/NO in insect hemocytes. The enhancement of larval survival during sepsis further demonstrates a physiological function of NO in the insect immune response. These data not only imply a further homology between the vertebrate and invertebrate immune responses; they also point to a form of secondary immune response within the innate system.

References

Beauvais, F., L. Michel and L. Dubertret. 1995. Exogenous nitric oxide elicits chemotaxis of neutrophils *in vitro*. J. Cell. Phys. 165:610-614.

Beckman, J. S. 1991. The double edged role of nitric oxide in brain function and superoxide-mediated pathology. J. Devel. Physiol. 15:53-59.

Bredt, D. S. and S. H. Synder. 1989. Nitric oxide mediates glutamate-linked enhancement of cGMP levels in the cerebellum. Proc. Natl. Acad. Sci. 86: 9030-9033.

Bredt, D. S. and S. H. Synder. 1992. Nitric oxide, a novel neuronal messenger. Neuron. 8:3-11.

Breer, H. and G. M. Shephard. 1993. Implication of NO/cGMP system for olfaction. Trends Neurosci. 46:5-9.

Caterina , M. J. and P. N. Deverotes. 1991. Molecular insights into eukaryotic chemotaxis. FASEB. 5:3078-3085

Champagne, D. E., R. H. Nussenzveig and J. M. C. Ribeiro. 1995. Purification, partial characterization, and cloning of nitric oxide-carrying heme proteins (nitrophorins) from the salivary glands of the blood sucking insect *Rhodnius prolixus*. J. Biol. Chem. 270:8691-8695.

Choi, S. K., H. K. Choi, K. Kadono-Okuda, K.Taniai, Y, Kato, M. Yamamoto, S. Chowdhury, J. Xu, A. Miyanoshita, N. C. Debnath, A. Asaoka and M. Yamakawa. 1995. Occurance of novel types of nitric oxide synthase in the silkworm, *Bombyx mori*. Biochem. Biophys. Res. Comm. 207:452-459.

Chomczynski, P. and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidunium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162:156-159.

Conte, A. and E. Ottaviani. 1995. Nitric oxide synthase activity in molluscan hemocytes. FEBS Lett. 365:120-124.

Dietmaier, W. and S. Fabry. 1995. Protocol: Di-/Trinucleotide Sticky End Cloning. PCR Application Manual, Boehringer Mannheim:136-138.

Dow, J. T., S. H. P. Maddrell, S. A. Davis, N. J. V. Skaer and K. Kaiser. 1994. A novel role for the nitric oxide-cGMP signaling pathway: the control of epithelial function in *Drosophila*. Am. J. Physiol. 226:R1716-R1719.

Engstrom, Y., L. Kadalayil, S-C. Sun, C. Samakovlis, D. Hultmark and I. Faye. 1993. κB-like motifs regulate induction of immune genes in *Drosophila*. J. Mol. Biol. 232:327-333.

Franchini, A., P. Fontanili and E. Ottaviani. 1995. Invertebrate immunocytes: relationship between phagocytosis and nitric oxide production. Comp. Biochem. Physiol. 110B:403-407.

Gillespie, J.P., M.R. Kanost and T. Trenczek. 1997. Biological mediators of insect immunity. In: *Annual Review of Entomology* (T. Mittler, F. Radovsky, V. Resh, eds.)42: 611-644. Annual Reviews Inc. Palo Alto, CA.

Green, S. J., M. S. Meltzer, J. B. Hibbs and C. A. Nacy. 1990. Activated macrophages destroy intracellular Leishmania major amastigotes by L-arginine-dependent killing echanism. J. Immunol. 144:278-283.

Grilli, M., J. Chiu and M. Lenardo. 1993. NF-κB and Rel: participants in a multiform transcriptional regulation system. Int. Rev. Cytology. 143:1-62.

Henry, Y., M. Lepoivre, J.-C. Drapier, C. Ducrocq, J. L. Boucher and A. Guissani. 1993. EPR characterization of molecular targets for NO in mammalian cells and organelles. FESEB J. 7: 1124-1134.

Hibbs, J. B., R. R. Taintor and Z. Vavrin. 1987. Macrophage cytotoxicity: Role for L-arginine deiminase and imino-nitrogen oxidation to nitrate. Science. 235: 479-476.

Ignarro, L. J. 1996. Nitric oxide as a communication signal in vascular and neuronal cells. In: *Nitric Oxide: Principles and Actions*. (J. Lancaster, ed.) 111-138. Academic Press Inc., San Diego, CA.

Ignarro, L. J. 1997. Activation and regulation of the nitric oxide-cyclic GMP signal trasnduction pathway by oxidative stress. In: *Oxidative Stress and Signal Transduction*. (H. J. Forman and E. Cadenas, eds.) 3-31. Chapman and Hall, Toronto. ON.

Ip, T., R. Kraut, M. Lavine and C.S. Rushlow. 1991. The dorsal morphogen is a sequence-specific DNA-binding protein that interacts with a long-range repression element in *Drosophila*. Cell. 64:439-446.

Ip, Y. T., M. Reach, Y. Engstrom, L. Kadalayil, H. Cai, S. Gonzalez-Crespo, K. Tatei and M. Levine. 1995. Dif, a dorsal-related gene that mediates an immune response in *Drosophila*. Cell. 75:753-763.

Kanost, M. R., M.K. Zepp, N.E. Ladendorff and L.A. Andersson. 1994. Isolation and characterization of a hemocyte inhibitor from the hemolymph of *Manduca sexta* larvae. Arch. Insect Biochem. Physiol. 27:123-136.

Kappler, C. M. M., M. Lagueux, E. Gateff, J. A. Hoffmann and J-M. Reichhart. 1993. Insect immunity, two 17bp repeats nesting a κB-related sequence confer inducibility to the diptericin gene and bind a polypeptide in bacteria-challenged *Drosophila*. EMBO J. 12:1561-1568.

Kuzin, B., I. Roberts, N. Peunova and G. Enikolopov. 1996. Nitric oxide regulates cell proliferation during *Drosophilia* development. Cell. 87:639-649.

Lamaitre, B., M. Meister, S. Govind, P. Georgel, R. Steward, J.M.Reichhart and J.A. Hoffman. 1995. Functional analysis and regulation of nuclear import of dorsal during the immune response in *Drosophila*. EMBO. J. 14:536-45

Lin, Y.-C., K. Brown and U. Siebenlist. 1995. Activation of NF- kB requires proteolysis of the inhibitor IκB-α: signal-induced phosphorylation of IkB-alpha alone does not release active NF-κB. Proc. Natl. Acad. Sci. 92: 552-556.

Marletta, M. A. 1993. Nitric oxide synthase structure and mechanism. J. Biol. Chem. 268:12231-12234.

Mayer, B. 1994. Nitric oxide/cyclic GMP-mediated signal transduction. In: *Molecular and Cell Biological aspects of Gastroenteropancreatic Neuroendocrine Tumor Disease* (B. Wiedenmann, L.K. Kvols, R. Arnold and E.-O. Riecken, eds.) 733:357-361. The New York Academy of Sciences, New York, NY.

Moncada, S., R. M. J. Palmer and E.A. Higgs. 1991. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharma. Rev. 43:109-142.

Muller, U. 1994. Ca^{2+}/calmodulin dependent nitric oxide synthase in *Apis mellifera* and *Drosophila melanogaster*. Eur. J. Neurosci. 7:2240-2248.

Muller, U. and G. Bicker. 1994. Calcium-activated release of nitric oxide and cellular distribution of nitric oxide-synthesizing neurons in the nervous system of the locust. J. Neurosci. 14:7521-7528.

Regulski, M. and T. Tully. 1995. Molecular and biochemical characterization of dNOS: a Drosophila Ca^{2+} / calmodulin-dependent nitric oxide synthase. Proc. Natl. Acad. Sci. USA. 92:9072-9076.

Ribeiro, J. M. C., J. M. H. Hazzard, R. H. Nussenzveig, D. E. Champagne and F. A. Walker. 1993. Reversible binding of nitric oxide by a salivary heme protein from a bloodsucking insect. Science. 260:539-541.

Sambrook, J., E. F. Fritsch and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, NY.

Siebenlist, U., G. Franzoso and K. Brown. 1994. Structure, regulation and function of NF-κB. Annu. Rev. Cell. Biol. 10:405-455.

Sun, S.-C., I. Lindstrom, J-Y. Lee, and I. Faye. 1991. Structure and expression of the attacin gene in Hyalophora cecropia. Eur. J. Biochem. 196:247-254.

Sun, S.-C. and I. Faye. 1992. Cecropia immunoresponsive factor, an insect immunoresponsive factor with DNA-binding properties similar to NF-κB. Eur. J. Biochem. 204:885-892.

Wu, K. K. 1995. Inducible cyclooxygenase and nitric oxide synthase. In: *Advances in Pharmacology*.(M. W. Anders, J. T. August, F. Murad and J. T. Coyle, eds.) 33: 179-208. Academic Press,Toronto, ON.

Chapter 5

The Relationship Between Nitric Oxide and Eicosanoids

Abstract

Nitric oxide synthase (NOS) has been previously shown to exist in insect hemocytes (see Chapter 4). Furthermore, nitric oxide (NO) has proven to play an important role in the modulation of the insect immune response. In the present work, a second class of immuno-modulators, the eicosanoids, were examined to study their effect on the inducible NOS in insect hemocytes (gNOS). Eicosanoid biosynthesis inhibitors were used in combination with the immuno-stimulator lipopolysaccharide (LPS) to measure NOS activity, levels of hemocyte NOS mRNA and the concentration of cGMP in hemocytes, a second messenger of NO. NOS activity in LPS-stimulated hemocytes was significantly reduced when co-treated with the eicosanoid biosynthesis inhibitors dexamethasone, eicosatetraynoic acid and indomethacin. Northern blot analysis revealed substantial decreases in relative NOS mRNA levels in LPS-induced hemocytes treated with dexamethasone and indomethacin. Dexamethasone also reduced the concentration of cGMP in LPS-induced hemocytes. These data suggest that either eicosanoid biosynthesis inhibitors or eicosanoids mediate the production of NO in insect hemocytes.

Introduction

Both eicosanoid and nitric oxide (NO) production increase during inflammatory processes in vertebrates (Wu, 1995), and both are recognized as important modulators of the vertebrate immune response. Recent evidence suggests that eicosanoids and NO are also immune modulators in insects (Stanley-Samuelson *et al.*, 1991; Miller *et al.*, 1994; Mandato *et al.*, 1997; Chapter 3). Such homologies suggest either the evolutionary conservation or the convergence of immuno-regulatory mechanisms between vertebrates and invertebrates.

Recent work with mouse and rat macrophages further suggests links between eicosanoids and NO (Salvemini *et al.*, 1993; Aeberhard *et al.*, 1995). Nononsteroidal anti-inflammatory drugs (NSAIDs), such as aspirin, ibuprofen and indomethacin have been shown to inhibit both prostaglandin synthesis and NO production. In addition to NSAIDs, glucocorticoids have also been used to inhibit both immune modulators. It has long been known that NSAIDs and glucocorticoids inhibit cyclooxygenase (COX-1 and COX-2), and consequently diminish prostaglandin (PG) synthesis (Wu, 1995). Aeberhard *et al.* (1995) reported that NSAIDs also inhibit the expression of the inducible nitric oxide synthase (iNOS) gene in rat alveolar macrophages. Other levels of interaction between NO and PGs include the inhibitory effect of PGE_2 on iNOS (Tetsuka *et al.*, 1994) and the activation of COX by NO (Salvemini *et al.*, 1993). Additionally, other investigators have reported that glucocorticoids also inhibit the induction of NOS in macrophages (Di Rosa *et al.*, 1990).

In this chapter, the putative relationship(s) between NO and eicosanoids in insect blood cells or hemocytes is investigated. Three distinct eicosanoid biosynthesis inhibitors, dexamethasone, indomethacin and the arachidonic analogue eicosatetraynoic acid (ETYA) all inhibit NO production, likely at the transcriptional level. Dexamethasone also diminished the production of cGMP, one of the main NO-induced second messengers. These results lend further support to the notion of an interaction between NO and PG biosynthesis pathways, and suggest a common ancestry between vertebrate and invertebrate regulatory pathways in the innate immune response.

Materials and Methods

Eicosanoid Inhibitors

The phospholipase A_2 (PLA_2) inhibitors dexamethasone and ETYA, and the cyclooxygenase inhibitor indomethacin were each dissolved in absolute ethanol and further diluted with mGIM such that the ethanol concentration was less than 0.5% (v/v). Larvae of the Greater Wax Moth, *Galleria mellonella*, were injected with 10 µl of LPS and inhibitor at concentrations of 50 µg/ml and 1 mM, respectively (40 replicates per group). Initial experiments demonstrated that ethanol (less than 1% v/v) had no effect on NOS activity in LPS-induced hemocytes (data not shown).

NOS Activity

Hemocytes from larvae treated with LPS and eicosanoid biosynthesis inhibitors were collected (see General Materials and Methods) and NOS enzymatic activity was assayed (see Chapter 4). Enzymatic activity is reported as pmol of ^{3}H-citrulline produced per minute of the reaction per mg of protein in the hemocyte homogenate. Statistical significance for this assay and all subsequent assay was determined by a paired student's T-test.

RNA Isolation and Northern Blot Analysis

RNA was extracted from activated hemocytes treated with eicosanoid biosynthesis inhibitors using the single step method of Chomczynski and Sacchi (1987), as discussed in the previous chapter. Northern blot analyses were performed

on isolated mRNA using the original 800 bp probe designed for the identification of the iNOS-like transcript and a probe for cytsolic actin (see Chapter 4). mRNA intensities were quantified by scanning densitometry (ImageQuant, Molecular Dynamics). Relative intensities of NOS mRNA were calculated by ratioing the intensity of each NOS band with its corresponding cytosolic actin control band to remove differences due to changes in RNA loading. These ratios were then used to calculate the percent differences between LPS-induced hemocytes and LPS-induced hemocytes treated with eicosanoid biosynthesis inhibitors.

cGMP Immunoassay

To determine whether eicosanoid biosynthesis inhibitors affected the concentration of cyclic guanosine monophosphate (cGMP) in induced hemocytes, larvae were injected with 10 µl of 50 µg/ml LPS, LPS + dexamethasone, or modified Grace's Insect Medium (mGIM) (40 replicates per treatment group). Eight hours post-injection, hemocytes were collected and homogenized in ice cold mGIM containing 1 mM dithiothreitol and 1mM 3-isobutyl-1-methyl-xanthine, which inhibit phosphodiesterase activity. This preparation was centrifuged at 16 000 g for 30 min at 4°C, and 100 µl of the supernatant assayed for cGMP in a correlated Enzymatic Immunoassay (EIA) (see Chapter 4). Concentrations are reported as pmol of cGMP per mg of cytosolic protein.

Results

Effect of NSAIDs and Dexamethasone on NOS Activity

All eicosanoid biosynthesis inhibitors tested significantly decreased NOS activity in LPS-treated hemocytes ($p>0.05$) (Fig. 5.1). The PLA_2 inhibitors dexamethasone and ETYA reduced NOS activity to 36 ± 5.4 pmol/mg/min and 32 ± 8.2 pmol/mg/min, respectively, compared to LPS-treated hemocytes (135.5 ± 8.3 pmol/mg/min). The cyclooxygenase inhibitor indomethacin reduced NOS activity 67% (44.3± 7.4 pmol/mg/min) compared to the LPS-treated hemocytes.

Effect of Dexamethasone on NOS mRNA

The effects of eicosanoid biosynthesis inhibitors on NOS mRNA expression were also examined by northern blot analysis. Injection of dexamethasone resulted in the largest decrease in NOS mRNA expression in LPS-induced hemocytes. The relative NOS mRNA intensity, as determined by scanning densitometry, decreased 80% (Fig. 5.2).

Effect of NSAIDs on NOS mRNA

The relative intensities of NOS mRNA were also determined for hemocytes treated with ETYA and indomethacin. The relative mRNA was decreased in LPS-induced hemocytes treated with either ETYA or indomethacin (20% and 71%, respectively) (Figs. 5.3 and 5.4).

Fig. 5.1: Effects of dexamethasone, ETYA and indomethacin on the induction of NOS activity. Hemocytes were collected from larvae co-injected with LPS with and without each inhibitor and NOS activity was determined 8 hours post-injection (40 larvae/treatment). The bars represent NOS activity measured in pmol of ^{3}H-citrulline produced per mg of hemocyte supernatant per minute (mean ± S.E.; n=9). The asterisk indicates a significant difference ($p<0.05$) from the control value.

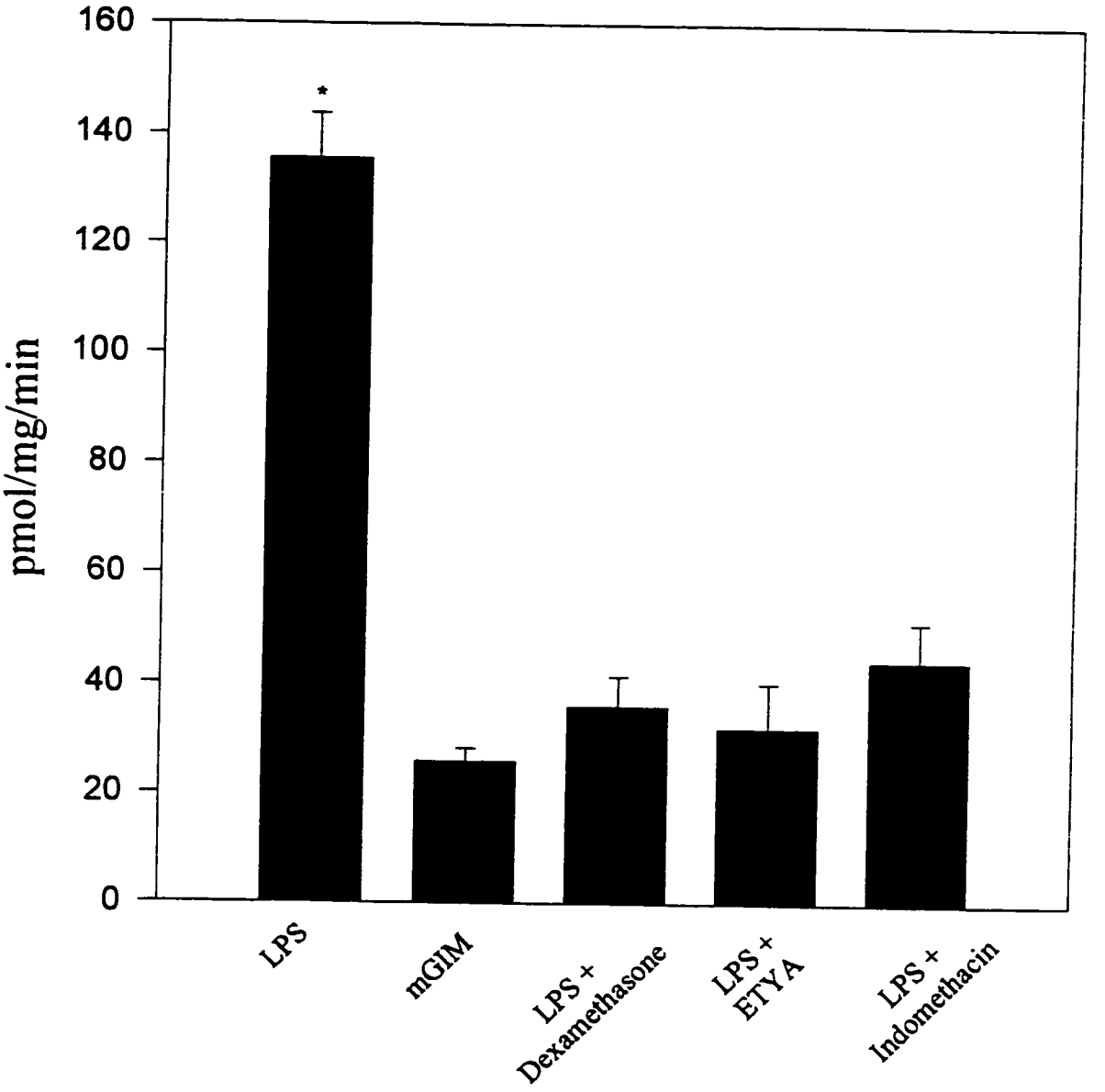
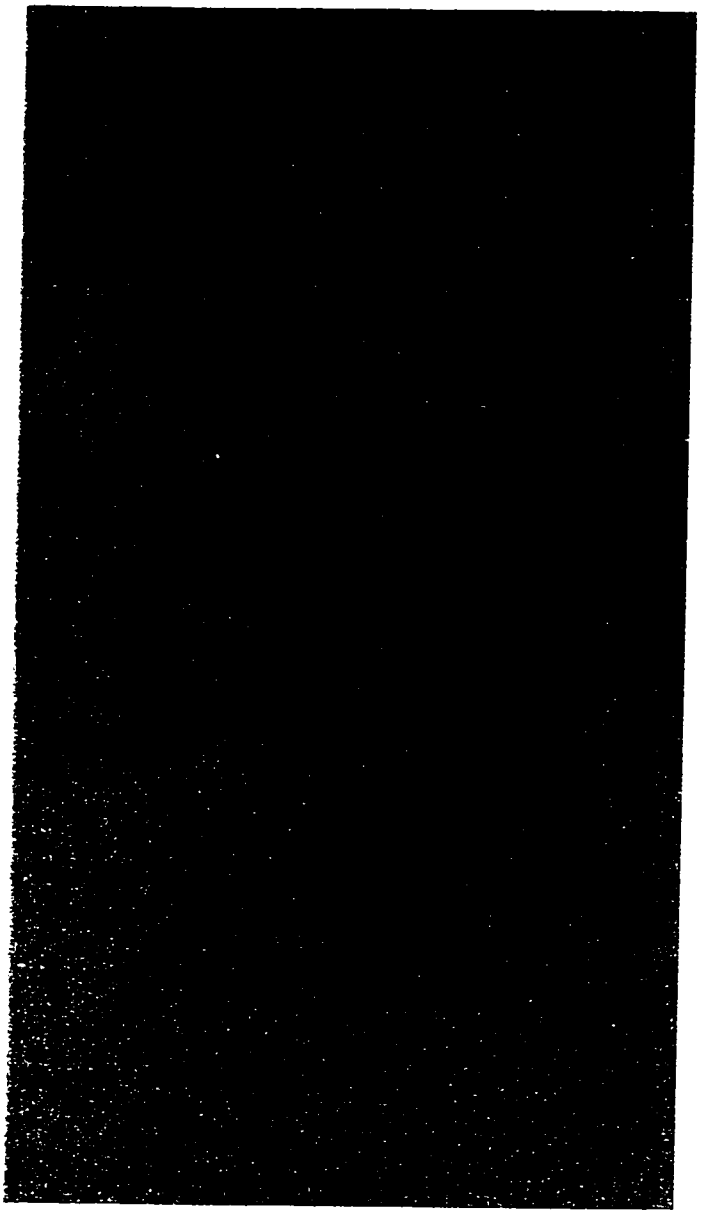

160
140
120
100
80
60
40
20
0
pmol/mg/min
*
LPS
mGIM
LPS + Dexamethasone
LPS + ETYA
LPS + Indomethacin

Fig. 5.2: Effect of dexamethasone on the induction of NOS mRNA. At eight hours post-injection with either mGIM, LPS or LPS and dexamethasone (30 larvae/ treatment) total RNA was collected from hemocytes and northern blot hybridization for NOS mRNA was performed. Lane 1: control hemocytes (mGIM-injected); Lane 2: hemocytes from larvae co-injected with LPS and dexamethasone; Lane 3: LPS-induced hemocytes. Dexamethasone decreased the relative mRNA intensity by 80% compared to the LPS-treatment alone (determined by scanning densitometry).

iNOS mRNA



Treatments mGIM LPS – Dexamethasone LPS

Cytosolic Actin

Fig. 5.3: Effect of ETYA on the induction of NOS mRNA. Eight hours post-injection with either mGIM, LPS or LPS and ETYA (30 larvae/treatment) total RNA was collected from hemocytes and northern blot hybridization for NOS mRNA was performed. Lane 1: control hemocytes (mGIM-injected); Lane 2: hemocytes from larvae co-injected with LPS and ETYA; Lane 3: LPS-induced hemocytes. ETYA decreased the relative mRNA intensity by 22% compared to the LPS-treatment alone (determined by scanning densitometry).


iNOS mRNA

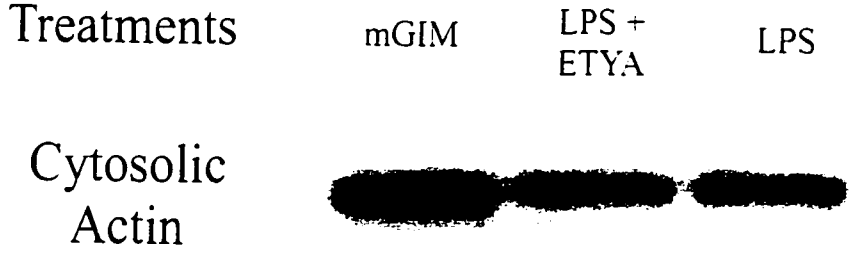
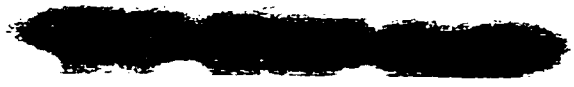

Treatments
mGIM
LPS + ETYA
LPS
Cytosolic Actin

Fig. 5.4: Effect of indomethacin on the induction of NOS mRNA. At eight hours post-injection with either mGIM, indomethacin and LPS or LPS alone (30 larvae/treatment) total RNA was collected from hemocytes and northern blot hybridization for NOS mRNA was performed. Lane 1: control hemocytes (mGIM-injected); Lane 2: hemocytes from larvae co-injected with LPS and indomethacin; Lane 3: LPS-induced hemocytes. Indomethacin injection decreased the relative mRNA intensity by 71% compared to the LPS-treatment alone (determined by scanning densitometry).

iNOS mRNA

Treatments	mGIM	LPS + Indomethacin	LPS

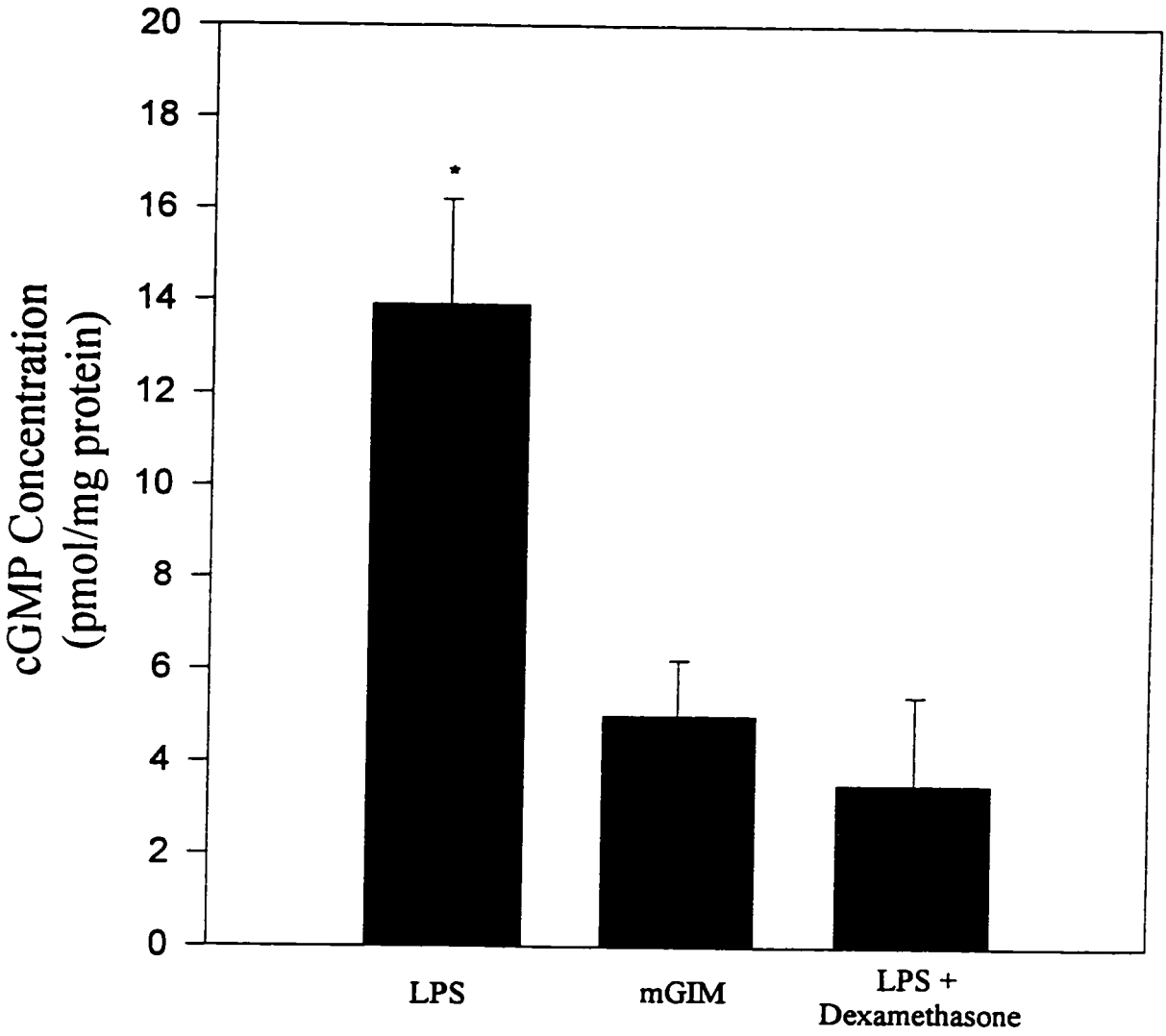
Cytosolic Actin



Effect of Dexamethasone on Hemocyte cGMP

As indicated in the Chapter 4, soluble guanylate cyclase activation is a sensitive indicator of NOS. The concentration of cGMP significantly decreased in LPS-induced hemocytes treated with dexamethasone ($p<0.05$). As indicated in Fig. 5.5, cGMP in dexamethasone-treated hemocytes was 3.52 ± 1.19 pmol/mg, compared to 13.9 ± 2.3 pmol/mg in LPS-induced hemocytes.

Fig. 5.5: Effect of dexamethasone on LPS-stimulation of cGMP. At eight hours post-injection with mGIM, LPS and dexamethasone of LPS (40 larvae/treatment) cytosolic protein was collect from hemocytes. Results from an EIA for cGMP showed no increase in cGMP concentrations in larvae injected with both LPS and dexamethasone compared to LPS alone. The bars represent the concentration of cGMP measured in pmol per mg of hemocyte supernatant (mean ± S.E). The asterisk indicates significant difference ($p<0.05$) from the control value.


cGMP Concentration
(pmol/mg protein)
20
18
16
14
12
10
8
6
4
2
0
*
LPS
mGIM
LPS +
Dexamethasone

Discussion

The presence of iNOS in hemocytes from LPS-treated insect larvae was demonstrated in the preceding chapter. The results of the present study indicate that, similar to recent findings on mouse macrophage NOS, eicosanoids mediate NOS activity in hemocytes. Three separate lines of evidence support this assertion. First, the eicosanoid biosynthesis inhibitors dexamethasone, indomethacin and ETYA all significantly reduced NOS activity, as measured by radioactive enzymatic assays. Second, these eicosanoid inhibitors all reduced the expression of NOS mRNA in LPS-stimulated hemocytes, as demonstrated by northern blot analysis. Third, dexamethasone significantly inhibited the production cGMP, a putative second messenger of NO. The actual mechanisms by which NSAIDs and glucocorticoids inhibit NOS are unknown, although at least two possibilities seem plausible: NSAIDs and glucocorticoids may affect transcription of the iNOS gene, and/or eicosanoid derivatives such as prostaglandins may influence iNOS mRNA stability (Scheinman *et al.*, 1995; Salvemini *et al.*, 1993).

With respect to the former possibility, experiments have demonstrated that NSAIDs and glucocorticoids inhibit the activation of nuclear factor kappa B (NF-κB) (Kopp and Ghosh, 1994). Many immunological responses in both invertebrates and vertebrates are regulated by the NF-κB/Rel transcription factor family, and there is evidence to suggest that NF-κB activation is the principle mechanism for the initiation of iNOS gene expression and the subsequent high output production of NO (Xie *et al.*, 1994).

NSAIDs have been determined to inhibit the activation of NF-κB in Jurkat T cell and mouse PD31 cells (Kopp and Ghosh, 1994). Scheinman *et al* (1995) demonstrated a similar repression of members of the NF-κB/Rel transcription factor family by the glucocorticoid dexamethasone. It is possible that an NF-κB analogue exists in insect hemocytes, and that the transcription of insect NF-κB could also be inhibited by glucocorticoids and NSAIDs. Clearly, future efforts need to be directed at uncovering such an analogue, which if present may modulate still other hemocyte functions.

Di Rosa *et al.* (1990) examined the effects of glucocorticoids on production of NO in macrophage cell line J774, and was demonstrated that at physiologically relevant concentrations of dexamethasone the release of NO from LPS-stimulated cells was inhibited. Furthermore, it was shown that this inhibition was related to the properties of dexamethasone, since other steroids such as progesterone had no effect. However, dexamethasone had no direct inhibitory effect on NO release once NOS was expressed, indicating that the mode of action must be prior to the synthesis of the enzyme.

Dexamethasone modulates the transcription of NOS in HeLa cells and 2B4 murine T cells by upregulating the gene which produces the inhibitory protein IκB-α (Scheinman *et al.*, 1995). Activation of the NOS gene requires the binding of the transcriptional activator NF-κB, which when bound to IκBα, is unable to enter the nucleus. Upon appropriate stimulation, NF-κB is released from its inhibitor molecule, but newly released NF-κB quickly re-associates the IκBα induced by

dexamethasone. Decreasing the free NF-κB concentration effectively blocks the activation of potentially all immune responses regulated by the NF-κB pathway. Similar experiments were carried out to examine the effects of NSAIDs on NOS activity. Aeberhard *et al* (1995) reported that indomethacin and other NSAIDs significantly inhibited the expression of iNOS in rat alveolar macrophages activated by LPS and gamma interferon. These researchers ascribed the inhibition of iNOS by NSAIDs to pretranslational control of enzymatic expression and not to direct inhibition of enzymatic activity.

With respect to the second mechanism by which NSAIDs or glucocorticoids may inhibit NOS, there is less empirical evidence to support the notion that eicosanoids may directly influence iNOS mRNA stability. The putative iNOS mRNA contains the 'AUUUA" motif in its 3' untranslated region, which is considered to be a mRNA instability determinant. Srivastava *et al.* (1994) determined that interleukin-1β stabilizes the COX-2 mRNA by phosphorylating cytosolic factors which bind to the AUUUA-rich 3' untranslated region. A similar mechanism may be involved in promoting the stability of iNOS mRNA (Tetsuka *et al.*, 1994).

There is a clear relationship in mammalian immunocytes between NOS and eicosanoid biosynthetic inhibitors. In this chapter the possibilities was examined that insect hemocyte NOS could also be inhibited by NSAIDs and glucocorticoids. The data presented demonstrate that NOS activity is indeed decreased in insect hemocytes treated with the eicosanoid biosynthesis inhibitor. Furthermore, the

expression of the NOS transcript was reduced in LPS-induced hemocytes treated with NSAIDs or glucocorticoids. It is unclear whether the decrease in NOS activity was due to inhibition of NOS transcription by the NSAIDs/glucocorticoids or due to a reduction in eicosanoid formation, which may have decreased NOS mRNA stability.

To distinguish between the two possibilities, a series of "rescue" experiments could be performed in which exogenous arachidonic acid or specific prostaglandins are co-injected with the eicosanoid biosynthesis inhibitors. If NOS activity or the concentration of NOS transcript increases upon the addition of exogenous arachidonic acid and PGs, this would imply that eicosanoids are influencing the stability of the NOS mRNA. If the NOS activity could not be rescued, this would indicate NSAIDs and glucocorticoids were mediating an effect at the level of transcription.

The hypothesis first put forth by Stanley-Samuelson *et al.*(1991) stating that eicosanoids influence the insect immune response has proven to be correct. However, the mode of action of eicosanoids still remains unclear. Assuming glucocorticoids and NSAIDs inhibit the production of NO, it is possible that the influence of eicosanoids on the insect immune response is not due to their direct presence, but rather is due to their mediation of the production of NO. Regardless of which mechanism applies, it is nevertheless interesting that such "cross-talk" between eicosanoid and NOS pathways exists in two such disparate animal phyla.

References

Aeberhard, E. E., S. A. Henderson, N. S. Arabolos, J. M. Griscavage, F. E. Castro, C. T. Barrett and L. J. Ignarro. 1995. Nonsteroidal anti-inflammatory drugs inhibit expression of the inducible nitric oxide synthase gene. Biochem. Biophys. Res. Comm. 208:1053-1059.

Chomczynski, P. and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidunium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162:156-159.

Di Rosa, M., M. Radomski, R. Carnuccio and S. Moncada. 1990. Glucocorticoids inhibit the induction of nitric oxide synthase in macrophages. Biochem. Biophys. Res. Comm. 172:1246-1252.

Kopp, E. and S. Ghosh. 1994. Inhibition of NF-κB by sodium salicylate and aspirin. Science. 265:956-958.

Mandato, C. A., S. J. Moore, W. L. Diehl-Jones and R. G. H. Downer. 1997. The effects of eicosanoids on prophenoloxidase activation, phagocytosis and cell spreading in *Galleria mellonella*. J. Insect Phys. 43:1-8.

Miller, J. S., Nguyen, T. and Stanley-Samuelson, D.W. 1994. Eicosanoids mediate insect nodule response to bacteria infection. Proc. Natl. Acad. Sci. 91: 12418-12422.

Salvemini, D., T.P. Misko, J.L. Masferrer, K. Seibert, M.G. Currie, P. Needleman. 1993. Nitric oxide and cyclooxygenase enzymes. Proc. Natl. Acad. Sci. 90: 7240-7244.

Scheinman, R.I., P.C. Cogswell, A.K. Lofquist and A.S. Baldwin. 1995. Role of trascriptional activation of IκB-α in mediation of immunosuppersion by glucocoticoids. Science. 270:283-286.

Srivastava, S. K., T. Tetsuka, D. Daphna-Iken and A. R. Morrison. 1994. IL-1β stabilizes COX II mRNA in renal mesangial cells: role of 3'-untranslated region. Am. J. Physiol. 267: F504-F508.

Stanley-Samuelson, D. W., Jensen, E., Nickerson, K.W., Tiebel, K., Ogg, C.L., and Howard, R.W. 1991. Insect immune response to bacterial infection is mediated by eicosanoids. Proc. Natl. Acad. Sci. 88:1064-1068.

Tetsuka, T., D. Daphna-Iken, S. K. Srivastava, L. D. Baier, J. DuMaine and A. R. Morrison. 1994. Cross-talk between cyclooygenase and nitric oxide pathways: prostaglandin E2 negatively modulates induction of nitric oxide synthase by interleukin 1. Proc. Natl. Acad. Sci. 91:12168-12172.

Wu, K. K. 1995. Inducible cyclooxygenase and nitric oxide synthase. In: *Advances in Pharmacology*. (J. T. August, M. W. Anders, F. Murad and J. T. Coyle, eds.) 33:179-208. Academic Press,Toronto, ON.

Xie, Q., Y. Kashiwabara and C. Nathan. 1994. Role of transcription factor NF-κB /rel in induction of nitric oxide synthase. J. Biol. Chem. 269:4705-4708.

Chapter 6

Conclusions

An Overview: Comparative Immunology

The strong functional similarities between the insect immune response and that of vertebrate innate response suggests a possible common lineage between these systems. Over a century ago, Metchnikoff was first to consider the concept of a conserved lineage in the immune system when he observed "it is the cellular defense systems that are most highly conserved among members of the animal kingdom" (Habicht, 1994).

It can be argued that there is no need for an elaborate inducible immune system in short-lived animals; and furthermore, there is no evidence to support the notion of an adaptive response in long-lived invertebrates. In view of this, Habicht (1994) concluded that some of the paradigms used by vertebrates do not apply to the invertebrate immune system. Upon examining the rooted phylogeny of several immune genes from both vertebrates and arthropods, Hughes (1998) determined that, based on sequence homology, immune systems have arisen independently in vertebrates and arthropods. Nevertheless, there is convincing evidence to support the notion of conservation between the vertebrate innate immune response and the insect immune system, at least at the functional level.

The immune system has evolved under selective pressures such that all multicellular organisms have the ability to recognize and neutralize invading microbes. The ancient innate immune system has developed germline-encoded receptors to distinguish the molecular patterns of microbial pathogens (Medzhitov and Janeway, 1997). It is generally accepted that this means of recognizing non-self

transcends taxonomic boundaries, and that the ancient innate immune system is a good starting point for understanding how more advanced immune systems have evolved. In this chapter, I examine the degree of conservation between proteins and signal transduction pathways and the modulation strategies used in both the vertebrate and invertebrate immune responses.

Conservation of Intrinsic Immune Proteins

Several proteins involved in both the vertebrate and invertebrate immune responses appear to have been conserved, and therefore, indicate a common ancestor. Of all the immune proteins discussed in Chapter 1, lysozyme and hemolin are perhaps the most useful to consider from a comparative perspective. The former is an example of a protein with both homologous structure and function in vertebrates and invertebrates, whereas the latter bears only superficial homologies.

The immune proteins with the strongest conservation are lysozymes. These antibacterial proteins are ubiquitous amongst the vertebrates and are also expressed in insects exposed to bacteria (Gillespie, 1997). Upon sequence analysis, vertebrate and insect lysozymes appear to be homologous and have a conserved immune function (Hughes, 1998).

The expression of hemolin in response to bacterial infection in insects is well documented (Sun *et al.*, 1990; Ladendorff and Kanost, 1991). Upon infection, hemolin binds to bacterial cell wall surfaces and hemocytes, and acts as an opsonin. However, it is not thought to function as a recognition molecule. Hemolin is a

member of the immunoglobulin superfamily, which includes many molecules involved in the vertebrate immune response, such as antibodies and T-cell receptors. Unlike most vertebrate immunoglobulins, no Fc receptors have been identified for hemolin. Phylogenetic analysis reveals that hemolin is most closely related to adhesion molecules found in vertebrate and invertebrate nervous systems. Thus, it appears that the immunological role of hemolin evolved independently of the vertebrate family of immunologulins (Hughes, 1998).

Conservation of Signalling Molecule Pathways

An additional example of an evolutionary conservation between immune systems is the Toll/Dorsal transduction pathway in insects and the interleukin-1 receptor/NF-κB system found in mammals. The protein Toll is a transmembrane receptor which plays a fundamental role in embryonic dorsal-ventral patterning in *Drosophila*, and has been demonstrated to be involved in the activation of many insect immune genes (Hashimoto *et al.*, 1988; Gillespie *et al.*, 1997). The extracellular domain of the invertebrate Toll receptor includes a region of leucine-rich repeats, while the cytoplasmic portion is homologous with the vertebrate interleukin-1 receptor (IL1R). The latter is involved in initiating the signalling of the NF-κB pathway, also has an extracellular domain of leucine-rich repeats, and is found in a number of vertebrate immunocytes (Hughes, 1998). As described in Chapter 4, ligand binding to the Toll receptor results in the release of the transcriptional activator Dorsal or dorsal related immune factor (DIF) from cactus,

which is homologous to the vertebrate inhibitory molecule IκB. These immuno-activated pathways are, therefore, common to both vertebrates and invertebrates, although it seems that the original role of Toll/Dorsal was in establishing embryonic polarity in insects.

Phylogenetic Conservation of Immune Modulators

The vertebrate immune system is a specific arrangement of many modulators which work in concert to provide both inducible and innate immune responses. While invertebrates are not capable of mounting an Ig-based adaptive response, they do produce an innate response in many respects similar to that of vertebrates. Herein, I present some of the functional homologies between the extrinsic modulators of vertebrate and invertebrate immunocyte activity.

Biogenic Amines

There is evidence to show that neurohormones can influence the behaviour of vertebrate immunocytes. Noradrenaline increases the motile behaviour of vertebrate macrophages and neutrophils, while 5-hydroxytryptamine (5-HT) and adrenaline increase the chemotatic activity of platelets (Rink and Hallam, 1984). These neurohormones elicit their effects via second messengers such as cAMP and inositol trisphosphate. Octopamine (a phenolic analogue of noradernaline) and 5-HT are two neurohormones commonly found in insect hemolymph which also have demonstrable immunoregulatory functions.

Similar to their modes of action in vertebrate immunocytes, these neurohormones mediate their effect in insect via second messengers such as Ca^{2+}, cAMP and inositol trisphosphate (Jahagirdar *et al.*, 1987; Baines and Downer, 1992; Baines *et al.*, 1992). It is, therefore, likely that the underlying cellular mechanisms used by the neurohormones are also homologous. For example, macrophage locomotory activity is based on changes in the actin subcortex caused by transient increases in intracellular Ca^{+2} (Marks and Maxfield, 1990; Hendy and Maxfield, 1993). Such fluctuations in intracellular Ca^{+2} have also been implicated in modulating neutrophil motility (Stossel, 1989). Similarly, transient Ca^{2+} oscillations and increases in intracellular Ca^{+2} have been observed in insect plasmatocytes treated with octopamine (Diehl-Jones and Mandato, unpublished data). The data presented in this thesis demonstrate at least functional homologies between vertebrate and invertebrate immunocytes in that a neuromodulator, octopamine, was shown to regulate hemocyte function. It is also shown that the mechanism by which hemocyte motility was effected is similar to that of vertebrate immunocytes. However, there is no evidence that this particular amine has a similar function in vertebrates or that, conversely, epinephrine enhances insect hemocyte motility.

Eicosanoids

Many vertebrate immune responses are modulated by eicosanoids, and based on this observation, Stanley-Samuelson (1991) initially put forth the hypothesis that eicosanoids may also mediate hemocytic immune responses in insects. The data

presented in this thesis confirm this hypothesis, and illuminate some of the specific hemocyte functions which are so modulated.

Eicosanoids have been detected in almost every mammalian tissue and have been demonstrated to be potent effectors of chemokinesis and chemotaxis associated with leukocyte motility and platelet aggregation (Stanley-Samuelson, 1987; von Euler, 1988). While all vertebrate immune cells are capable of producing eicosanoids, macrophages and monocytes seem to be the major source of endogenous prostaglandins and other eicosanoids (von Euler, 1988). Prostaglandins work as paracrines, thereby eliciting effects on adjacent cells by binding to specific G protein-linked eicosanoid receptors.

Eicosanoids have also been detected in the hemolymph of insects and, similar to vertebrates, are synthesized in immune tissues including both fat body and hemocytes. Fat body preparations incubated with labile polyunsaturated fatty acids (eicosanoid precursor) synthesize substantial amounts of four prostaglandins (PG), $PGF_{2\alpha}$, PGE_2, PGD_2 and PGA_2 (Stanley-Samuelson, 1994). Similar experiments demonstrated that hemocytes were also capable of synthesizing the same PGs (Stanley-Samuelson, 1994). Presumably, the eicosanoids in hemolymph and hemocytes also work via specific G protein-linked eicosanoid receptors. This suggests that different G proteins could be activated by specific ligand-occupied receptors. G proteins, in turn, can stimulate and inhibit intracellular second messenger systems including phospholipase C and adenylate cyclase, as well as gate Ca^{2+} channels (Smith, 1989).

Although it is now apparent that eicosanoids mediate several aspects of invertebrate immunity, including nodule formation, phagocytosis, hemocyte spreading and prophenoloxidase activation (Mandato *et al.*, 1997), future work should be aimed at delineating the specific intracellular pathways. An understanding of the specific second messenger system(s) activated by eicosanoids during the insect immune response will enable a more precise dissection of not only which eicosanoids are involved, but also how other immunomodulatory inputs are integrated.

Nitric Oxide

In the last decade, NO has arguably proven to be the most important signalling molecule in vertebrates. Aside from the effects of NO on vertebrate neurological and circulatory physiology, NO has proven to be an effective modulator of the vertebrate innate immune response. NOS has been discovered in nearly all vertebrate immunocytes and its influence as a paracrine signalling molecule is rivalled only by the eicosanoids.

Other investigators have provided evidence that NO is also an important signalling molecule in invertebrates. In this thesis, I demonstrate that NO also plays a role in the insect immune system, indicating yet another functional homology between vertebrate and invertebrate immunocytes. In both cases, LPS induces large quantities of NO, which confer bactericidal properties in both vertebrates and insects.

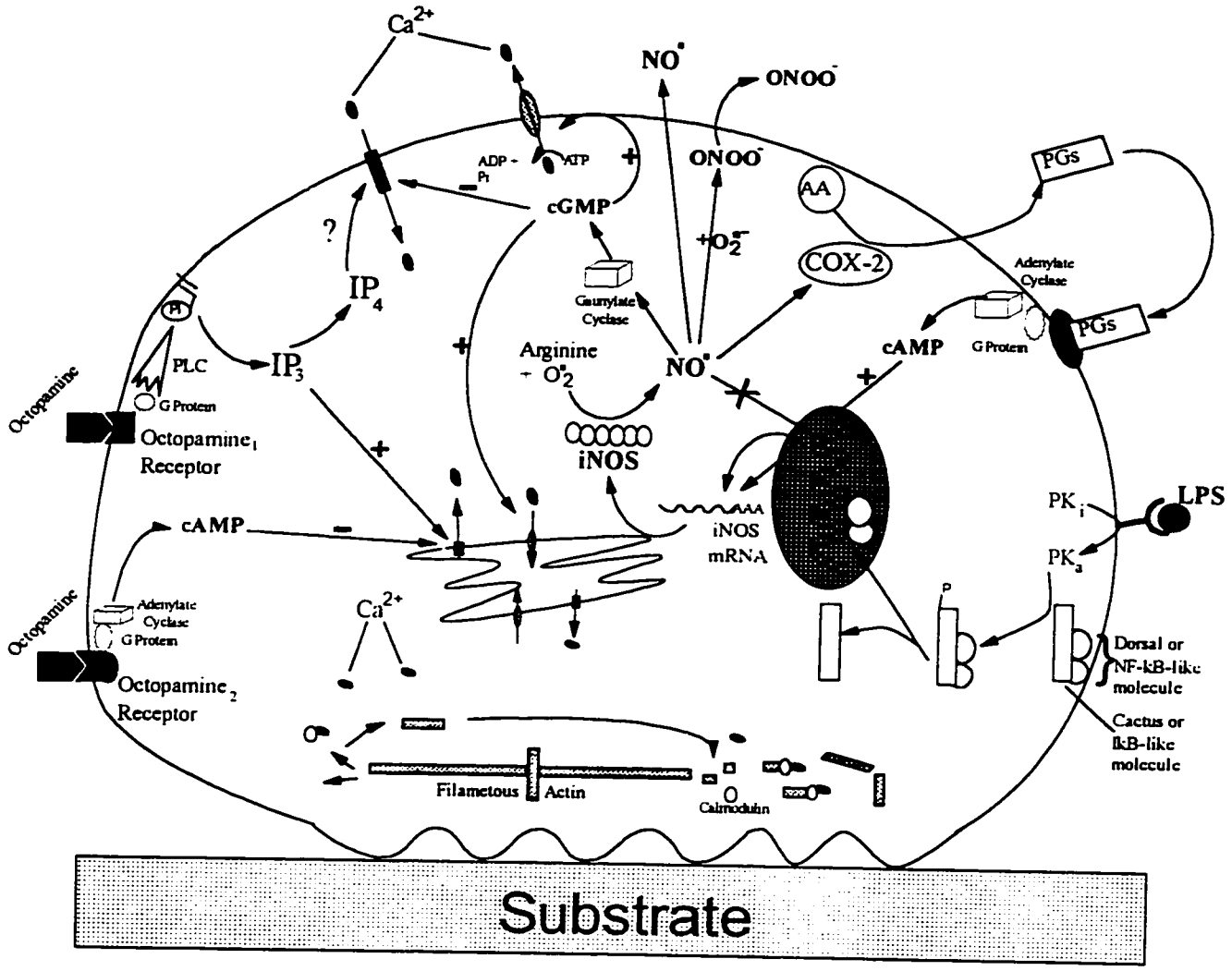
Yet another homology exists between these phyla in that NO acts as a second messenger which stimulates gaunylate cyclase to produce cGMP. The increase in cGMP is reported to elicit chemotaxis and migration in vertebrate neutrophils (Beauvais *et al.*, 1995), although the function of the increased cGMP in insect hemocytes is unknown. The effect of cGMP and NO on hemocyte migration was examined, and there were no significant changes in motility or migration (data not shown). This observation indicates that, while many aspects of NO production and signalling are conserved between vertebrates and invertebrates, some down-stream effects are not. This invites further investigations into the cellular effects of NO/cGMP and other related pathways in hemocytes.

A Unifying Model of Immunomodulators

In chapters 2 through 4, I have presented evidence that biogenic amines, eicosanoids and NO each modulate some aspect of hemocyte activity. These modulators, their presumed modes of actions and their interactions are presented in Figure 6.1.

As depicted in the model, octopamine increases the concentration of cytosolic Ca^{2+} in hemocytes (Jahagirdar *et al.*, 1987 and Diehl-Jones and Mandato, unpublished data). Upon binding to the octopamine$_1$ receptor, octopamine could stimulate a G protein-linked phospholipase C-β (PLC-β), which in turn hydrolyses inositol trisphosphate (IP_3) from the plasma membrane. The newly released IP_3 presumably diffuses to the endoplasmic reticulum (ER), where it binds to IP_3-gated

Fig. 6.1: Modulators of Insect Immunity and their Presumed Interactions.


Ca2+
NO•
ONOO-
ADP + Pi
ATP
cGMP
ONOO-
AA
PGs
?
IP4
+O2•-
COX-2
Adenylate Cyclase
Guanylate Cyclase
PI
PLC
IP3
G Protein
cAMP
G Protein
PGs
Arginine + O2
NO•
Octopamine
Octopamine1 Receptor
iNOS
iNOS mRNA
cAMP
PKi
LPS
PKa
Adenylate Cyclase
G Protein
Ca2+
Octopamine
Octopamine2 Receptor
Dorsal or NF-kB-like molecule
Cactus or IkB-like molecule
Filametous Actin
Calmodulin
Substrate

Ca^{2+} channels, allowing the efflux of free Ca^{2+} into the cytosol. Concurrent with this, IP_3 likely becomes phosphorylated to inositol tetrakisphosphate (IP_4), which has a slower and longer lasting effect on intracellular Ca^{2+} concentration by gating Ca^{2+} channels on the plasma membrane (Alberts *et al.*, 1994).

Another second messenger by which octopamine might affect intracellular Ca^{2+} concentration is cAMP. Orr *et al* (1985) demonstrated the presence of an octopamine-sensitive adenylate cyclase on hemocyte membranes. Octopamine binding to $octopamine_2$ receptors would likely stimulate adenylate cyclase through a G protein-mediated mechanism, resulting in the inhibition of Ca^{2+} efflux from the ER. Depending upon the number of receptors and their affinity for octopamine, these two receptors working in concert could regulate intracellular Ca^{2+} concentrations. Different effects on distinct hemocyte populations could be elicited in accordance with the number and type of octopamine receptors.

Intracellular Ca^{2+} levels may also be influenced by the production of eicosanoids. The synthesis of eicosanoids is not restricted to a central endocrine organ; rather, most immunocytes are capable of synthesizing eicosanoids (Smith, 1989). Eicosanoids can therefore act as local hormones or "autocoids" and mediate their effects on themselves or neighbouring cells at very low concentrations (Smith, 1989). Most eicosanoid actions are expressed thorough a G protein-linked eicosanoid receptor. G proteins have been shown to stimulate and inhibit adenylate cyclase, activate phospholipase C and open or close Ca^{2+} channels (Stanley-Samuelson, 1994).

It has been suggested that another role for eicosanoids is the regulation of iNOS mRNA level. Tetsuka *et al.* (1994) hypothesized that eicosanoids might interact with factors that modulate iNOS mRNA stability. iNOS mRNA has the "AUUUA" motif in its 3' untranslated region, which is thought to be a determinant of mRNA instability . As depicted in Figure 6.1, eicosanoid-mediated increases in cAMP might stimulate cAMP-dependent kinases to phosphorylate the 3' untranslated region of the iNOS mRNA, thus conferring greater stability to the iNOS transcript (Tetsuka *et al.*, 1994). The down-stream effect of this would be an increase in NO production.

LPS is a major cell wall component of Gram-negative bacteria and triggers a cascade of antibacterial responses in mammalian macrophages (Weinstein *et al.*, 1991). Charalambidis *et al.* (1996) examined the effect of LPS on insect (medfly) hemocytes and found the signal transduction to be very similar to that of macrophages. As well, binding of LPS to a hemocyte surface receptor appeared to initiate an increase in protein-tyrosine phosphorylation. I propose that such an increase in phosphorylation in insect hemocytes could result in the initiation of transcription in the NOS pathway (see Fig. 6.1). In mammalian immunocytes, LPS and cytokines initiate iNOS transcription through a series of phosphorylation steps and subsequent proteolysis of the inhibitory molecule IκB-α. A similar mechanism may be responsible for the activation of LPS-induced NOS in insect hemocytes. Furthermore, I cannot rule out the possibility that some as yet undiscovered insect

cytokines released upon insect handling or wounding work synergistically with LPS to mediate the activation of NOS in insect hemocytes.

One other tenet of the model is that an increase in phosphorylation is involved in starting a dorsal/NF-κB-like signalling pathway (see Chapter 4). As illustrated in figure 6.1, following the binding of a dorsal/NF-κB-like molecule to the NOS gene, transcription and RNA processing, the NOS mRNA is exported from the nucleus to the cytosol. After translation of the NOS mRNA and given the appropriate substrates, the NOS enzyme produces NO in insect hemocytes. The NO molecule may act alone or combine with superoxide to produce bactericidal activity. Ostensibly, the NO/peroxynitrite diffuses from the hemocyte, and due to its powerful oxidant capacity, destroys micorooganisms. Alternatively, or in addition to the above, NO may work as a signalling molecule in insect hemocytes. The primary target of NO signalling is the heme domain of the enzyme guanylate cyclase. NO has been demonstrated to increase the concentration of cGMP in hemocytes, and this second messenger has been speculated to regulate Ca^{2+}/inositol phosphate pathways. In part this could be accomplished by the stimulation of Ca^{2+} ATPase pumps in the plasma membrane or in the endoplasmic reticulum; both are enzymes which remove Ca^{2+} from the cytosol (Hirata and Murad, 1994), as shown in figure 6.1.

Another potential target for NO is the active site of the enzyme cyclooxygenase (COX) (Salvemini *et al.*, 1993). Although the pathways leading to COX activation by NO are not entirely clear, it is known that, similar to guanylate cyclase, COX has an iron-heme centre at the active site. In this schema, NO

induction of prostaglandins may be a key regulatory event in the immune response in both vertebrates and insects.

Where do such insights point us? At an applied level, increased knowledge of these immunomodulators can enhance our efforts to use intrinsic features of the insect cellular immune response to control pest insects. As an example, DeKeyser *et al.* (1994) have developed octopamine analogues that effectively hyper-stimulate the insect immune response in crop pest insects. At a theoretical level, my findings are provocative in that they strengthen the assumption that the insect immune response is a template for the vertebrate innate immune response. However, the most relevant implication of the work in this thesis may be in demonstrating the efficacy of an insect model for investigating aspects of basic cellular and molecular physiology.

References

Alberts, B., D. Bray, J. Lewis, M. Raff, K. Roberts and J. Watson. 1994. The immune system. In: *Molecular Biology of the Cell.* 721-785. Garland Publishing, Inc., New York, NY,.

Baines, D., T. DeSantis and R.G.H. Downer. 1992. Octopamine and 5-hydroxytrypamine enhance the phagocytic and nodule formation activities of cockroach (*Periplanteta americana*) haemocytes. J. Insect Physiol. 38:905-914.

Baines, D. and R. G. H. Downer. 1992. 5-Hydroxytrypamine-sensitive adenylate cyclase affects phagocytosis in cockroach hemocytes. Arch. Insect Biochem. Physiol. 21:303-316.

Beauvais, F., L. Michel and L. Dubertret. 1995. Exogenous nitric oxide elicits chemotaxis of neutrophils *in vitro*. J. Cell. Phys. 165:610-614.

Charalambidis, N. D., C. G. Zervas, M. Lambropoulou, P. G. Katsoris and V. J. Marmaras. 1995. Lipopolysaccharide-stimulated exocytosis of nonself recognition protein from insect hemocytes depend on protein tryosine phosphorylation. Eur. J. Cell Biol. 67:32-41.

DeKeyser, M. A., D. S. Mitchell and R. H. G. Downer. 1994. Improved synthesis and spectral characterization of D 5281, an oxadianzine miticid/insecicide. J. Agr. Food Chem. 42:1783-1785.

Gillespie, J. P., M. R. Kanost and T. Trenczek. 1997. Biological mediators of insect immunity. In: *Annual Review of Entomology*. (T. E. Mittler, F. J. Radovsky and V. H. Resh, eds.) 42:611-644. Annual Reviews Inc., Palo Alto, CA.

Habicht, G. S. 1994. Primordial immunity: foundation for the vertebrate immune system. In: *Primordial Immunity: Foundation for the Vertebrate Immune System*. (G. Beck, E. L. Cooper, G. S. Habicht and J. J. Marchalonis, eds.) 712: ix-xi. The New York Academy of Sciences, New York, NY.

Hashimoto, C., K. L. Hudson and K. V. Anderson. 1988. The toll gene of *Drosophila*, required for dorsal-ventral embryonic polarity, appears to encode a transmembrane protein. Cell. 52:269-279.

Hendy, B. and F. R. Maxfield. 1993. Regulation of neutrophil motility and adhesion by intracellular calcium transients. Blood Cells. 19:143-164.

Hirata, M. and F. Murad. 1994. Interrelationships of cyclic GMP, inositol phosphates and calcium. In: *Advances in Pharmacology*. (F. Murad, ed.) 26:195-216. Academic Press Inc., San Diego, Ca.

Hughes, A. L. 1998. Protein phylogenies provide evidence of radical disontinuity between arthropod and vertebrate immune sytems. Immunogenetics. 47: 283-296.

Jahagirdar, A. P., G. Milton, T. Viswanatha and R. G. H. Downer. 1987. Calcium involvement in mediating the action of octopamine and hypertrehalosemic peptides on insect hemocytes. FEBS. 219:83-87.

Ladendorff, N. E. and M. R. Kanost. 1991. Bacteria-induced protein P4 (hemolin) from *Manduca sexta*: a member of the immunoglobulin superfamily which can inhibit hemocyte aggregation. Arch. Insect Biochem. Physiol. 18:285-300.

Mandato, C . A., S. J. Moore, W. L. Diehl-Jones and R. G. H. Downer. 1997. The effects of eicosanoids on prophenoloxidase activation, phagocytosis and cell spreading in *Galleria mellonella*. J. Insect Phys. 43:1-8.

Marks, P. W. and F. R. Maxfield. 1990. Transient increases in cytosolic calcium appear to be required for the migration of adherent human neutrophils. J. Cell Biol. 110:43-52.

Medzhitov, R. and C. A. Janeway. 1997. Innate immunity: the virtues of a nonclonal system of recognition. Cell. 91:295-298.

Orr, G. L., J. W. D. Gole and R. G. H. Downer. 1985. Characterization of an octopamine-sensitive adenylate cyclase in haemocyte membrane fragments of the American cockroach *Periplanta americana* L. Insect Biochem. 15:695-701.

Rink, T. J. and T. J. Hallam. 1984. What turns platlets on ? TIBS. 9:215-219.

Salvemini, D., T. P. Misko, J. L. Masferrer, K. Seibert, M. G. Currie add P. Needleman. 1993. Nitric oxide and cyclooxygenase enzymes. Proc. Natl. Acad. Sci. 90:7240-7244.

Smith, W. L. 1989. The eicosanoids and their biochemical mechanisms of action. Biochem. J. 259:315-324.

Stanley-Samuelson, D. W. 1987. Physiological roles of prostaglandins and other eicosanoids in invertebrates. Biol. Bull. 173:92-109.

Stanley-Samuelson D. W., E. Jensen, K. W. Nickerson, K. Tiebel, C.L. Ogg and R. W. Howard. 1991. Insect immune response to bacterial infection is mediated by eicosanoids. Proc. Natl. Acad. Sci. 88:1064-1068.

Stanley-Samuelson, D. W. 1994. Prostaglandins and related eicosanoids in insects. In: *Advances in Insect Physiology*. (P. D. Evans, ed.) 24:115-212. Academic Press, Toronto, ON.

Stossel, T. 1989. From signal to pseudopod. J. Biol. Chem. 264:18261-19264.

Sun, S-C., I. Lindstrom, H. Boman, I. Faye and O. Schmidt. 1990. Hemolin: an insect-immune protein belonging to the immunoglobulin superfamily. Science. 250: 1729-1732.

Tetsuka, T., D. Daphna-Iken, S. K. Srivastava, L. D. Baier, J. DuMaine and A. R. Morrison. 1994. Cross-talk between cyclooygenase and nitric oxide pathways: prostaglandin E2 negatively modulates induction of nitric oxide synthase by interleukin 1. Proc. Natl. Acad. Sci. 91:12168-12172.

von Euler, U. S. 1988. Biology of prostanoids. In: *Prostaglandins: Biology and Chemistry of Prostaglandins and Related Eicosanoids*. (P. B. Curtis-Prior, ed.). 1-7. Churchill Livingstone, London, England.

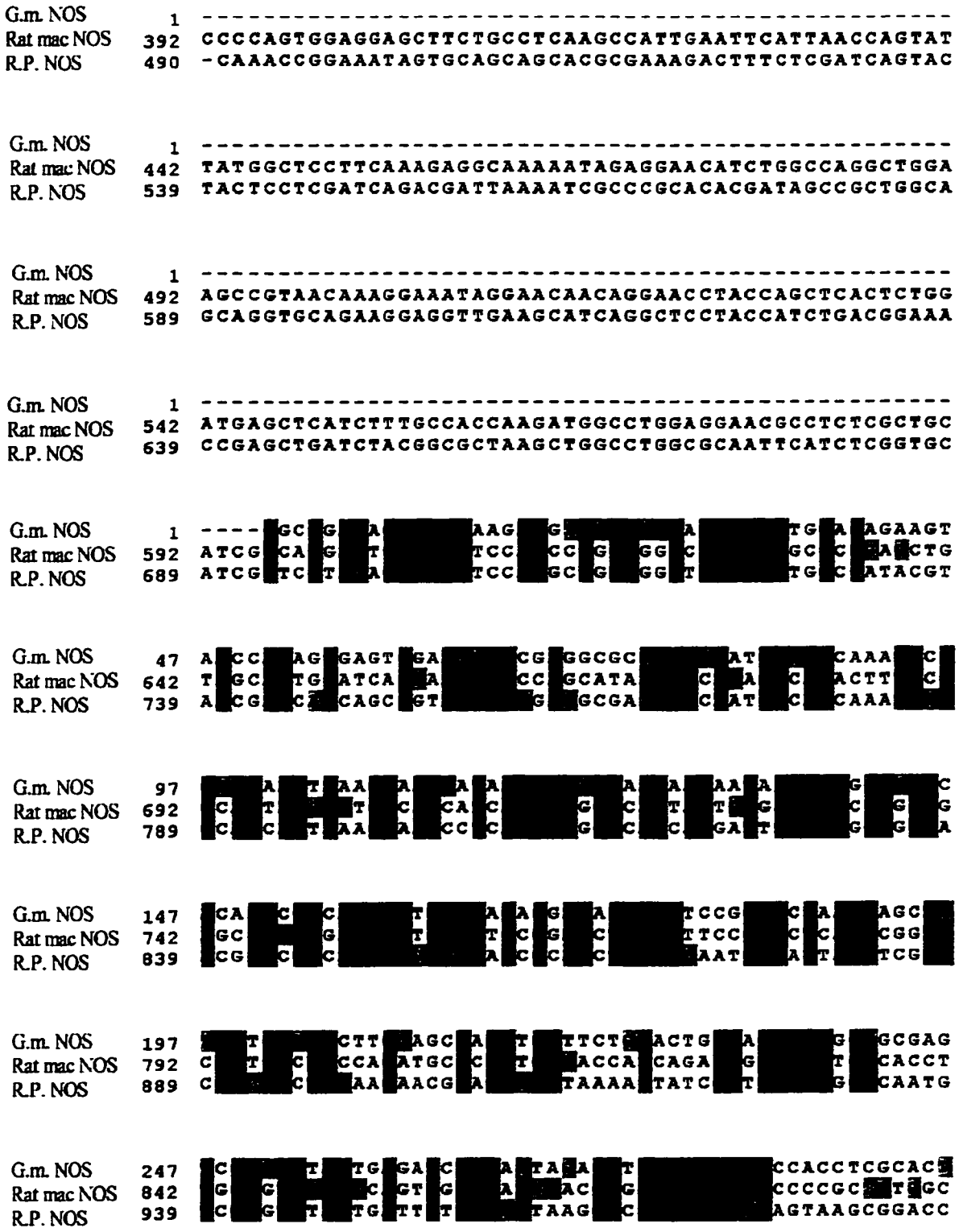
Weinstein, S., M. R. Gold and A. DeFranco. 1991. Bacterial lipopolysaccharide stimulates protein-tyrosine phosphorylation in macrophages. Proc. Natl. Acad. Sci. 88:4148-4152.

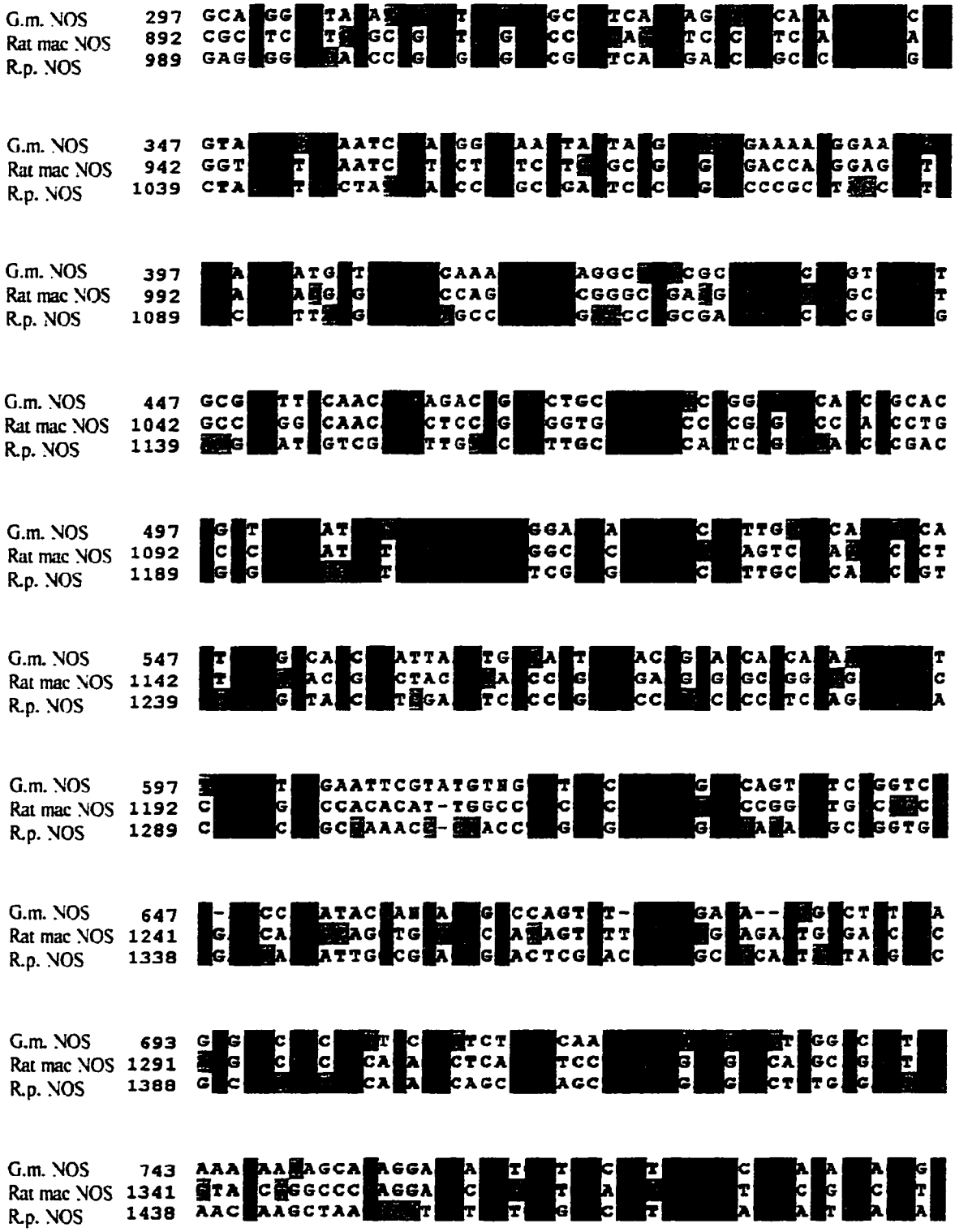
Appendices

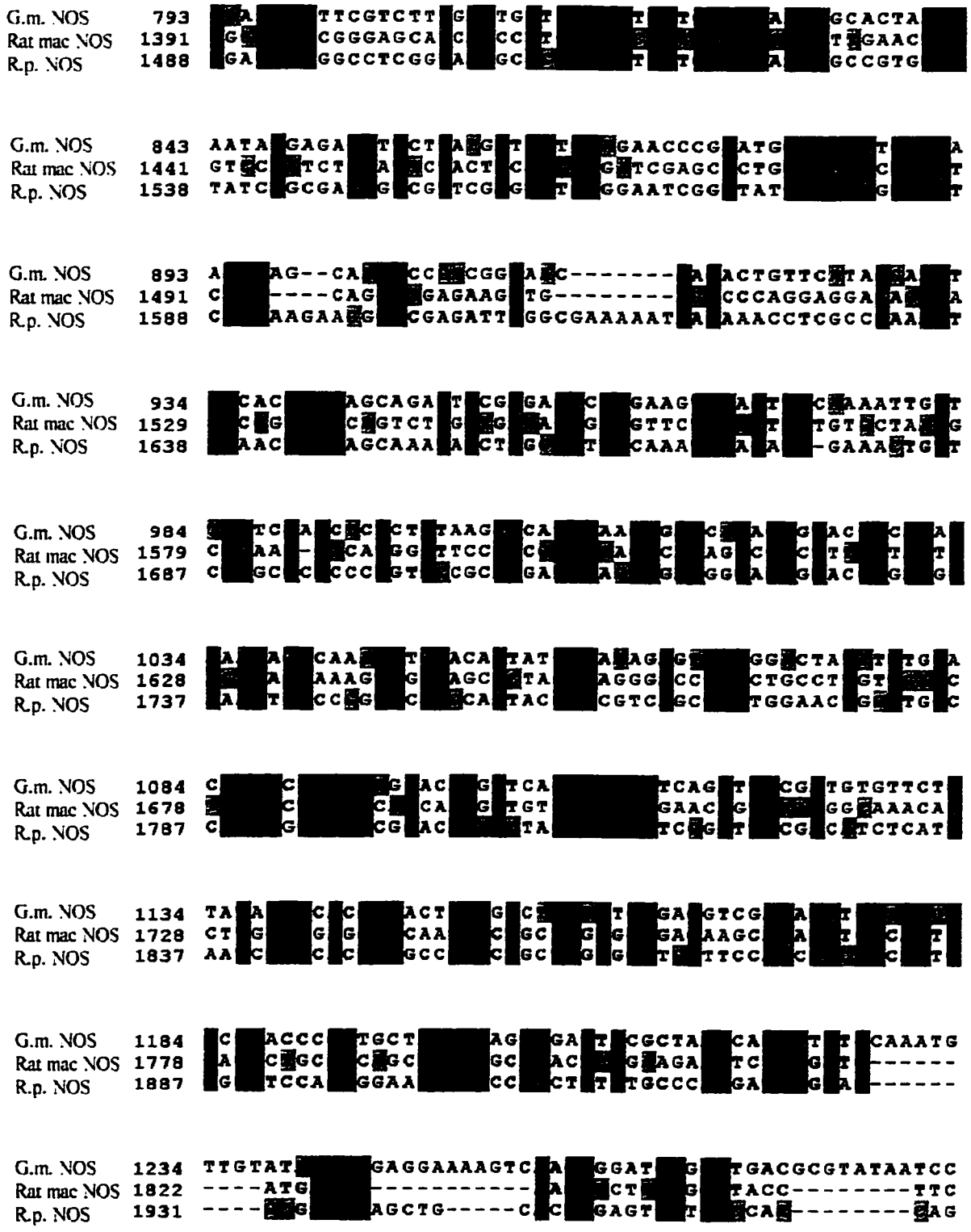
Appendix A: Sequence Comparison

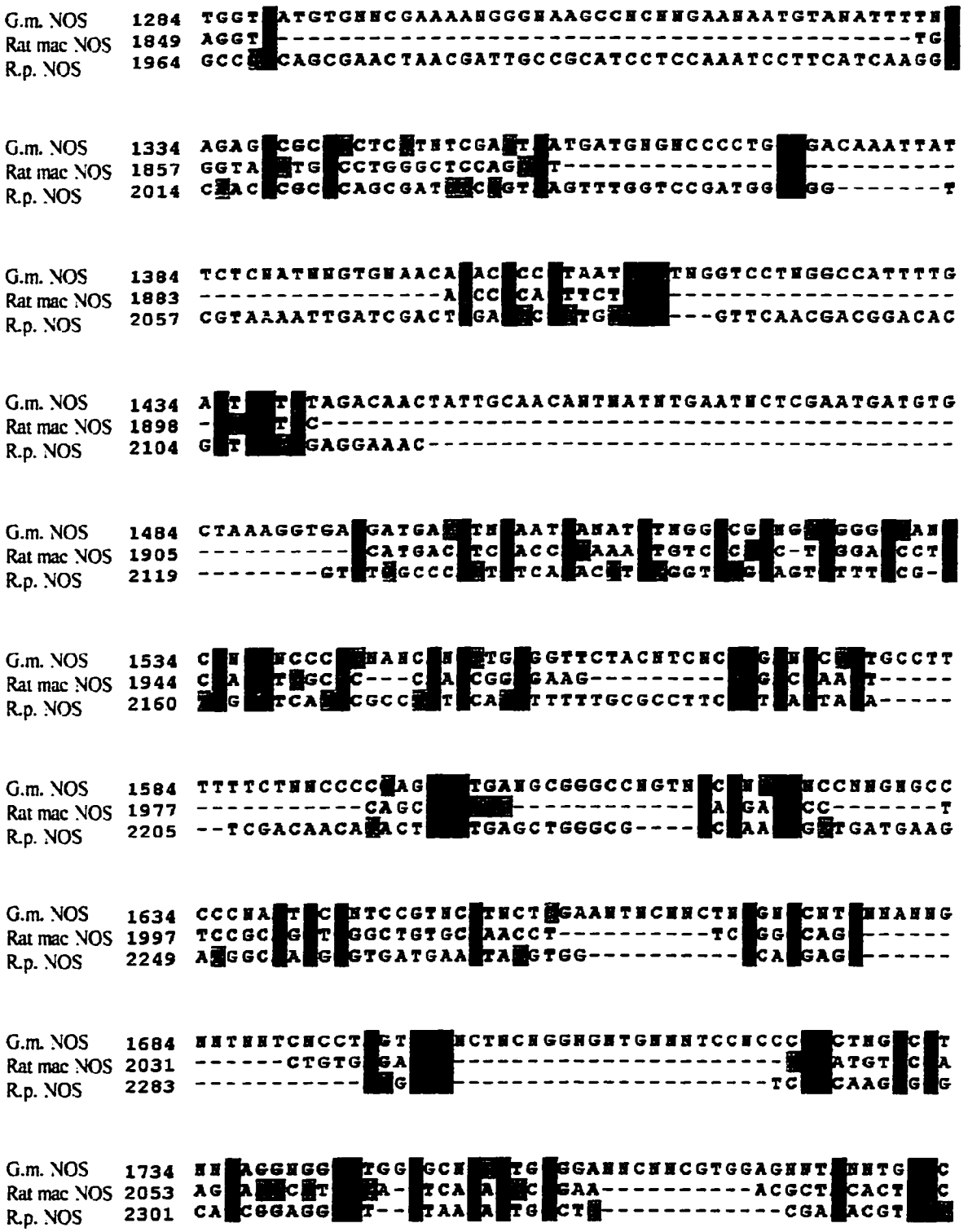
The appendix contains nucleotide comparisons between the partial NOS gene sequence from *Galleria mellonella* hemocytes (labelled G.m. NOS), the sequence for the Assassin Bug, *Rhodnius prolixus* NOS (R. p. NOS; U59389) and the sequence for the *Rattus norvegicus* macrophage NOS (Rat mac NOS; X59949). The *Galleria mellonella* NOS partial sequence shows 61% homology with the mouse macrophage NOS sequence and 55% homology with the *Rhodnius prolixus* NOS sequence. The alignment and comparison of the three genes performed by using MSASHADE which is a C-program based on the Pascal BOXSHADE program created by Kay Hofmann and Micheal D. Brown.

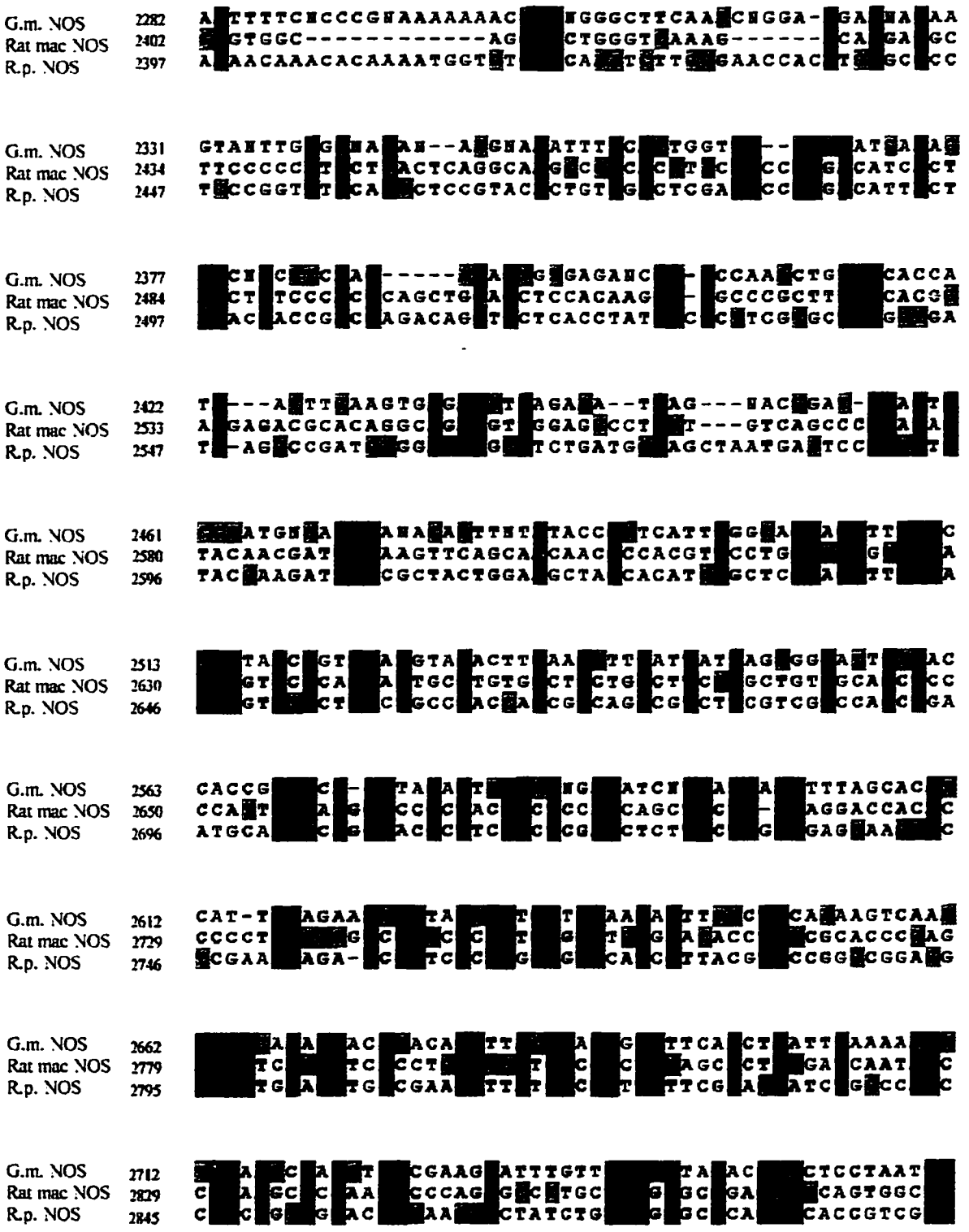
```
G.m. NOS      1 ----------------------------------------------------
Rat mac NOS   1 ----------------------------------ATGGCTTGCCCTT
R.p. NOS      1 GCTTGCGGGTGGGAAGATGGCGGACACGACCACGGTTGTGGTGGAGCGGC
                                                     ---

G.m. NOS      1 ----------------------------------------------------
Rat mac NOS  14 GGAAGTTTCTCTTCAGAGTCAAATCTTACCAAGGTGACCTGAAAGAGGAA
R.p. NOS     51 GTGAAGTTGCTGAAGGGCGCGAAAGTAGCAAGGCGAACCATATCGGTGAG

G.m. NOS      1 ----------------------------------------------------
Rat mac NOS  64 AAGGACATTAACAACAACGTGGAGAAAACCCCAGGTGCTATTCC------
R.p. NOS    101 GAGCGCCGTGGGTATGATGTGTCACGGAAGCGGTGCAGCATATCGGTGCA

G.m. NOS      1 ----------------------------------------------------
Rat mac NOS 108 CAGCCCAACAACACAGGATGACC---------------CTAAGAGTCACA
R.p. NOS    151 CGGCGGGGGGACGGAGGGCGGTGGAGGCAATATGAGGACCAACTATCGGG

G.m. NOS      1 ----------------------------------------------------
Rat mac NOS 143 AGCATCAAAATGGTTTCCCCCAGTTCCTCACTGGGACTGCACAGAATGTT
R.p. NOS    201 AGCTGTCACCGGCCTCGTTGCGGATCCATCGGAAATCGTCGCACGATATT

G.m. NOS      1 ----------------------------------------------------
Rat mac NOS 193 CCAGAATCCCTGGACAAGCTGCATG-------------------TGACTC
R.p. NOS    251 CGCAATACGCTGCTCGGGCCGGACGGTGAGGTGCTGCATCTGCATGACCC

G.m. NOS      1 ----------------------------------------------------
Rat mac NOS 224 C-----------------------------------ATCGACCCGCCCACAGC
R.p. NOS    301 TTCCGGGAAGGGTGGCGACGGGATGGGCAAGATGCCGGCCGTCGTCAAAC
                                                    -- -- -  - -

G.m. NOS      1 ----------------------------------------------------
Rat mac NOS 242 ACGTGAGGATCAAAAACTGGGGCAATGGAGAGATTTTTCACGACACCCTT
R.p. NOS    351 CGATCAAGTTGAAGAGCATTGTCACCAAGGCGGAATCGTACGACACCATG

G.m. NOS      1 ----------------------------------------------------
Rat mac NOS 292 CACCACAAGGCCACCTCGGATATCTCTTGCAAGTCCAAATTATGCATGGG
R.p. NOS    401 CATGGCAAGGCGAGCGATGTAATGTCATGCTCGCGGGAAGTGTGCATGGG

G.m. NOS      1 ----------------------------------------------------
Rat mac NOS 342 GTCCATCATGAACTCCAAGAGTTTGACCAGAGGACCCAGAGTCAAGCCCA
R.p. NOS    451 AAGCGTTATGACGCCGCACGTCATCGGTACGGAAACGCG-----------
```

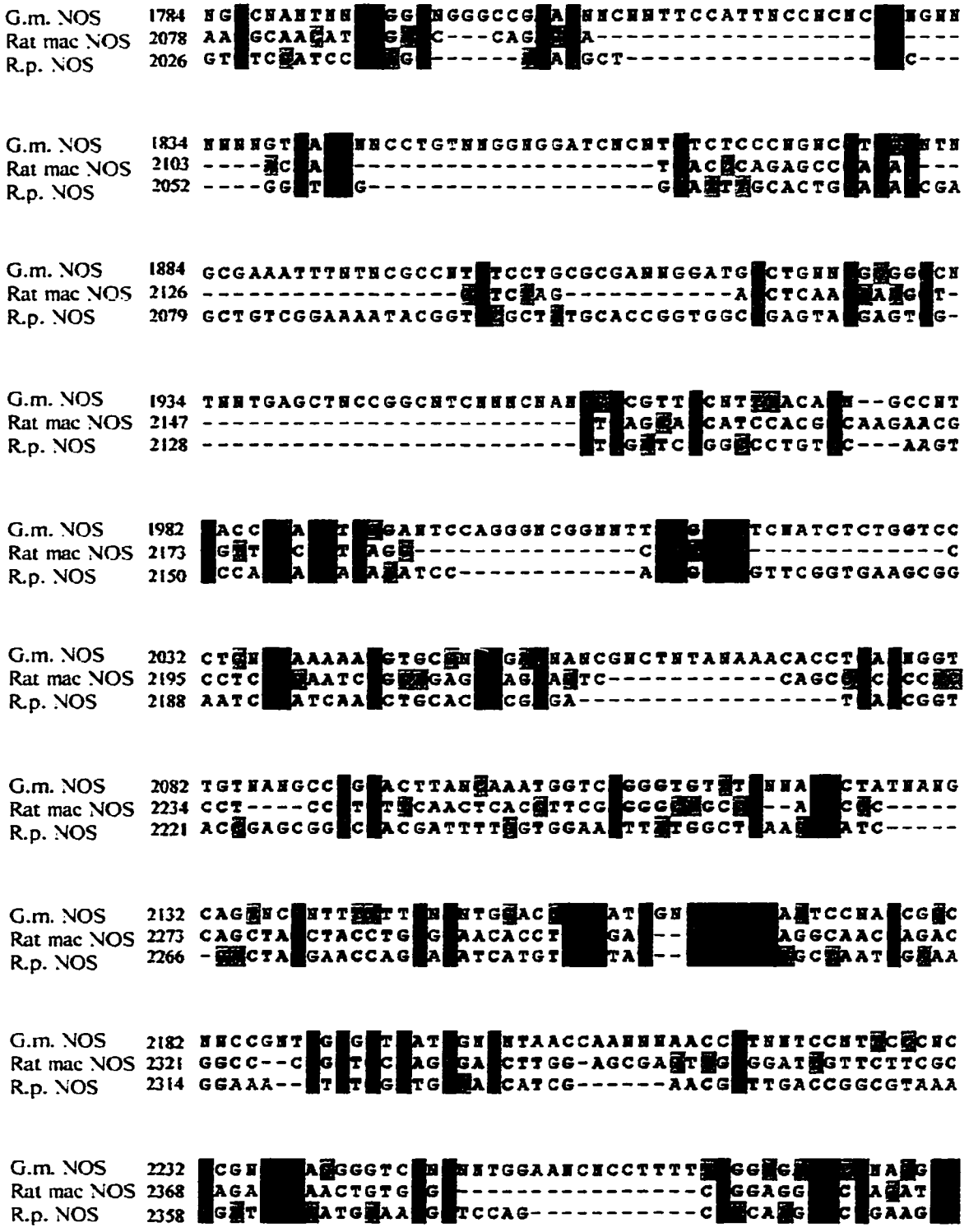

G.m. NOS 1 ----------
Rat mac NOS 392 CCCCAGTGGAGGAGCTTCTGCCTCAAGCCATTGAATTCATTAACCAGTAT
R.P. NOS 490 -CAAACCGGAAATAGTGCAGCAGCACGCGAAAGACTTTCTCGATCAGTAC
G.m. NOS 1 ----------
Rat mac NOS 442 TATGGCTCCTTCAAAGAGGCAAAAATAGAGGAACATCTGGCCAGGCTGGA
R.P. NOS 539 TACTCCTCGATCAGACGATTAAAATCGCCCGCACACGATAGCCGCTGGCA
G.m. NOS 1 ----------
Rat mac NOS 492 AGCCGTAACAAAGGAAATAGGAACAACAGGAACCTACCAGCTCACTCTGG
R.P. NOS 589 GCAGGTGCAGAAGGAGGTTGAAGCATCAGGCTCCTACCATCTGACGGAAA
G.m. NOS 1 ----------
Rat mac NOS 542 ATGAGCTCATCTTTGCCACCAAGATGGCCTGGAGGAACGCCTCTCGCTGC
R.P. NOS 639 CCGAGCTGATCTACGGCGCTAAGCTGGCCTGGCGCAATTCATCTCGGTGC
G.m. NOS 1 ----
Rat mac NOS 592 ATCG
R.P. NOS 689 ATCG
G.m. NOS 47
Rat mac NOS 642
R.P. NOS 739
G.m. NOS 97
Rat mac NOS 692
R.P. NOS 789
G.m. NOS 147
Rat mac NOS 742
R.P. NOS 839
G.m. NOS 197
Rat mac NOS 792
R.P. NOS 889
G.m. NOS 247
Rat mac NOS 842
R.P. NOS 939

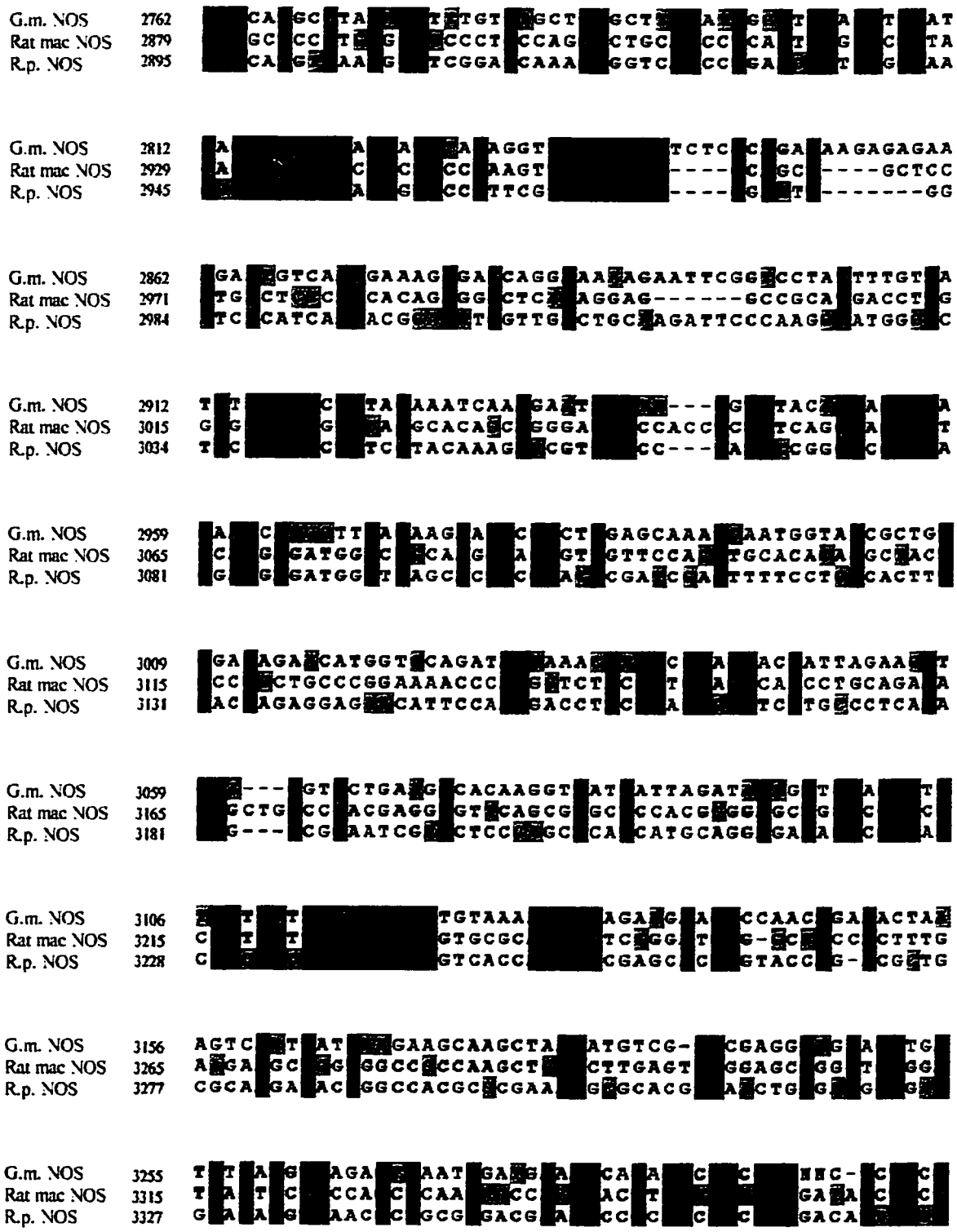

G.m. NOS 297
Rat mac NOS 892
R.p. NOS 989
G.m. NOS 347
Rat mac NOS 942
R.p. NOS 1039
G.m. NOS 397
Rat mac NOS 992
R.p. NOS 1089
G.m. NOS 447
Rat mac NOS 1042
R.p. NOS 1139
G.m. NOS 497
Rat mac NOS 1092
R.p. NOS 1189
G.m. NOS 547
Rat mac NOS 1142
R.p. NOS 1239
G.m. NOS 597
Rat mac NOS 1192
R.p. NOS 1289
G.m. NOS 647
Rat mac NOS 1241
R.p. NOS 1338
G.m. NOS 693
Rat mac NOS 1291
R.p. NOS 1388
G.m. NOS 743
Rat mac NOS 1341
R.p. NOS 1438

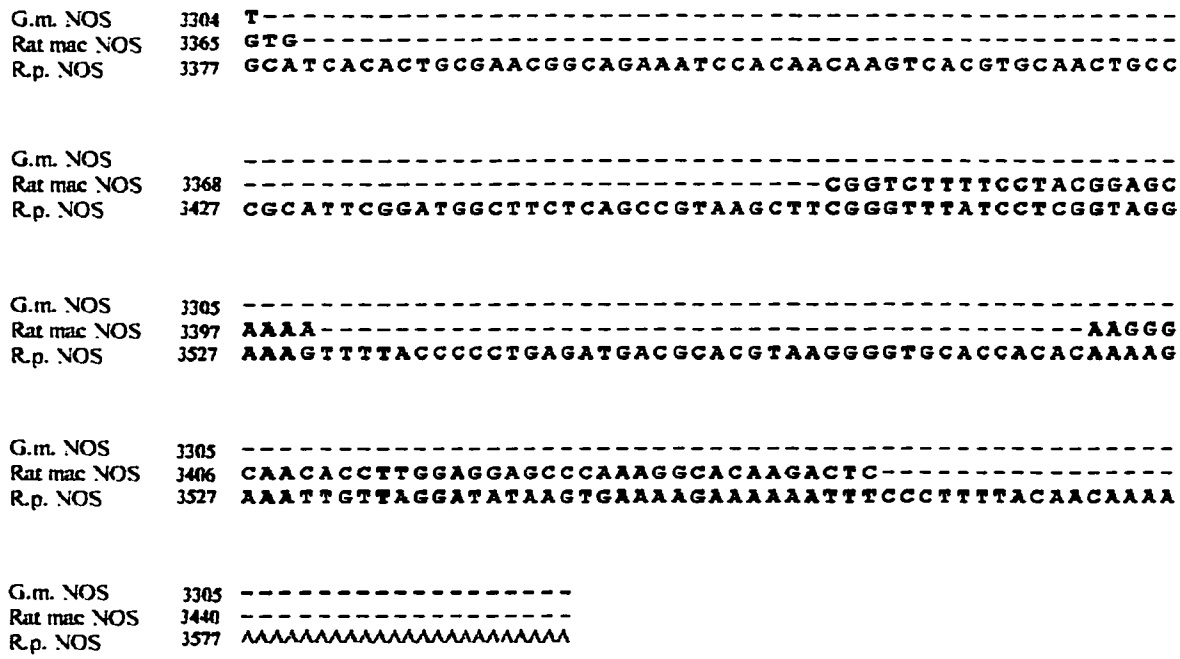

G.m. NOS 793
Rat mac NOS 1391
R.p. NOS 1488
G.m. NOS 843
Rat mac NOS 1441
R.p. NOS 1538
G.m. NOS 893
Rat mac NOS 1491
R.p. NOS 1588
G.m. NOS 934
Rat mac NOS 1529
R.p. NOS 1638
G.m. NOS 984
Rat mac NOS 1579
R.p. NOS 1687
G.m. NOS 1034
Rat mac NOS 1628
R.p. NOS 1737
G.m. NOS 1084
Rat mac NOS 1678
R.p. NOS 1787
G.m. NOS 1134
Rat mac NOS 1728
R.p. NOS 1837
G.m. NOS 1184
Rat mac NOS 1778
R.p. NOS 1887
G.m. NOS 1234
Rat mac NOS 1822
R.p. NOS 1931


G.m. NOS 1284
Rat mac NOS 1849
R.p. NOS 1964
G.m. NOS 1334
Rat mac NOS 1857
R.p. NOS 2014
G.m. NOS 1384
Rat mac NOS 1883
R.p. NOS 2057
G.m. NOS 1434
Rat mac NOS 1898
R.p. NOS 2104
G.m. NOS 1484
Rat mac NOS 1905
R.p. NOS 2119
G.m. NOS 1534
Rat mac NOS 1944
R.p. NOS 2160
G.m. NOS 1584
Rat mac NOS 1977
R.p. NOS 2205
G.m. NOS 1634
Rat mac NOS 1997
R.p. NOS 2249
G.m. NOS 1684
Rat mac NOS 2031
R.p. NOS 2283
G.m. NOS 1734
Rat mac NOS 2053
R.p. NOS 2301


G.m. NOS 2282
Rat mac NOS 2402
R.p. NOS 2397
G.m. NOS 2331
Rat mac NOS 2434
R.p. NOS 2447
G.m. NOS 2377
Rat mac NOS 2484
R.p. NOS 2497
G.m. NOS 2422
Rat mac NOS 2533
R.p. NOS 2547
G.m. NOS 2461
Rat mac NOS 2580
R.p. NOS 2596
G.m. NOS 2513
Rat mac NOS 2630
R.p. NOS 2646
G.m. NOS 2563
Rat mac NOS 2650
R.p. NOS 2696
G.m. NOS 2612
Rat mac NOS 2729
R.p. NOS 2746
G.m. NOS 2662
Rat mac NOS 2779
R.p. NOS 2795
G.m. NOS 2712
Rat mac NOS 2829
R.p. NOS 2845


G.m. NOS 1784
Rat mac NOS 2078
R.p. NOS 2026
G.m. NOS 1834
Rat mac NOS 2103
R.p. NOS 2052
G.m. NOS 1884
Rat mac NOS 2126
R.p. NOS 2079
G.m. NOS 1934
Rat mac NOS 2147
R.p. NOS 2128
G.m. NOS 1982
Rat mac NOS 2173
R.p. NOS 2150
G.m. NOS 2032
Rat mac NOS 2195
R.p. NOS 2188
G.m. NOS 2082
Rat mac NOS 2234
R.p. NOS 2221
G.m. NOS 2132
Rat mac NOS 2273
R.p. NOS 2266
G.m. NOS 2182
Rat mac NOS 2321
R.p. NOS 2314
G.m. NOS 2232
Rat mac NOS 2368
R.p. NOS 2358


G.m. NOS 2762
Rat mac NOS 2879
R.p. NOS 2895
G.m. NOS 2812
Rat mac NOS 2929
R.p. NOS 2945
G.m. NOS 2862
Rat mac NOS 2971
R.p. NOS 2984
G.m. NOS 2912
Rat mac NOS 3015
R.p. NOS 3034
G.m. NOS 2959
Rat mac NOS 3065
R.p. NOS 3081
G.m. NOS 3009
Rat mac NOS 3115
R.p. NOS 3131
G.m. NOS 3059
Rat mac NOS 3165
R.p. NOS 3181
G.m. NOS 3106
Rat mac NOS 3215
R.p. NOS 3228
G.m. NOS 3156
Rat mac NOS 3265
R.p. NOS 3277
G.m. NOS 3255
Rat mac NOS 3315
R.p. NOS 3327


G.m. NOS 3304 T----------
Rat mac NOS 3365 GTG----------
R.p. NOS 3377 GCATCACACTGCGAACGGCAGAAATCCACAACAAGTCACGTGCAACTGCC
G.m. NOS ----------
Rat mac NOS 3368 ----------CGGTCTTTTCCTACGGAGC
R.p. NOS 3427 CGCATTCGGATGGCTTCTCAGCCGTAAGCTTCGGGTTTATCCTCGGTAGG
G.m. NOS 3305 ----------
Rat mac NOS 3397 AAAA----------AAGGG
R.p. NOS 3527 AAAGTTTTACCCCCTGAGATGACGCACGTAAGGGGTGCACCACACAAAAG
G.m. NOS 3305 ----------
Rat mac NOS 3406 CAACACCTTGGAGGAGCCCAAAGGCACAAGACTC----------
R.p. NOS 3527 AAATTGTTAGGATATAAGTGAAAAGAAAAAATTTCCCTTTTACAACAAAA
G.m. NOS 3305 ----------
Rat mac NOS 3440 ----------
R.p. NOS 3577 AAAAAAAAAAAAAAAAAAAAA

Appendix B: Primer Sequences

Original Set of Degenerate Primers

NOS 1 -- 5'-TTATG(tc)CCIGCIGA(tc)TGG(ga)TITGG-3'
NOS 2 -- 5'-GTCACC(ga)AA(gat)AT(ga)TC(tc)TG(tc)TA-3'
NOS 3 -- 5'-TAATG(tc)AT(act)GGI(ac)GI AT(act)CA(ga)TGG-3'
NOS 4 -- 5'-CGTAGGIGGIACIA(gt)CCAIA(tc)CCA-3'

Note: (1) nucleotides in brackets represent redundencies
(2) I = Inosine

Second Set of Primers

NOS A -- 5'-GCTAATGCTTGCCGTCTGTGC-3'
NOS B -- 5'-TTAAAGGACAGTGCTCTGGTCG-3'
NOS C -- 5'-TTAGTACGCCACAGAAACAGG-3'
NOS D -- 5'-GCTAGCTCCTAATTTCCACATG-3'
NOS E -- 5'-GCTATCTAAATGGTGCAATGC-3'
NOS F -- 5'-GCTAGCCGTTACCAAATGTCG-3'

Appendix C: Raw Sequence Analysis

Clone N1-- sequencing primer: m13-reverse (NOS3→)

NTGCGATGGTGCANnCGGAGGATACAGTGGAAGAAgTTACAAATATTCG
ATTGCAGAGAAGTAACCACAGCGAGTGGAATGTTCGAGGCGCTCTGTAA
TCATATTAAATACGCCACAAATAAAGGAAATATAAGGTCAGCAATAACA
ATATTCCCGCAACGCACAGACGGCAAGCATGACTACAGGATATGGAATC
CGCAGCTAATCAGCTATGCTGGTTACTTGGAGCCAGATGGTTCTGTACTG
GGAGATCCGGCGCGAGTCGAATTTACTGAGATCTGCATTAGACTTGGCT
GGAAGCCACCTCGCACTGCATGGGATATATTACCTCTAGTGCTGTCAGCA
GATGGCAAAGATCCCGAGTACTTCGAAATCCCACGGGAAATTATTATGG
AAGTGAAAATGGAACACCCCAAGTATGATTGGTTCAAAGAGCTAGGCTT
ACGCTGGTACGCGTTGCCTGCGGTTTCCAACATGAGACTGGACTGCGGT
GGTCTGGAATTCACCGGCACCGCTTTCAATGGCTGGTACATGGGAACAG
AgATCGGTTGTCGCAACTTCAGTGACGCCAACCGATTAAATGTTATTGAG
ACAGTAgCAACAAAAATGGGTTTGGATACgAATTCgTATGTGTCTCTCTG
GAAGGACAGTGCTCTGGTCNAATCAATATAACANTACTGCCANTTTCCA
CAGAAACACTCTCTATANTGGAACACCATTCCCTTCTGAACAATCATAAA
ACATTTGGACAATGACC

Clone N1 -- sequencing primer m13-forward (←NOS 4)

agGGGGGAGNCCAgaCCAATCGGCAGGACAtcCTCCTCTGCTTTTGTTTTCA
TTGTCCAAATGTTTTATGAATTGCTCAGAAGCGGAATGGTGGTCCACTAT
AGAGACGTTGTCTCTGTGGAAACTGTGCAGTACTGCTATATTGACTTCGA
CCAGAGCACTGTCCTTCCAGAGAGACACATACGAATTCGTATCCAAACC
CATTTTTGTTGCTACTGTCTCAATAACATTTAATCGGTTGGCGTCACTGA
AGTTGCGACAACCGATCTCTGTTCCCATGTACCAGCCATTGAAAGCGGTG
CCGGTGAATTCCAGACCACCGCAGTCCAGTCTCATGTTGGAAACCGCAG
GCAACGCGTACCAGCGTAAGCCTAGCTCTTTGAACCAATCATACTTGGG
GTGTTCCATTTTCACTTCCATAATAATTTCCCGTGGGATTTCGAAGTACT
CGGGATCTTTGCCATCTGCTGACAGCACTAGAgGTAATATATCCCATGCA
GTGCGAgGTGGCTTCCAGCCAAGTCTAATGCAGATCTCAGTAAATTCGAC
TCGCGCCGGATCTCCCAgTACAGAAcCATCTGGCTCCAAGTAACCAgCAT
AgCTGAtTAgCTGCGGATTCCATATCCTGTAgTCATGCTTGCCGTCTGTGC
GTTGCGGGAATATgGTnATTGCTGAACTTATATTCCTTATTTGTGGCTTTT
AATATGATACNAACGCCCCAACATTCCCTCCCTGTGGTTACTTCTCTGCA
ATCAATATTGTACTTCTNCCCTGTTTCCNCCNATGCCTAATTCTAATCAG
GTCAAACTGTTTNCNGTGTAAATGNTNTCCGCCCCAATNCCCCCACTTCA
AACCGAANCTAANTNTAACCCGGGGGGCNAANAATTACAACNCNNTAAT
GNTTCNCCCCGCCCNTTNCNTCGGAAACNTCNC

Clone N2-- sequencing primer: m13-reverse (NOS 1 →)

aCgATTATGGCCGGCgGATTGGGTGTGGATAGTACGCCAATGTCTTCGTC
TTTGACTGCTGTGTTTCATCAGGAAATGGCACTATACAATATGAGACCTT
CTTATGATTATCAAGAACCCGCATGGAAGACTCACAAATGGAGCAAGAC
CGACGGCAACAAGACTGTTCATAGGAAGTTCCACTTCAAGCAGATTGCG
AGAGCCGTGAAGTTTACTTCCAAAATTGTTTGGTCGAGCTCTCTCTAAGC
GCATCAAAGCGACCATACTGTACGCCACAGAAACAGGCAAATCTGAACA
GTATGCCAAAGAGTTGGGGACTATATTTGGACACGCCTTCAATGCACAG
GTTCACTGCATGTCAGATTACGATGTGTTCTCTATAGAACACGAAACTCT
AGTCTTGATTGTGACGTCGACATTTGGTAACGGCGAAcCCCCTGCTAATG
GAGAGGATTTCGCTAAACATCTTTTCCAAATGTTGTATAATGAGAGGAA
AAgTCaAGAgGATGGGCATGACGCGTaTAATCCTGGTaATTATAAACTACC
aACACCaAAgTCTTTAATGCGAACAAACAGTTTAATGGCaCTAATATTGAT
TATAAgAAACAGTTGTCgCGTTTGGAATCgAACAAAAgtagCaTagCaGGATC
GTCGACAGTTGAACAAATAGGACCCTCCTAATGTTTGCTTTGCNTATTTG
CNTNNGATCTANCCCTATCCGAATTCTGTTNCTTTNGGAAGAATATAACA
AATCTNGGCGATCTTGGAGGCAAAGAAATTTAAAATTGGCTGTNGAAAT
AAATTTTGGACGGAACACNTTTCNAANTGGTCTCAATATTTNCCCTTGCT
TGCAAACTTCTGTTGGACAAATTTTTNATNNNNATCCNNAANCTTNCTTC
CTNCNCTACTGAAAAANGTCAATGGAAACCTTNCTGATCC

Clone N2 -- sequencing primer m13-forward (←NOS 2)

ACGAAGAGNNCTCgTGGTATCTGTTTTCATCcATTAACTCTAACATGAAA
TCATCTACTTCCTCGTCCGACATTTTAGCTTGCTTCTTAATAATTGACTTT
AGTTTCTGTTGGACTTCTTCTGCCATTTTACAATCGCCACAAACATAAAA
GTGTCCATCTTTATCTAATAATAACCTTGTGACTTCAGCACCTTCATCTTC
TAATAGTTCTTGGACATGTTTTTTATCTGCACCATGTTCTCTCGACAGCG
CTACCATTGCTTTGCTCAAGACGCCTTCTTGTAAAGCTTGCTTCTTTTCTT
CTGTATACAAATCCATTCTTTGATTTCTACAGCCAAAAAATAACAAAATA
GGACCGAATTCTTTTTCCTGCTCTCTTTCCTTGACATTCTTTCTCTCTTCT
CTGCGATGATGCCAGAAACCTCTAAATGGTGCAATGCCTGATCCAGGTC
CAACCAATATAAGCGGAGCAGACAAATCTTTAGGCATGTGGAAATTAgG
AGCACGTCTAATAAAAACAAATACTTCGTCACCTGGTTTCAAATTTTTTA
AATAAGTTGAACACACTCCGTAATGTGTAGGTCCTTTGCCATTTTGACTT
TTGTAGAcAACTATTGCaACAGTAATATGAATTCTCGAATGATGTGCTAA
AGGTGATGANGATATCNAATAAAATCTAGGTGCAGCGGTGGTAACAATG
CCGCTAATAATGAAACTTGAAGTCTACATGAACGGATATCTGCCAAAAC
TTCTGCCAAATGAAGGGTATANAAATGTNTCCATCNCATACGCACTGAA
TCCGTNCTAATTTCTAATCTCTCACTTCAAATTATGGTGGCACAGTTTGG
CAGGNTCTCACACTGTGTTGAAGGNGGTGTTTGATTTCAAAACCAATGA
AAATTTNCTTNTGTNCCGCAANTACTTCTNTTCTTCCNGTTTGAAGCCCN
TAGGTTTTTTTNC

Clone N3 -- sequencing primer NOS B (NOS B→)

TNNNNNNTCNaTaTaGCAGTACTGCACAGTTTCCACAGAGACAACGTCTC
TATAGTGGACCACCATTCCGCTTCTGAGCAATTCATAAAACATTTGGACA
ATGAAAACAAAAGCAGAGGAGGATGTCCTGCCGATTGGATCTGGATAGT
ACCGCCAATGTCTTCGTCTTTGACTGCTGTGTTTCATCAGGAAATGGCAC
TATACAATATGAGACCTTCTTATGATTATCAAGAACCCGCATGGAAGACT
CACAAATGGAGCAAGACCGACGGCAACAAGACTGTTCATAGGAAATTCC
ACTTCAAGCAGATTGCGAGAGCCGTGAAGTTTACTTCCAAATTGTTTGGT
CGAGCTCTCTCTAAGCGCATCAAAGCGACCATACTGTaCGCCACAGAAAC
AGGCaAATCTGAACAGTATGCCAAAGAGTTGGGGACTATATTTGGACAC
GCCTTCAATGCACAGGTTCACTGCATGTCAGATTACgATGTGTTCTCTAT
AGAACACGAAACTCTAGTCTTGATTGTGACGTCgACATTTGGTAACGGNN
NNAAAA

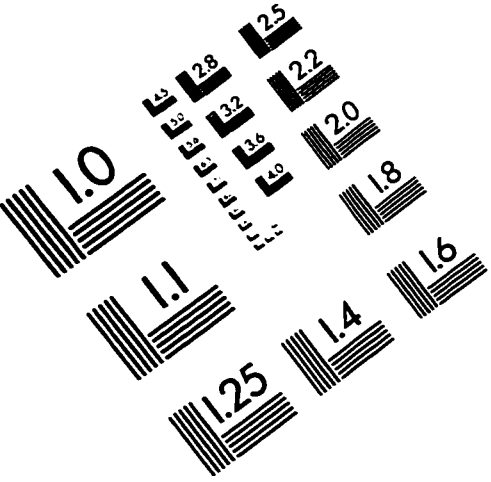
Clone N4 -- sequencing primer NOS C (NOS C→)

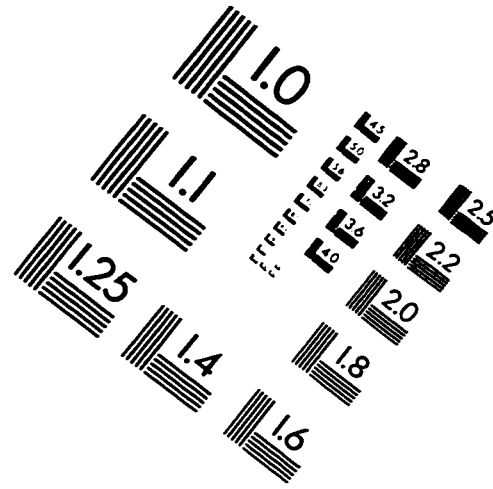
NNCTTTGGGGNCTGTNTNAATGTGNNCGAAAANGGGNAAGCCNCNNGA
ANAATGTANATTTTNCAGAGTCGCGTCTCATNTCGAATAATGATGNGNC
CCCTGGTGACAAATTATTCTCNATNNGTGNAACACACTCCGTAATGTGTN
GGTCCTNGGCCATTTTGACTTTTGTAGACAACTATTGCAACANTNATNTG
AATNCTCGAATGATGTGCTAAAGGTGATGATGATATNGAATAANATCTN
GGTCGCNGCGGGGGTANCCCNGCNCCCCTNANCANCTTGAGGTTCTACN
TCNCGGGANGCGCTGCCTTTTTTCTNNCCCCGAGGGGTGANGCGGGCCN
GTNGCGNGCGNCCNNGNGCCCCCNAATCCGNTCCGTNCATNCTCGAANT
NCNNCTNCGNGCNTCNNANNGNNTNNTCNCCTAGTCGTNCTNCNGGNG
NTGNNNTCCNCCCCGCTNGTCGTNNCAGGNGGTGTGGTGCNGGTTGCGG
ANNCNNCGTGGAGNNTANNTGTCCNGCCNANTNNGGGGGNGGGCCGAA
CNNCNNTTCCATTNCCNCNCGTNGNNNNNNGTAAGCNNCCTGTNNGGN
GGATCNCNTCTCTCCCNGNCCTGGGNTNGCGAAATTTNTNCGCCNTCTC
CTGCGCGANNGGATGCCTGNNCGGGGCCNTNNTGAGCTNCCGGCNTCN
NNCNANCCCCGTTGCNTTTACACNGCCNTTACCCAACATGGGANTCCAG
GGNCGGNNTTTGGAATTCNATCTCTGGTCCCTCNCAAAAAATGTGCGNT
GGAANANCGNCTNTANAAACACCTGAANGGTTGTNANGCCTGGACTTAN
CAAATGGTCAGGGTGTTTGNNAGGCTATNANGCAGTNCCNTTTTTTGNG
NTGGACCGGGATTGNTTTCCCAATCCNACCGGCNNCCGNTTGGGGTAAT
GGNTNTAACCAANNNAACCTTNNTCCNTTCCCNCCCGNCCAAGGGGTCT
NCNNTGGAANCNCCTTTTTTGGGGATTTTNAGGGAAATTTTCNCCC

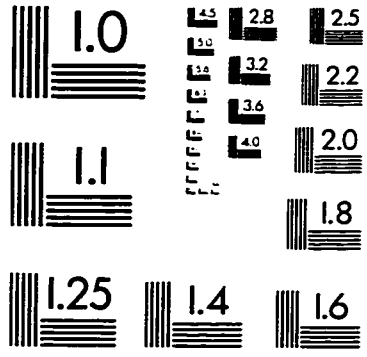
Clone N5 -- sequencing primer NOS D (NOS D→)

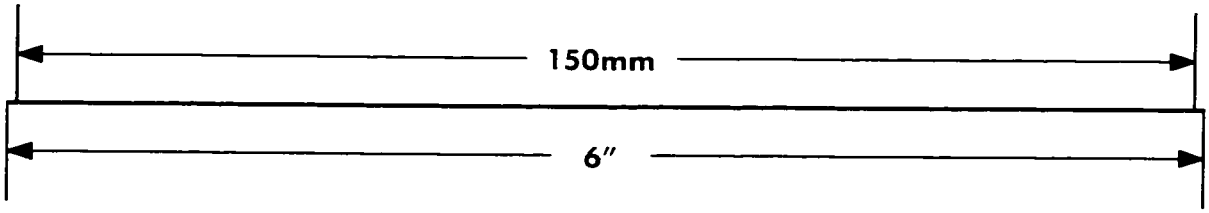
GNNNNNNNNTTNNNNNNNNGNNTgTCTGCTCCGCTTATATTGGTTGGAC
CTGGATCAGGCATTGCACCATTTAGAGGTTTCTGGCATCATCGCAGAGA
AGAGAGAAAGAATGTCAAGGAAAGAGAGCAGGAAAAAGAATTCGGTCC
TATTTTGTTATTTTTTGGCTGTAGAAATCAAAGAATGGATTTGTATACAG
AAGAAAAGAAGCAAGCTTTACAAGAAGGCGTCTTGAGCAAAGCAATGGT
AGCGCTGTCGAGAGAACATGGTGCAGATAAAAAACATGTCCAAGAACTA
TTAGAAGATGAAGGTGCTGAAGTCACaAGGTTATTATTAGATAAAGATG
GACACTTTTATGTTTGTGGCGATTGTAAAATGGCAGAAGAAGTCCAACA
GAAACTAAAGTCaATTATTAAGAAGCAAGCTAAAATGTCGGACGAGGAA
GTAGATGATTTCaTGTTAGAGTTAATGGATGAAAACAGATATCACGAAGA
CATATTTGGCATAACACTTAGAACCGCTCAAGTGCAAAGTGCATCGCGA
GACATTGCTAAGCGAACTCGTCAGGAGTCCATCCAATCTCAATCGTAAA
AAACAATAATAATTTaTTGCAGATATTGACTGATTTTGAATGTnCCAtATTc
CAATTCTGTagACCNTCCANAATCNTGTTGTTTNTACNTANGGCTNTANG
GNTTATTAATTCCTTTTTCAATTNTAANTNTTTTCNTNNAANTTTTCCAAC
NTCCAANTTCTAAAACNANTAAAACNCNTANNCNNTTATTTTTNGTCCTA
CNCCTNTANTTNTGGTANAAAGCCNAAGCTTTNAATNAATNACCCCNCC
CTCCCCTNAAATTNNNNNNNTTTTTTTTTTNNNAAANTTTTNANTNNTTA
AAAAAAAAGNTNAANCCNNNCCNNNNTTNCCNNNTNNANNNNTTNCCN
GNNNNNNNCC

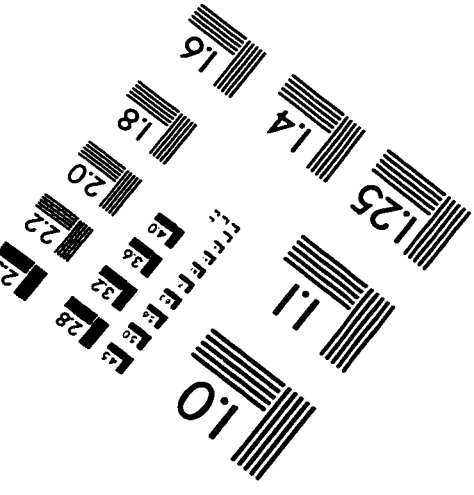
IMAGE EVALUATION TEST TARGET (QA-3)

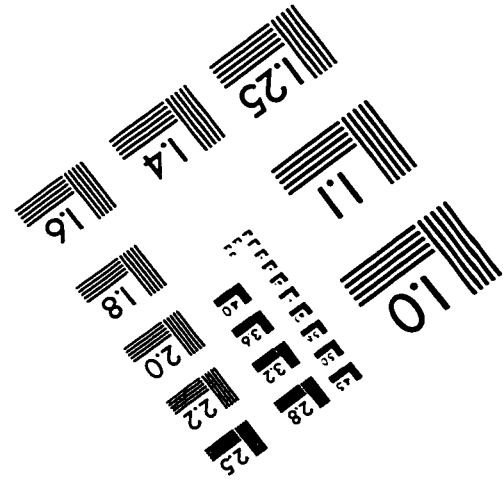












APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989