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Nitric Oxide Regulates Cell Proliferation during Drosophila Development

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Summary

Cell division and subsequent programmed cell death in imaginal discs of Drosophila larvae determine the final size of organs and structures of the adult fly. We show here that nitric oxide (NO) is involved in controlling the size of body structures during Drosophila development. We have found that NO synthase (NOS) is expressed at high levels in developing imaginal discs. Inhibition of NOS in larvae causes hypertrophy of organs and their segments in adult flies, whereas ectopic expression of NOS in larvae has the opposite effect. Blocking apoptosis in eye imaginal discs unmasks surplus cell proliferation and results in an increase in the number of ommatidia and component cells of individual ommatidia. These results argue that NO acts as an antiproliferative agent during Drosophila development, controlling the balance between cell proliferation and cell differentiation.

Introduction

Organ development requires a tightly controlled program of cell proliferation followed by growth arrest and differentiation and, often, programmed cell death. The balance between the number of cell divisions and the extent of subsequent programmed cell death determines the final size of an organ (reviewed by Bryant and Simpson, 1984; Raff, 1992). Although much of the cellular machinery that determines the timing of onset and cessation of cell division per se is well understood (reviewed by Hunter and Pines, 1994; Morgan, 1995; Sherr and Roberts, 1995; Weinberg, 1995), little is known about the signals that cause discrete groups of cells and organs to terminate growth at the appropriate cell number and size.

During Drosophila development, the structure, size, and shape of most of the organs of the adult fly are determined in the imaginal structures of the larvae (Cohen, 1993; Fristrom and Fristrom, 1993). Imaginal discs, specialized groups of undifferentiated epithelial cells that are recruited during embryogenesis, are formed in the first larval instar as integuments of the larval epidermis. Disc cells divide rapidly throughout the larval development and cease proliferating at the end of the third instar period. In leg, wing, and haltere discs, progression through the cell cycle stops in G2 phase 3–4 hr before puparium formation. It resumes 15–18 hr later (12–14 hr after pupariation) and then stops again in a defined spatial pattern after 12–14 hr (10–14 hr of pupal development) (Fain and Stevens, 1982; Graves and Schubiger, 1982; Schubiger and Palka, 1987). Although most of the dividing cells in the late larvae and in the early pupae are already committed to their adult fate, they do not develop a fully differentiated phenotype until growth arrest is firmly established. Thus, cell proliferation is temporally separated from cell differentiation, which takes place later during metamorphosis. Experiments with transplanted imaginal discs suggest that cessation of cell proliferation in these structures is controlled by mechanisms that, while intrinsic to the disc, are not completely cell-autonomous (Bryant and Schmidt, 1990; Cohen, 1993). The signaling pathways that control coordinated temporary growth arrest in larvae and pupae and subsequent terminal growth arrest in pupae and adults are not known, but they probably involve as yet undetermined inter- and intracellular second messenger molecules.

Nitric oxide (NO) is a diffusible multifunctional second messenger that has been implicated in numerous physiological functions in mammals, ranging from dilation of blood vessels to immune response and potentiation of synaptic transmission (reviewed by Bredt and Snyder, 1994a; Nathan and Xie, 1994; Garthwaite and Boulton, 1995). NO is produced from arginine by nitric oxide synthase (NOS) in almost all cell types. A group of three chromosomal genes, giving rise to numerous isoforms of NOS, have been cloned from mammalian cells (reviewed by Knowles and Moncada, 1994; Wang and Marsden, 1995), and recently a Drosophila NOS gene, whose coding structure resembles the gene for the mammalian neuronal isoform, has been isolated (Regulski and Tully, 1995). NO can act in cells as an efficient antiproliferative agent by suppressing DNA synthesis (Garg and Hassid, 1989; Lepoivre et al., 1990; Kwon et al., 1991). This ability of NO to prevent DNA synthesis and cell division initiates growth arrest during nerve growth factor–driven neuronal differentiation of cultured cells and switches the program from proliferation to differentiation (Peunova and Enikolopov, 1995). Here, we show that NO controls cell number and thus the size of anatomical structures in an intact animal. During Drosophila development, NOS activity appears in imaginal discs of the third instar and reaches high levels at the time when cells of the discs enter a state of temporary cytostasis. Inhibition of NOS at this stage results in excessive growth of the structures of the body of the adult flies. Conversely, ectopic expression of NOS in larvae results in reduced growth of adult structures. The antiproliferative action of NO becomes even more obvious if the effect of programmed cell death is blocked. Our data suggest that NO acts as an antiproliferative agent

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during cell differentiation in Drosophila and that induction of NOS is a crucial step in Drosophila development. We propose that NO can play a broader role as a general regulator of cell proliferation and differentiation during organism development and morphogenesis.

Results

NOS Is Expressed in Imaginal Discs during Larval Development

At the end of the third instar, cells of imaginal discs undergo temporary cell cycle arrest. Cytostasis is released 12–14 hr after pupariation and is established once again (this time permanently) in the late pupae and the pharate adult. The ability of NO to halt cell division reversibly and establish temporary growth arrest makes it a plausible candidate for mediating cytostasis in imaginal discs. To investigate this possibility, we examined imaginal discs of the third instar and early pupae for NOS presence. Drosophila NOS (dNOS) gene, which is preferentially expressed in the adult head, has recently been cloned and characterized (Regulski and Tully, 1995). However, different NOS–related mRNA species are present in the embryo, larvae, and adult flies (M. Regulski, personal communication; Y. Stasiv and G. E., unpublished data). These mRNAs may be produced by the cloned dNOS gene or by other potential Drosophila NOS genes, making the detection of the relevant RNA species difficult. Therefore, to visualize the expression of NOS in Drosophila during larval development, we used histochemical staining for the NADPH–diaphorase (reduced nicotinamide adenine dinucleotide phosphate–diaphorase) activity of NOS, which reflects the distribution of the total enzyme activity in a tissue (Dawson et al., 1991; Hope et al., 1991; Muller, 1994).

NADPH–diaphorase staining was observed in all imaginal discs, imaginal rings, histoblasts, and the brain of the larvae, beginning in the third instar. Staining became more intense as development proceeded, and in late third instar larvae and early pupae, a highly specific and reproducible pattern of very intense staining was evident (Figure 1). In the leg imaginal disc, NADPH–diaphorase staining was initially seen at the very beginning of the third instar. Staining was confined to the center of the disc, corresponding to the presumptive distal tip of the leg. As the discs matured, diaphorase staining intensified, and in the late third instar it nearly obliterated the distinction between individual concentric rings of epithelial folding normally seen in axial view. At the end of the third instar stage, the staining of the center of the disc (distal tip), which stained most darkly at the beginning of the third period, was weaker in comparison with the surrounding cells. Later in development, when the discs began to evert in the prepupae, diaphorase staining of the forming leg became less intense, and a distinct characteristic pattern of staining of individual segments became evident. At 2–4 hr after puparium formation, intense NADPH–diaphorase staining was observed in the presumptive tibia, first and second tarsal segments, and the proximal part of the fifth tarsal segment of the forming leg (Figure 1e). Staining was much weaker in the third and fourth segments, and areas of intense staining were unevenly distributed throughout the regions of presumptive femur. Weak staining was also present in the coxa and body wall. The progression of staining patterns throughout the larval development was highly specific and reproducible. The staining of the imaginal discs corresponding to the first, second, and third pairs of legs was very similar. As with the leg imaginal discs, other imaginal discs (Figure 1), imaginal rings, and histoblasts (data not shown) exhibited increasingly intense NADPH–diaphorase staining as larval development proceeded. Wing, eye, haltere, and genital discs in the third instar had distinct and reproducible patterns of intense staining, which gradually decreased in a specific spatial pattern during early pupal development.

These results demonstrate that there is a gradual and specific accumulation of NOS in developing imaginal discs, which reaches highest levels at the time when the progression through the cell cycle slows down.

Synthesis of DNA Is Affected by Manipulations of NOS Activity

If NO acts as an antiproliferative agent during Drosophila development at stages when the cells of imaginal discs enter temporary cytostasis, then its action might directly affect DNA synthesis in the discs. Inhibition of NOS would then be expected to relieve the block and increase the number of cells in S phase; conversely, high levels of NO would lead to a decrease in the number of dividing cells. To test this hypothesis and to map the extent and distribution of the antiproliferative effect of NO, we monitored DNA synthesis in larval and prepupal discs while manipulating the levels of NOS activity. To inhibit NOS activity, we injected specific NOS inhibitors in developing larvae. To increase the levels of NOS, we induced expression of NOS transgene in transformed larvae carrying the mouse NOS2 cDNA gene (Lowenstein et al., 1992) under the control of the heat-shock promoter. NOS2 is a calcium-independent form of NOS that is capable of efficient constitutive NO production. We labeled imaginal discs with 5-bromo-deoxyuridine (BrdU) and compared the extent and distribution of labeling of S-phase nuclei in leg imaginal discs from larvae after inhibition of NOS, from NOS2–transformants after heat-shock induction, and from control untreated larvae (Figure 2). The data in Figure 2 show that there were significantly more BrdU-labeled cells in imaginal discs of larvae in which NOS activity was suppressed by L-nitroarginine methyl ester (L-NAME) than in control untreated larvae (or larvae treated with the inactive isomer D-NAME; data not shown). In contrast, there were markedly fewer BrdU-labeled cells in imaginal discs from induced NOS–transformed flies than in uninduced controls. At the same time, these changes in the number of BrdU-labeled cells after inhibition or ectopic expression of NOS appeared to be evenly distributed over the entire disc.

These data indicate that modulation of NOS activity affects the number of cells in S phase in imaginal discs, which is consistent with the observations that NO suppresses DNA synthesis and cell division.

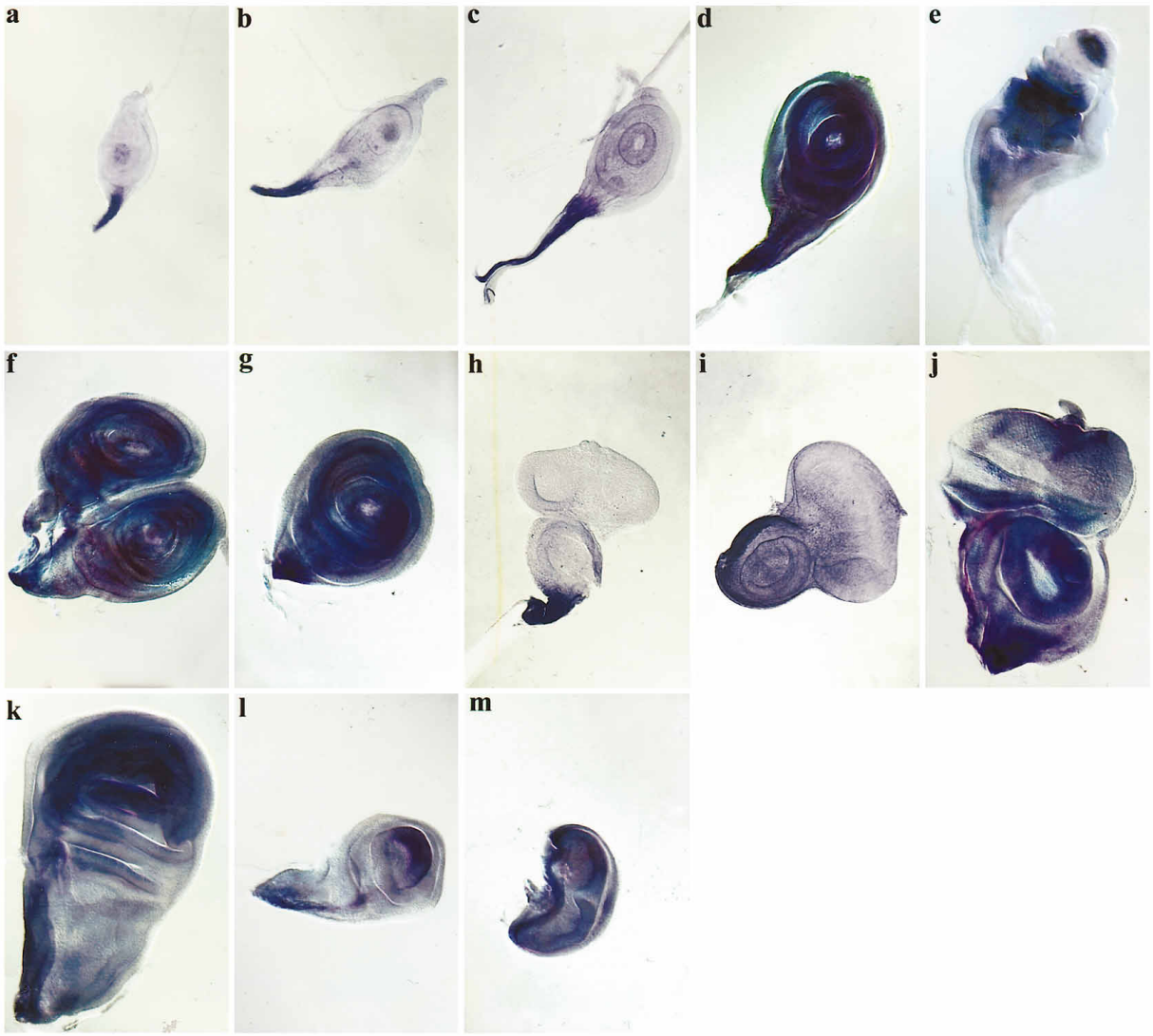

Figure 1. Diaphorase Staining of Developing Imaginal Discs

Panels show imaginal discs from larvae stained for NADPH–diaphorase.
(a–e) Second leg discs; discs were isolated 48 (a), 72 (b), 84 (c), and 96 (d) hr after egg laying and 2 hr after pupariation (e).
(f) First leg discs from 96 hr-old larvae.
(g) Third leg disc from 96 hr-old larvae.
(h–j) Eye-antennal discs from 72 (h), 84 (i), and 96 (j) hr-old larvae.
(k) Wing disc from 96 hr-old larvae.
(l) Haltere disc from 96 hr-old larvae.
(m) Genital disc from 96 hr-old larvae. All panels are to the same scale.

Inhibition of NOS Results in Hypertrophy of Leg Segments

The highest levels of diaphorase staining occur during the period of development when DNA synthesis and the rate of cell division in most of the imaginal disc cells slow down. The strong antiproliferative properties of NO and the specific pattern of diaphorase staining seen in mature imaginal discs implied that NO might act as a growth arrest agent in these structures, capable of inhibiting DNA synthesis and supporting temporary cytostasis during the switch to metamorphosis. If NO indeed acts as an antiproliferative agent during the late stages of larval development, then inhibition of NOS might result in excessive growth of organs and tissues, whereas ectopic overexpression of the NOS gene might have the opposite effect.

To test this hypothesis, we inhibited NOS activity by injecting specific NOS inhibitors in the developing larvae at the end of the third instar, several hours before metamorphosis. Most of the larvae completed metamorphosis successfully, giving rise to adult flies within the normal time frame. The resulting adults differed from normal flies in many respects, the most dramatic being enlargements of the appendages and other structures of the

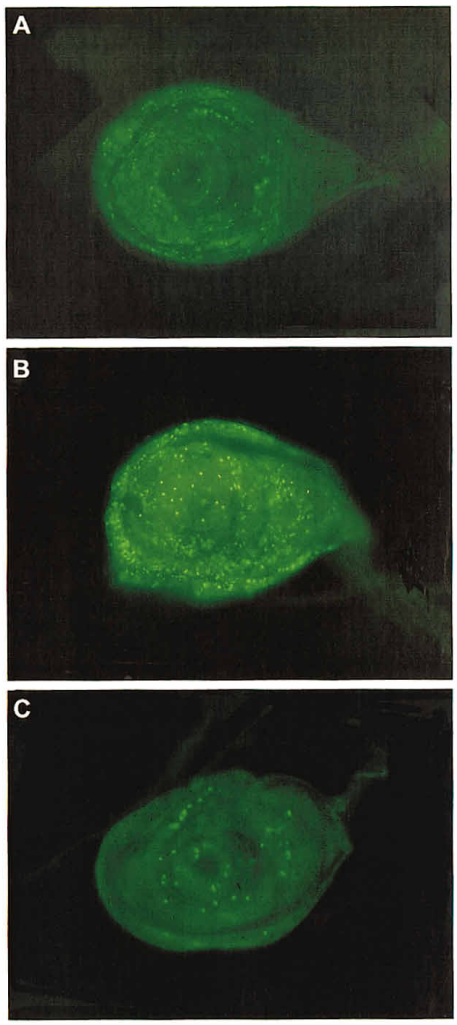

Figure 2. DNA Synthesis in Imaginal Discs after Inhibition and Ectopic Expression of NOS

Imaginal discs from late third instar were labeled with BrdU and stained with anti-BrdU antibodies. The number of fluorescing nuclei reflects the number and distribution of cells in S phase.
(A) Third leg imaginal disc from a control larva.
(B) Third leg imaginal disc from a larva treated with NOS inhibitor L-NAME. The distribution of BrdU-labeled nuclei in imaginal discs after treatment with inactive isomer D-NAME was identical to that of untreated controls.
(C) Third leg imaginal disc from an hs-mNOS transgenic larva treated with heat-shock.

fly body. The changes included, first, hypertrophy of the femur, tibia, and the segments of the tarsus; second, overgrowth of the tissues originating from the genital disc (in extreme cases, these cells contributed to more than one-quarter of the fly body); third, an increase in the overall surface of the wings; fourth, overgrowth of the cells of tergites and sternites; fifth, hypertrophy of the humerus; sixth, occasional duplications of some areas of the eye; seventh, occasional malformation of genital structures, legs, and eyes; and eighth, occasional ectopic formation of misplaced body structures.

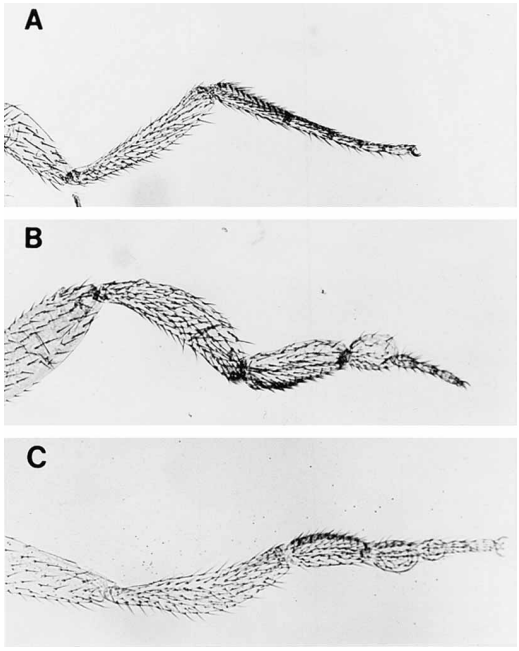

Figure 3. Overgrowth of Adult Leg Segments after Inhibition of NOS

Third instar larvae were treated with NOS inhibitors L-NAME and ETU. Legs from adult flies are shown.
(A) Third leg from a control untreated fly.
(B) Third leg from a fly treated with ETU at the third instar stage.
(C) Third leg from a fly treated with L-NAME at the third instar stage.
Note that the segments most affected in adults are those that stain particularly strongly for NADPH–diaphorase in larvae.

The changes were most profound in and most often affected the legs of the adults. The hypertrophy was particularly strong in the third pair of legs, where the diameter of certain segments increased 3–4-fold (Figure 3). The number of bristles and the number of rows of bristles also increased, confirming that hyperproliferation of the cells had occurred. The leg segments most strongly affected were those (first and second tarsal segments, tibia, and femur) whose primordia had the highest levels of NOS at the larval and prepupal stages. The changes affected mainly the anteroposterior and dorsoventral but not the proximodistal axes, so that the length of the affected segments remained the same. Identical changes were observed when two structurally unrelated inhibitors of NOS, 2-ethyl-2-thiopseudourea (ETU) and L-NAME (but not D-NAME) were used, indicating that the observed effect resulted specifically from blocking NOS activity.

In summary, our data show that inhibition of NOS at the late stages of larval development results in excessive cell proliferation and increased size of the structures of the body of the adult fly.

Ectopic Expression of a Mouse NOS Transgene Results in Reduced Size of Leg Segments

The ability of NO to inhibit DNA synthesis and cell proliferation suggests that overexpression of NOS in developing larvae may lead to diminished cell proliferation in the imaginal discs (see Figure 2) and to a reduction in

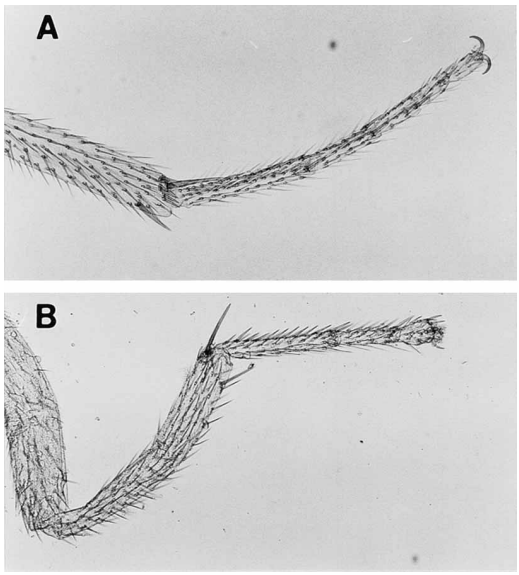

Figure 4. Reduced Growth of Adult Leg Segments after Ectopic Expression of NOS from a Transgene

Transgenic larvae carrying mouse NOS2 gene under a heat-shock promoter were exposed to elevated temperature.
(A) Third leg from a control untreated fly.
(B) Third leg from a fly treated with heat shock after pupariation. Note that the segments most affected in adults are those that stain weakly for NADPH–diaphorase in larvae.

the size of organs of the adult fly. We tested transformed flies that expressed the mouse NOS2 transgene under the control of the heat-shock promoter. Transgenic larvae were heat-shocked within 1 hr after pupariation to induce ectopic expression of NOS before the final cell divisions took place. This resulted in a reduction in the size of the limbs of the fly (Figure 4). The distal segments of the legs were affected most frequently and to the greatest degree. In extreme cases, the whole tarsus was shortened 1.5–2-fold, and the third, fourth, and fifth segments were fused together with poorly defined boundaries. The number of bristles in a row on the affected segments also decreased, although the number of rows did not change. The segments of the adult leg most often affected by the overexpression of NOS (third, fourth, and fifth tarsal segments) were those that were not affected by the NOS inhibitors and whose precursors exhibited particularly low levels of diaphorase staining in the early prepupal stages. The most terminal structures of the appendage, including the tarsal claw, remained intact in these defective legs. This suggests that the observed reduction in size was due to incomplete growth of the distal area of the developing appendage, rather than to complete loss of its distal structures. In contrast to the results on NOS inhibition, the changes affected only the proximodistal axis, while the diameter of the affected segments remained the same. In addition to the reduction in the size of the leg segments, changes included a decrease in the overall surface of the wings, cuts in the wings, and reduced size of tergites and sternites.

We conclude from these results that ectopic expression of NOS at the late stages of larval development results in a decrease in cell proliferation and a reduction in the size of the structures of the body of the adult fly.

Inhibition of Apoptosis Unmasks Excessive Proliferation

In leg imaginal discs, the changes in the number of S-phase nuclei after manipulation of NOS activity directly correlated with the changes in the size of the adult limbs (see Figure 2). However, in the eye imaginal disc, we consistently detected an increase in the number of cells in S phase after inhibition of NOS (data not shown), but the resulting adult eye usually appeared normal. We tested the possibility that the apparently normal eye phenotype occurred as a result of programmed cell death, which counteracts excessive cell proliferation induced by NOS inhibition and restores the normal number of cells in the eye during metamorphosis. To suppress programmed cell death, we used GMR-P35 flies (Hay et al., 1994; donated by Drs. B. Hay and G. Rubin), in which apoptosis in the developing eye is largely prevented by expression of recombinant baculovirus p35 protein. p35 is a strong inhibitor of apoptosis, which acts by inhibiting the interleukin-1β-converting enzyme–like proteases and is able to prevent apoptosis in multiple contexts. GMR-P35 flies express p35 under the transcriptional control of multimerized glass-binding site from the Drosophila Rh1 promoter (Hay et al., 1994). *glass* promoter directs expression of the transgene in all cells in and posterior to the morphogenetic furrow in the eye disc (Ellis et al., 1993; Hay et al., 1994).

When NOS was inhibited in GMR-P35 larvae, the eyes of the adult flies showed numerous changes, reflecting the excessive proliferation of various cell types in the developing eye. The most dramatic of these changes was in the number of ommatidia in the adult eye, which increased from the nearly invariant complement of 750 in wild-type flies (747 +/− 4) and untreated GMR-P35 flies (748 +/− 6), to nearly 820 (818 +/− 21) after NOS inhibition in GMR-P35 flies (Figure 5A). This, together with the elevated number of cells per ommatidium (see below), caused an increase in the overall size of the eye. Other changes in p35-expressing flies after inhibition of NOS compared with the control GMR-P35 flies included, first, more ommatidia with an irregular shape (perhaps because of the uneven increase in the number of various cell types); second, more ommatidia with an irregular arrangement of the rows; and third, more ommatidia of a smaller size.

Another manifestation of the inhibition of NO production in GMR-P35 flies was an increase in the number of pigment, cone, and bristle cells. Wild-type ommatidia contain, in addition to eight photoreceptor cells, a set of four cone cells and two primary pigment cells, surrounded by an array of six secondary pigment cells, three tertiary pigment cells, and three bristles (Wolff and Ready, 1993). The number of photoreceptor and accessory cells is normally constant, and variations in this arrangement in the eyes of the normal flies are very rare. In GMR-P35 flies, the number of secondary and tertiary pigment cells was increased from 12 to 25 (25 +/− 4) cells per sample area (defined as described in the legend to Figure 5; see also Hay et al., 1995) as a

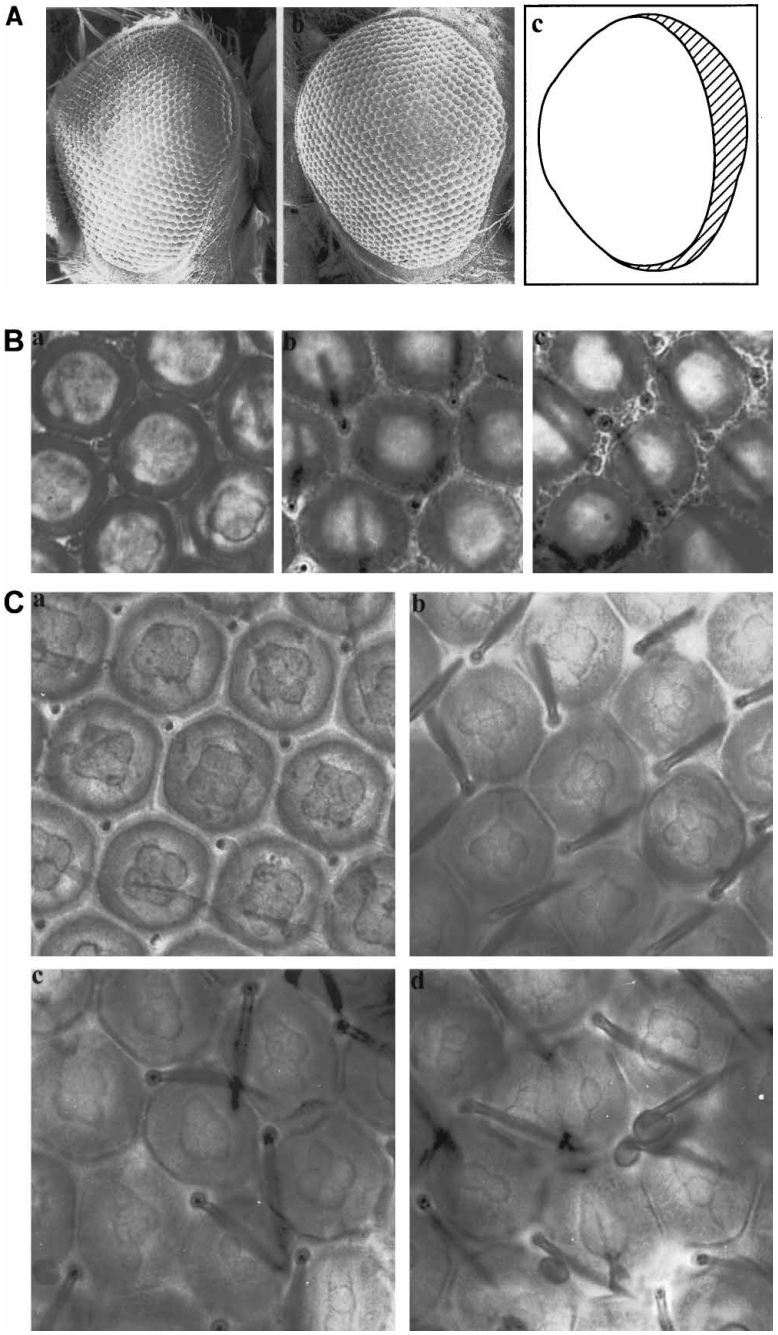

Figure 5. Eye Phenotypes of Flies Expressing p35 Inhibitor of Apoptosis after Inhibition of NOS

(A) Scanning electron micrographs of adult eyes. a, control GMR-P35 flies; b, GMR-P35 flies treated with NOS inhibitor L-NAME at the larval stage; c, overlay of the contours of a and b. Photographs were taken from a similar angle. Note the increased surface and convexity of the adult eye after treatment with NOS inhibitor at the larval stage. Series of electron micrographs were used to quantitate ommatidia in the adult eyes. The number of ommatidia in untreated GMR-P35 flies was the same as in the wild-type flies and constituted approximately 750 per adult eye versus approximately 820 after NOS inhibition.

(B) Cobalt sulfide–stained 55 hr-old pupal eyes from a control fly (a), GMR-P35 fly (b), and GMR-P35 fly after inhibition of NOS in the late third instar (c). The focus was adjusted mostly on the secondary and tertiary pigment cells. Note numerous extra secondary and tertiary pigment cells and bristles per sample area in (c). Sample area is defined as in Hay et al. (1995) and corresponds to the hexagonal area bounded by straight lines linking the cone cells of the six surrounding ommatidia.

(C) Cobalt sulfide–stained 70 hr-old pupal eye from a control fly (a), GMR-P35 fly (b), and GMR-P35 fly after inhibition of NOS in the late third instar (c and d). The focus was adjusted on the cone and primary pigment cells. Note clusters of six or more cone cells in c and ommatidia with one, two, or three cone cells per sample area in d. Also note ommatidia with irregular shape and row arrangement.

result of suppressed programmed cell death. Inhibition of NOS in these flies resulted in a further increase in the number of secondary and tertiary pigment cells to more than 35 (36 +/− 8) per sample area (Figure 5B). This number exceeds the maximal number of pigment cells saved from programmed cell death in untreated GMR-P35 flies (Figure 5B; Hay et al., 1994, 1995) and suggests that extra pigment cells arise as a result of excessive cell proliferation caused by inhibition of NOS combined with suppression of cell death caused by p35.

The number of ommatidia with extra primary pigment cells in GMR-P35 flies after inhibition of NOS was also increased in comparison with control flies, although it only slightly exceeded the levels in untreated GMR-P35 flies (data not shown). Furthermore, the number of bristles was increased in some areas of the eye in GMR-P35 flies after NOS inhibition, up to 4–5 per ommatidium instead of the three seen in normal flies and untreated GMR-P35 flies, and these were often mislocated (Figure 5B). Similarly, the number of cone cells was increased from four in normal and untreated GMR-P35 ommatidia to five and six in many ommatidia of GMR-P35 flies after NOS inhibition (Figure 5C (c)). We have also found clusters of ommatidia containing one, two, or three cone

cells (Figure 5C (d)), which may correspond to improperly formed supernumerary ommatidia (see above) that did not attain the proper set of cells.

We conclude that prevention of apoptosis by baculovirus p35 protein in the developing eyes of transgenic flies revealed excessive proliferation of various cell types after NOS inhibition in larvae, which was otherwise masked by programmed cell death in the larvae and pupae.

Discussion

Transformation of imaginal precursors in adult structures during fly metamorphosis involves transition from cell proliferation to cell differentiation. Cessation of cell division is a necessary, although not sufficient, condition for cell differentiation to proceed. A temporary cytostasis occurs at the end of the larval period, and permanent arrest of cell division occurs during pupal development. NO, a diffusible messenger molecule, is capable of efficiently blocking cell division. We have recently shown that induction of NOS initiates a switch to growth arrest prior to differentiation of cultured neuronal cells (Peunova and Enikolopov, 1995). Thus, NOS can act as a permissive factor, making the further development of the fully differentiated phenotype possible. We now report that NOS acts as an antiproliferative agent during normal Drosophila development. Our results indicate that NO is an important growth regulator in the intact developing organism.

Throughout larval development, there is a gradual and spatially specific accumulation of NADPH–diaphorase activity in developing imaginal discs, reflecting an increase in overall NOS content. At the time temporary cytostasis is being established in imaginal discs, NADPH–diaphorase staining becomes particularly intense, and it gradually decreases during prepupal and pupal development (Figure 1). Besides the imaginal discs, other structures with intense NADPH–diaphorase staining include imaginal rings, histoblasts, and the brain. These structures undergo radical changes during metamorphosis before giving rise to adult organs. Their development includes periods of rapid cell division alternating with periods of cytostasis and thus must employ mechanisms for coordinated cessation of DNA synthesis and cell division in a spatially defined pattern. Since NO can prevent cell division and can diffuse and act within a limited volume, we considered the hypothesis that NO acts to induce coordinated growth arrest during Drosophila development. If, indeed, NO actively exerts its antiproliferative activity during the development of imaginal discs, then inhibition of NOS before the temporary cytostasis is established at the end of the larval period could lead to the reversal of the arrest of cell division and induce additional divisions, which in turn could lead to increased size of structures of the body of the adult fly. Conversely, excessive or ectopic production of NO in larvae could cause premature cessation of cell division and lead to a reduction in the size of the structures in the adults. Both predictions were confirmed in experiments in which NOS activity was manipulated in the developing fly. NOS inhibition in larvae caused an increase in the number of cells in some parts of the adult body and an increase in their size, whereas ectopic expression of the NOS transgene during development caused a decrease in the number of cells in some structures in the adult and a decrease in their size, probably by partial fusion and reduction. In the developing leg, the segments that were most often affected when NOS activity was inhibited and the segments that were most often affected when the activity was ectopically induced were nonoverlapping and complementary. Most importantly, their distribution matched the distribution of NOS in the imaginal discs (Figure 6), thereby supporting the hypothesis that NO plays a causative role in growth arrest in normal development.

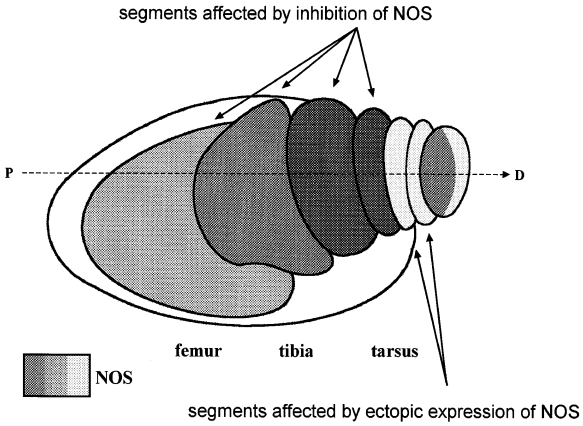

Figure 6. Changes of the Leg Segments in Adult Fly Correspond to the Distribution of NOS in the Imaginal Discs of the Larvae

Segments that were most often and most strongly affected in adults (enlarged after NOS inhibition and reduced after ectopic NOS expression in larvae) are indicated. "P-D" marks the proximodistal axis. Shading of the segments on the scheme corresponds approximately to the intensity of NADPH–diaphorase staining.

The antiproliferative properties of NO suggest that NOS acts in development through its influence on DNA synthesis and cell division. Our experiments with BrdU incorporation in leg discs with elevated and diminished production of NO (Figure 2) corroborate this hypothesis and suggest a direct link between synthesis of NO, number of S-phase cells, and the final size of the organ. In accordance with this notion, in many instances no BrdU incorporation was observed in regions highly enriched in NOS (Figures 1 and 2; unpublished data). The mechanisms for the NO–mediated arrest of the cell cycle (both temporary and terminal) are unknown, but they probably involve the conventional cellular machinery for growth arrest, e.g., cell cycle–dependent kinases and their inhibitors. Consistent with this, we observed changes in expression of these proteins when cultured cells were treated with NO (N. P. and G. E., unpublished data). An intriguing feature of imaginal disc cells is that they stop dividing and accumulate in G2 phase in the late third instar, preceding the period of temporary cytostasis (Fain and Stevens, 1982; Graves and Schubiger, 1982; Schubiger and Palka, 1987). This parallels a tendency of NO–treated (Peunova and Enikolopov, 1995) and nerve

growth factor–treated (Buchkovich and Ziff, 1994) PC12 cells to accumulate in G2 phase. Interestingly, imaginal discs are released from the G2 block and reenter S phase 12–15 hr after pupariation, at the time when diaphorase staining is greatly diminished. These correlations between imaginal discs cells and NO–treated cells support the idea that NO can be a major inducer of cytostasis in the cells of imaginal discs in the prepupal stage.

The final number of cells in an organ or a segment is determined both by cell multiplication and cell death, which the forming structures of the fly undergo as a normal step in development (especially at the late stages of pupal development). Results shown in Figure 2 indicate that the changes in the size of the leg segments after manipulation of NOS activity were correlated directly with the changes in DNA synthesis and the number of dividing cells. Furthermore, we did not detect any significant changes in apoptosis in the larval and prepupal leg discs after inhibition or ectopic expression of NOS, compared with the control discs, when cell death was monitored by acridine-orange staining or by the TUNEL assay (B. K. and G. E., unpublished data). This suggests that it is cell multiplication, rather than changes in programmed cell death, that leads to the changes in the size of the appendage.

On the other hand, the apoptotic death may conceal excessive cell proliferation in other developing organs. We tested whether the potential excessive cell proliferation can be unmasked in the absence of programmed cell death. We used transgenic flies in which programmed cell death in the developing eye was suppressed by recombinant p35, an inhibitor of apoptosis, to reveal excessive proliferation after NOS inhibition. We found that under these circumstances, several cell types and structures are overrepresented, the most noticeable change being an overall increase of the size of the eye due to the increased number of ommatidia. In addition, other cell types (e.g., secondary and tertiary pigment cells, cone cells, and cells of the bristles) proliferated after NOS inhibition to levels higher than those achieved by blocking apoptosis by p35 (Hay et al., 1994). These data demonstrate that the removal of suppressive influence of NO leads to an increased size of the adult organ, unless this effect is masked by programmed cell death, and indicate that final cell number in the adult organ is under dual control by both cell proliferation and programmed cell death. Furthermore, these data provide independent support for the hypothesis that NO directly regulates the cell number during development.

After inhibition of NOS with either of two structurally unrelated compounds, excessive growth was observed in most of the structures of the adult flies that derive from imaginal discs and histoblasts, albeit to varying extents for different organs. The most obvious changes were observed in the segments of the legs whose primordia showed the highest levels of NOS. We have not detected any substantial number of instances in which a duplication of a larger structure (for example, segments of the legs or wings) occurred. This indicates that extra proliferation of cells under the influence of NOS inhibitors occurs after the developmental fate is determined for most of the cells in the imaginal discs. This suggests that in most cases NO may be more important for the induction of growth arrest and subsequent differentiation of already committed cells than for the developmental commitment and establishment of the cell identity in the embryo or larvae.

We found it intriguing that only some of the axes of the developing structures were affected by manipulations of NOS activity. For instance, in developing legs only the anteroposterior and dorsoventral axes, but not the proximodistal axis, were affected by inhibition of NO production. In contrast, when NOS was ectopically expressed, only the proximodistal axis was affected. These results suggest that a gradient of NO may be involved in the process of establishing the polarity of the axes of the developing organ.

In summary, we have demonstrated that inhibition of NOS in larvae leads to enlargement of organs in adults and, conversely, that ectopic expression of NOS in larvae leads to a reduction in the size of organs in adults. Also, the distribution of affected segments in the adult leg corresponds to the distribution of NOS in the larvae, and the changes in segment size can be directly correlated to changes in DNA synthesis in imaginal discs after manipulations of NOS activity. The increased cell proliferation that occurs in response to NOS inhibition is masked in some structures by programmed cell death, and it can be revealed by suppressing apoptosis. Taken together, these results demonstrate that activation of NOS is a crucial step in Drosophila development. They confirm the hypothesis that NO acts as an antiproliferative agent during cell differentiation and organism development and controls the cell number in an intact developing organism.

How general is the phenomenon of the NO–mediated growth arrest in organism development? NOS expression can be induced to high levels in a large number of tissues and cell types by appropriate stimulation (Bredt and Snyder, 1994a; Forstermann et al., 1995). In most cases, the pattern of NOS distribution in a developing organism differs strongly from the distribution in the adult organism. Furthermore, transient elevation of NOS expression in a given tissue often coincides with the cessation of division of committed precursor cells. The developing mammalian brain provides an especially apt demonstration of this (Bredt and Snyder, 1994b; Blottner et al., 1995). A strong elevation of NOS activity in the developing cerebral cortical plate and hippocampus at days 15–19 of prenatal development correlates with the time course of cessation of precursor cells proliferation, tight growth arrest, and cell differentiation; notably, NOS activity goes down after the proliferation of committed neuronal precursors is completed. NOS levels are also transiently increased in developing lungs, bones, blood vessels, and nervous system (Blottner et al., 1995; Collin-Osdoby et al., 1995; Cramer et al., 1995; Shaul, 1995; Wetts et al., 1995). Elsewhere, NOS activity is greatly elevated in regenerating tissues when cessation of cell division is crucial for prevention of the unregulated growth (Roskams et al., 1994; Blottner et al., 1995; Decker and Obolenskaya, 1995; Hortelano et al., 1995). In all these cases, a transient elevation of NOS activity might trigger a switch from proliferation to growth arrest and differentiation, thus contributing to the proper morphogenesis of the tissue and the organ. It is noteworthy

that mutant mice in which one of the alternative translation initiation sites of neuronal *NOS* was disrupted by homologous recombination, leading to partial reduction of activity of this isoform (Huang et al., 1993; Brenman et al., 1996), developed hypertrophy of the stomach, which expanded to several times its normal size.

We propose that production of NO is required during embryonic development and during tissue regeneration in the adult organism for the proper control of cell proliferation. The antiproliferative properties of NO may be particularly important in situations in which terminal differentiation of committed cells is temporally separated from cell proliferation and is strictly dependent on cessation of cell division. Given the multiplicity of the NOS isoforms and their overlapping tissue distribution, it is conceivable that any group of cells in the embryo and fetus can be exposed to NO action. Furthermore, recent data showing that NO can be transferred within the organism by hemoglobin (Jia et al., 1996), raise the possibility that a developing mammalian embryo can also be supplied with NO exogenously by the mother.

NO is a readily diffusible molecule, and it may therefore exert its antiproliferative properties not only in the cell that produces it but in the neighboring cells as well (Gally et al., 1990). This property is important when one considers mechanisms for the coordinated development of a group of neighboring cells committed to form a particular structure. These cells have to generate an intrinsic signal that tells them to stop dividing in a coordinated fashion after they have reached a certain number. This cooperation and coordination is achieved in many instances by tightly controlled paracrine regulation, which involves signaling between adjacent cells via gap junctions or secreted proteins. We propose here that yet another way of coordinating developmental decisions in groups of cells is by diffusible antiproliferative second messenger molecules, which can spread without a need for surface receptors or specialized systems for secretion and exert their influence within a limited domain. An efficient source of readily diffusible molecules may induce synchronized changes in the adjacent cells within a limited volume of a tissue. Moreover, several adjacent cells producing easily diffusible antiproliferative messenger molecules may share the total pool of these molecules, produced by the neighbors as well as by themselves. If a particular threshold level of a signal is needed to initiate a signaling chain that eventually leads to growth arrest, then the cells in this group could stop dividing when a certain number of cells and, therefore, a certain local concentration of messenger molecules is reached. In this way, by organizing groups of cells in functional clusters and coordinating their decisions on proliferation and differentiation, NO may instruct the developing structures to terminate their growth when they attain the appropriate size and shape and thus may participate in tissue and organ morphogenesis.

Experimental Procedures

Drosophila Stocks

Drosophila melanogaster Oregon R strain was used for most of the experiments described. Transgenic GMR-P35 flies (3.5 and 2.1 alleles; Hay et al., 1994) were a gift from B. Hay and G. M. Rubin. Transgenic flies carrying mouse macrophage NOS (NOS2) gene under heat-shock promoter (hs-mNOS20(2) and hs-mNOS15(2) alleles) were generated by P-element–mediated germline transformation. A 4100 bp NotI fragment from the plasmid CL-BS-mac-NOS containing the entire mouse macrophage NOS gene (Lowenstein et al., 1992; a gift of C. Lowenstein and S. H. Snyder) was cloned into the NotI site in the P element vector pP{CaSpeR-hs} (Thummel and Pirrotta, 1992; gift of G. Halder), placing it under the control of the Drosophila hsp70 promoter. The construct was coinjected into embryos (Spradling, 1986) with the helper P element phs-π-Δ2-3 (Misra and Rio, 1990). A set of two independent homozygous transformed lines was established. Expression of NOS2 transgene after heat-shock treatment of larvae and adult flies was confirmed by diaphorase staining and by protein and RNA analysis. In control experiments, identical regimens of heat-shock treatment of nontransformed flies did not induce any anatomical changes per se.

Histochemistry and Electron Microscopy

NADPH–diaphorase staining was performed as described by Dawson et al. (1991) and Hope et al. (1991), with minor modifications. Fixation-insensitive NADPH–diaphorase staining reflects activity of various NOS isoforms in mammals and Drosophila (Dawson et al., 1991; Hope et al., 1991; Muller, 1994; Regulski and Tully, 1995). Imaginal discs were mounted in 80% glycerol and photographed in a Zeiss Axiophot microscope under Nomarski optics. Cobalt-sulfide staining of the pupal retinae was carried out as described by Wolff and Ready (1991). BrdU labeling to identify cells in S phase was performed essentially as described by Schubiger and Palka (1987) and by Baker and Rubin (1992), with minor modifications. Imaginal discs were removed, rinsed, and incubated in Schneider's media in 50 μg/ml solution of BrdU for 30–40 min at room temperature. They were fixed in 4% formaldehyde, treated with 1:1 mixture of heptane and formaldehyde, rinsed, depurinated by 1 M HCl, blocked by 1% sheep serum, and incubated with anti-BrdU antibodies (Beckton-Dickinson). After extensive washing, discs were incubated with fluorescein-coupled anti-mouse secondary antibodies (Boehringer-Mannheim). After rinsing, individual imaginal discs were dissected away, dehydrated in ethanol, and mounted in Vectashield mounting media (Vector Laboratories). Scanning electron microscopy was performed at the SUNY-Stony Brook Microscopy Center, essentially as described by Kimmel et al. (1990). The number of ommatidia was determined both by analyzing series of scanning electron micrographs and by analyzing adult heads under the blue fluorescent light in a Zeiss Axiophot microscope.

Microinjection of Larvae

For inhibition of NOS, third instar larvae were injected with L-nitroarginine methyl ester (L-NAME), its inactive enantiomer D-nitroarginine methyl ester (D-NAME; both from Sigma), and 2-ethyl-2-thiopseudourea (ETU; Calbiochem). Chemicals were dissolved in Schneider's solution at concentrations of 0.1 M for L-NAME and D-NAME and 0.01 M for ETU and mixed with Freund's adjuvant (Sigma) in 1:3 ratio. Amounts of 5–10 nl were microinjected in staged late third instar using a glass needle. Timing of the injection of NOS inhibitors that gave the highest efficiency (as determined by the changes in the phenotype of the adults) was determined in trial experiments and was found to be most efficient when performed 5–12 hr before pupariation. This treatment did not affect the onset of pupariation and hatching.

Ectopic Expression of NOS

For regulated ectopic expression of NOS, larvae carrying the mouse NOS2 cDNA under the control of Drosophila heat-shock promoter were treated with heat shock at 36°C for 40 min within the first hour after puparium formation. For BrdU-labeling experiments, third instar larvae were treated with heat shock 5–8 hr before puparium formation.

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