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Nitric Oxide, Cell Multiplication, and Cell Survival

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Arrest of cell division is crucial for cells to enter a program of terminal differentiation. In the developing organ or a differentiating tissue, growth arrest defines roughly the size of the cellular population that is further committed to become a domain of differentiated cells. Eventually, the balance between the number of cell divisions and the extent of subsequent programmed cell death determines the final size of a domain, a tissue, or an organ (for review, see Bryant and Simpson 1984; Raff 1992, 1996). Mitogenesis, cytostasis, and survival of neuronal cells can be induced and maintained by the same or by different growth or trophic factors. The signaling pathways that coordinate proliferation, growth arrest, and survival of cells and groups of cells in developing organisms are not known, but they probably involve as yet undetermined inter- and intra-cellular second messenger molecules.

Nitric oxide (NO) is a diffusible multifunctional transcellular messenger that has been implicated in numerous physiological functions in mammals, ranging from contraction of muscles and dilation of blood vessels to immune response and potentiation of synaptic transmission (for review, see Bredt and Snyder 1994a; Nathan and Xie 1994; Garthwaite and Boulton 1995). NO is a highly reactive molecule that can spread from its original source and act on adjacent cells within a restricted volume, displaying properties similar to both paracrine and autocrine effectors. NO is produced from arginine by nitric oxide synthase (NOS). Three chromosomal NOS genes have been cloned from mammalian cells (for review, see Knowles and Moncada 1994; Wang and Marsden 1995). These loci give rise to numerous NOS isoforms, which differ in their tissue distribution, dependence on calcium, and mechanism of activation.

Besides its short-term physiological effects, NO can directly affect cell division and cell survival. Depending on the biochemical milieu, NO can act in some systems as a hazardous agent, inducing mutagenesis, cytotoxicity, and apoptosis. However, in other systems, NO can act as a delicate antiproliferative instrument, inducing temporary cytostasis, which is reversed if the concentration of NO decreases. These antiproliferative properties of NO may contribute to the cellular decision-making processes that control cessation of division, entry to a differentiation program, suppression or induction of programmed cell death, etc.

Here, we consider our recent studies to understand the role of NO in development. We present evidence that NO has a dual role during neuronal cell differentiation—at first it acts as an antiproliferative agent, inducing growth arrest in dividing cells, and later, when the growth arrest is firmly established and the cells have undergone differentiation, it helps to suppress programmed cell death, thus mediating the survival effects of growth factors. Moreover, we have found that the antiproliferative features of NO enable it to control cell number during the development of an intact animal. Our results suggest that NO can play a broader role as a general regulator of cell proliferation and cell survival during organism development and morphogenesis.

NO INITIATES A SWITCH FROM PROLIFERATION TO CYTOSTASIS DURING DIFFERENTIATION OF NEURONAL CELLS

When naive PC12 cells are exposed to nerve growth factor (NGF), they show few signs of neuronal differentiation within the first 3–4 days. However, after 5–6 days, the cells stop dividing, enter a cytostatic state, and develop a differentiated phenotype: They change the pattern of expressed genes, start to express a variety of differentiation markers, and, as a most visible trait, they send out processes, building a dense network of extended and branched neurites (Greene and Tishler 1976; Greene et al. 1987). Thus, the response of PC12 cells to NGF involves a proliferative phase that is followed by growth arrest and differentiation. The switch from proliferation to cytostasis is critical for the cells to establish a differentiated phenotype after exposure to NGF. If cells cannot establish growth arrest, as happens in mutant PC12-U2 cells, they cannot acquire a differentiated phenotype and instead continue to proliferate (Burstein and Greene 1982). We have found that production of NO is crucial for the NGF-induced neuronal differentiation of PC12 cells (Peunova and Enikolopov 1995). Our experiments suggest that the cytostatic effect of NGF is mediated by NO and that induction of NOS is an important step in the commitment of neuronal precursors during differentiation.

NGF Treatment Induces NOS Activity

When untreated PC12 cells were tested for NOS by the NADPH-diaphorase cytochemical reaction, which reflects total NOS activity, no staining was observed.

After the cells were treated with NGF, however, they gradually started to acquire an intense NADPH-dependent blue color after diaphorase staining, indicating that NOS accumulates in PC12 cells in response to NGF treatment. This increase in diaphorase staining was specific for NGF and did not appear after addition of fetal calf serum or epidermal growth factor (EGF). However, staining was increased after addition of the fibroblast growth factor (bFGF), which, like NGF, induces neuronal differentiation in PC12 cells. Importantly, the NGF-treated cells that were first to undergo initial morphological changes characteristic of the differentiated phenotype were also the first to show bright staining. This suggests that elevation of NOS activity precedes development of the differentiated phenotype. Induction of NOS was confirmed using in vitro assays of NOS activity, immunocytochemistry, reverse-transcriptase polymerase chain reaction (RT-PCR), and RNase protection assays of NOS transcripts. The results of these assays indicate that during the early stages of NGF action, a substantial part of the total NOS activity is due to the induction of the Ca^{++}-independent *NOS2* gene and that the neuronal NOS1 form is activated much later, following the establishment of the growth arrest (see also Hirsch et al. 1993).

NO Can Act as an Antimitogenic Agent in PC12 Cells

NO can inhibit DNA synthesis in several cell types (Garg and Hassid 1989; Lepoivre et al. 1990; Kwon et al. 1991; Hogan et al. 1992). It acts as an antimitogenic agent in PC12 cells as well. Several NO donors, sodium nitroprusside (SNP), *S*-nitroso-*N*-acetylpenicillamine (SNAP), and *S*-nitroso-*N*-penicillamine (SNOP), suppressed DNA synthesis in a concentration-dependent manner (albeit with different potencies), indicating that NO is an active inhibitor of DNA replication in PC12 cells. This inhibition, which resulted in a decrease in cell division, was not due to cytotoxicity, since the cells resumed proliferation once the source of NO was removed. NO-mediated inhibition of DNA synthesis overcomes the increase of DNA synthesis induced by serum, NGF, EGF, bFGF, insulin-like growth factor, and various growth factors in combination with NOS inhibitors (see below).

The results of the fluorescence-activated cell sorting (FACS) analysis showed that NO induces specific changes in the cell-cycle phase distribution of PC12 cells. After treatment with NO donors, cells tended to accumulate specifically in G_2 phase, and the proportion of cells in S phase decreased. Remarkably, within a specific range of concentrations of NO donors, the proportion of PC12 cells in G_2 and S phases was similar to levels reached after prolonged treatment with NGF (Buchkovich and Ziff 1994; Peunova and Enikolopov 1995). These results with externally added NO donors suggest that NO molecules produced intracellularly during the course of NGF action could play a similar role in promoting NGF-induced cytostasis.

Inhibition of NOS Activity Prevents Cytostatic Action of NGF

After several days of NGF treatment, the proliferation of PC12 cells ceases and differentiation occurs. If NO produced by the NGF-induced NOS in PC12 cells can indeed act as a crucial antiproliferative factor, then inhibition of the enzyme should uncouple the proliferative and cytostatic components of NGF action and prolong the proliferative phase while suppressing the cytostatic effect of NGF. NOS inhibitors *N*-nitro-L-arginine methyl ester (L-NAME), *N*-monomethyl-L-arginine (L-NMMA), and 2-ethyl-2-thiopseudourea (ETU) indeed reversed the cytostatic action of NGF when applied together with the growth factor, and forced the cells to continue to proliferate instead of ceasing to divide after several days of NGF treatment. In the absence of NGF, similar concentrations of inhibitors did not have a detectable effect on PC12 cell growth; in particular, they did not accelerate cell proliferation. Addition of NOS inhibitors also did not hinder one of the most important early features of NGF action on neuronal cells—rapid induction of a set of immediate early genes (IEGs). Induction of IEGs is believed to trigger further steps in a cascade of gene activity, ultimately leading to the development of the differentiated phenotype. We tested a panel of IEGs (c-*fos*, c-*jun*, *fra-1*, *egr-1*, *nur77*, and others) by RNase protection and found that addition of the NOS inhibitor L-NAME did not affect either their basal level of expression or their induction by NGF. This proves that the NOS inhibitors do not merely prevent the initial steps of NGF action (such as binding to the receptors, activation of the receptors, induction of the MAP kinase signaling pathway) and, in addition, suggests that the NOS-dependent stage(s) occurs later in the development of the differentiated phenotype.

The most visible consequence of NGF action on PC12 cells is neurite outgrowth, and this was also affected by NOS inhibition. Under normal conditions, almost every cell gradually extends neurites in response to NGF, but when PC12 cells were treated with a combination of NGF and NOS inhibitor, the number of cells with neurites decreased dramatically, in accordance with the observation that most of the cells continued to divide and did not enter the differentiated state. In summary, our data show that inhibition of NOS reverses the NGF-dependent growth arrest, indicating that NO mediates the antiproliferative action of NGF in neuronal cells.

NOS Action Is Dependent on Cell Proliferation

To determine whether in the course of response to NGF there is a critical phase during which the cells are particularly susceptible to NOS action, PC12 cells were treated with NOS inhibitor L-NAME before or after the addition of NGF. L-NAME was able to reverse the cytostatic action of NGF only when applied before NGF addition or within 2–3 days of NGF treatment;

after that the inhibitor had no effect. Note that this time is shorter than the time needed for full manifestation of cytostasis and development of the neuronal phenotype. This suggests that the critical time window for NO action is within the first days of NGF addition, after which NOS may relegate its antiproliferative functions to the other, perhaps more general, systems of growth arrest.

To test if the crucial phase of NOS action in NGF-treated cells is cell-cycle specific, we uncoupled the mitogenic and differentiating phases of NGF action by suppressing proliferation of PC12 cells. Cells were brought to G_0 by growth on a medium with low (0.5%) serum and then transferred either to low-serum medium again or to high (15%)-serum medium and treated with combinations of NGF and L-NAME. When the cells were transferred to the low-serum medium (which does not permit cells to cycle), the NOS inhibitor could not prevent neurite outgrowth even at very high concentrations (10 mM). In contrast, when starved cells were transferred to the high-serum medium, where they could resume proliferation, the same concentrations of L-NAME were quite effective at suppressing the neurite outgrowth. This implies that if growth arrest is established by means other than NO production, then NO-mediated induction of cytostasis is not evoked and NOS inhibitors do not affect the action of NGF. Taken together, these results argue that the critical period of NOS action is dependent on the cell cycle progression and that it probably occurs in a different phase of the cycle from the decision to enter the differentiation program.

Mutant Cells' Capacity to Differentiate Can Be Rescued by NO

Mutant PC12-U2 cells retain the early steps of the response to NGF but have lost the capacity to execute the later steps (Burstein and Greene 1982). They do not stop dividing after NGF treatment and, as a consequence, they do not develop the fully differentiated phenotype; in particular, they do not send out processes. To investigate whether this defect is connected to NO production, we tested whether the phenotype could be overcome by the addition of NO. Although neither NGF nor NO alone had any effect on the phenotype of U2 cells, in combination these treatments restored the differentiated neuronal phenotype. The mutant cells stopped dividing and grew extensive branched neurites, similar to wild-type PC12 cells. Thus, it was possible to rescue the defect in mutant cells and to restore the complete differentiation program by complementing NGF action with NO.

To confirm further the involvement of NOS in the differentiation of mutant U2 cells, the induction of NOS by NGF in these cells was examined by immunofluorescence and by RT-PCR analysis. Inducible NOS (NOS2) was expressed at much lower (almost tenfold) levels in U2 cells than in the wild-type PC12 cells after 4 days of NGF treatment. However, the levels of neuronal NOS (NOS1) in U2 cells were indistinguishable from those in PC12 cells. Together, these data demonstrate that the NGF-treated U2 cells are defective in NGF-dependent NOS2 induction and that this defect can be rescued by the external application of NO. Importantly, the *NOS2* gene is expressed in U2 cells, although at low levels, and it can be induced by means other than NGF. This suggests that the defect in U2 cells resides in the signaling regulatory pathway rather than in the gene for NOS2 itself.

In summary, we present evidence for a causative role for NO action in NGF-induced growth arrest in PC12 cells. Our experiments argue that NO, produced by the NGF-induced NOS, serves as a switch between the proliferative and cytostatic phases of NGF action in neuronal cells and that NOS activation is necessary for the further development of the differentiated phenotype. Our results suggest a role for *NOS* as a growth arrest gene in the commitment cascade during NGF-mediated differentiation of neuronal cells.

NO MEDIATES SURVIVAL OF FULLY DIFFERENTIATED NEURONAL CELLS

Differentiated neuronal cells become dependent on growth factors for survival. NGF is a survival factor for sympathetic neurons and for PC12 cells, and when NGF-differentiated PC12 cells are deprived of growth factors, they undergo apoptosis. Our studies indicate that during the early stages of NGF action, NO initiates growth arrest. Here we present evidence that during the later stages of NGF action, in fully differentiated PC12 cells, NO has an additional role as a mediator of the survival functions of NGF.

Fully Differentiated PC12 Cells Become Dependent on NO

When growth factors are removed from NGF-differentiated PC12 cells, these cells undergo programmed cell death by apoptosis. This process can be blocked by reapplying NGF. Since NGF-induced NO production remains very high during the late stages of NGF action, after growth arrest has been already established, we examined whether part of the NGF action as a survival agent could be supported by NO. We have found that NO, like growth factors, can inhibit apoptosis of differentiated PC12 cells after NGF withdrawal. When NO-releasing compounds were added to fully differentiated cells before or immediately after withdrawal of NGF, cell death was markedly reduced, demonstrating that NO can partially substitute for NGF in preventing apoptosis. This suggests that endogenously produced NO, like that produced by NO donors, could contribute to cell survival. It may mediate some of the survival functions of NGF and may be necessary for the suppression of cell death. Indeed, inhibition of NOS in fully differentiated cells led

to massive death by apoptosis, as documented by several criteria (changes in morphology, acridine orange and DAPI staining, TUNEL assay, and FACS analysis). Importantly, no apoptosis was observed when a similar treatment was applied to naive PC12 cells or to cells that had been treated with NGF for 2–4 days and hence had not yet established growth arrest and had not differentiated (also see above). Similarly, inhibition of NOS did not impair the survival of mutant U2 cells, which retain the early steps of the response to NGF but have lost the capacity to differentiate. Together, the results with differentiated, naive, and mutant cells indicate that fully differentiated PC12 cells, in parallel with increased dependence on NGF, develop a dependence on NO production for their survival, such that inhibition of NOS activity leads to cell death, and addition of NO can suppress cell death.

NO Activates Anti-apoptotic Machinery

A possible explanation of the role of NO in preventing apoptosis may involve its antiproliferative properties. NO may prevent cells from traversing the cell cycle, thereby keeping them from reaching a critical point where mitogenic signals contradict the established cytostatic condition of the cells, prompting them to undergo apoptosis (Evan et al. 1992; Ferrari and Greene 1994). In addition, inhibition of apoptosis by NO may involve direct activation of an anti-apoptotic program by NO; for example, by changing the levels of expression of proteins that prevent or promote apoptosis. We tested whether treatment of PC12 cells with NO changes the expression of the Bcl-2 family of proteins (Bcl-2, Bcl-x_L, Bcl-x_S, Bax, Bad, Bak, Bag-1) involved in regulating programmed cell death (for review, see Steller 1995; White 1996; see also Boise et al. 1993; Oltvai et al. 1993; Yang et al. 1995) and how these changes relate to those induced by NGF. Prolonged treatment of PC12 cells with NGF leads to an increase in the level of Bcl-x_L, a product that, like Bcl-2, can prevent apoptosis in many cell types. At the same time, the amount of Bax, a protein that counters the ability of Bcl-2 and Bcl-x_L to repress apoptosis, decreased with a similar time course. In contrast, the levels of these proteins are unchanged in the mutant U2 cells after NGF treatment, indicating that changes in the expression of apoptosis-related genes are connected to withdrawal from the cell cycle and differentiation, and not just to prolonged NGF treatment. Thus, NGF-induced differentiation of PC12 cells changes the expression of genes involved in the cell death program in favor of products that prevent apoptosis. This may lead to a switch of the cell program toward prevention of cell death, in line with the known role of NGF as a survival factor.

To determine whether NO can mediate part of the effect of NGF on the apoptosis machinery, we subjected PC12 cells to sustained treatment with NO donors. The changes in expression of a panel of apoptosis-related proteins were very similar to the changes evoked by NGF treatment. When the same donors were added to U2 cells (NGF-treated and untreated), the changes observed were identical to those seen in wild-type PC12 cells; most importantly, the levels of Bcl-x_L were increased, whereas the levels of Bax were decreased. Thus, like NGF, NO is able to induce an anti-apoptotic program directly, and this ability of NO may contribute to the survival functions of NGF.

Our results indicate that action of NO is critical in both the early and late phases of NGF-driven neuronal differentiation, i.e., before and after induction of growth arrest. To examine the contribution of different NOS isoforms in these two phases of differentiation, we followed the expression of various NOS genes by Western blot analysis and by RT-PCR. We found that differentiation of PC12 cells is accompanied by a switch from expression of the inducible form (NOS2) soon after addition of NGF (before growth arrest is established), to the neuronal form of NOS (NOS1), which reaches the maximal levels after the growth arrest is established and, perhaps, is more directly associated with the survival functions.

Dual Role of NO in Neuronal Cells

Our results argue that there is a dual role for NO during NGF-driven differentiation of neuronal cells (Fig.1). NO acts as an initiator of growth arrest during the early stages of NGF action and as a mediator of the survival effects of growth factor during the later stages. In the proliferative phase of NGF action, the NOS gene(s) is induced as a delayed response to the growth factor stimulation. This produces enough NO to inhibit DNA synthesis and, probably, to activate further checkpoints, thereby blocking further progression of the cell cycle, completing the proliferative phase of NGF action, and switching the cells to the cytostatic phase. Thus, NO acts here as a permissive factor for the remaining program of differentiation traits (such as neurite outgrowth), which can only occur after cell division has ceased; in addition, NO probably induces some of the later differentiation markers directly by promoting gene activity (Peunova and Enikolopov 1993).

Later, once growth arrest is established and the cells become fully differentiated and dependent on growth factors, NO also mediates the cells' survival both by enforcing the growth arrest of differentiated cells and by activating an anti-apoptotic program. Perhaps the switch from expression of the calcium-independent form of NOS to the calcium-dependent form underlies the dual role for NOS as a trigger of cytostasis in undifferentiated cells and as an inhibitor of apoptosis in differentiated cells. Given the unconventional properties of NO, it is possible that NO diffuses from the cell that produces it to promote cessation of growth in adjacent cells and that this phenomenon contributes

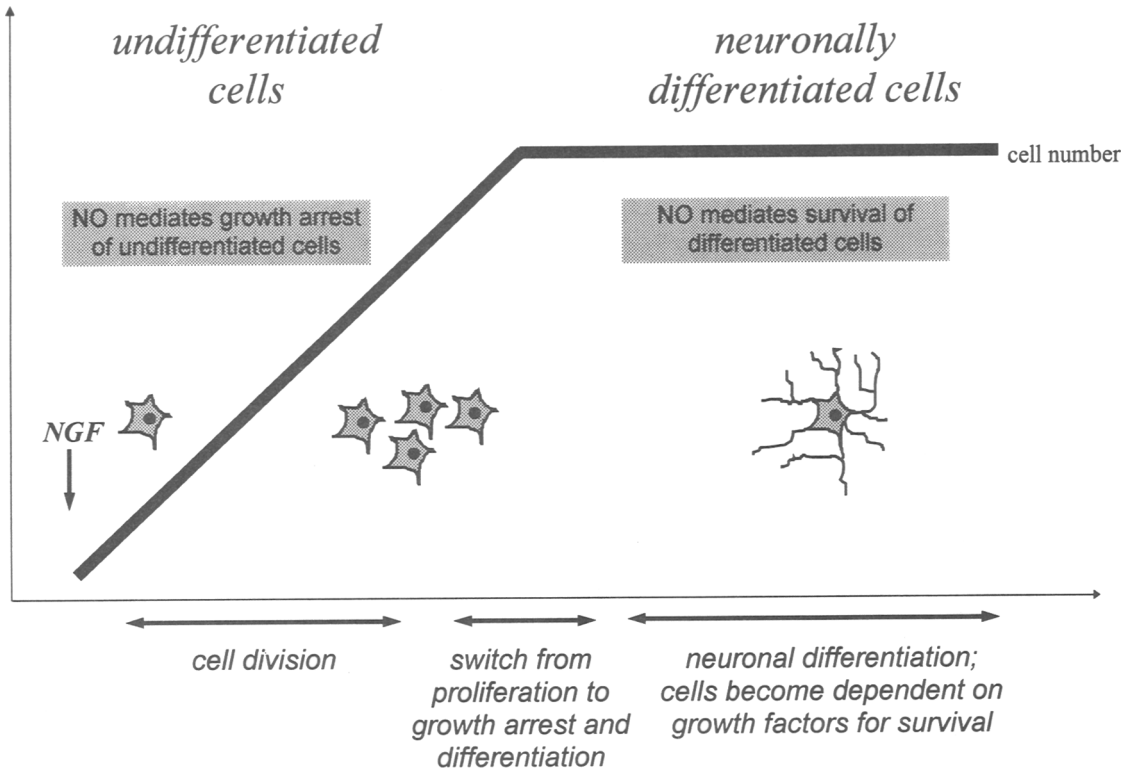

Figure 1. Schematic summary of NGF action and NOS functions. The model relates NGF-induced NOS activity to proliferation, differentiation, and survival of PC12 cells. In the early stages of the NGF action, in dividing undifferentiated cells, NO mediates transition to the cytostatic phase and neuronal differentiation. At the later stages, in fully differentiated cells, NO mediates part of the survival functions of NGF and prevents cells from programmed cell death both by enforcing the growth arrest and by activating an anti-apoptotic program.

to the synchronization of development and/or survival of a domain of neuronal precursors.

In the brain, NO production is associated with the death of neurons by necrosis and apoptosis after an ischemic stroke and, perhaps, neurodegenerative diseases (Choi 1993; Bredt and Snyder 1994a; Gross and Wolin 1995). Paradoxically, NOS-positive neurons in the brain are remarkably resistant to damage, and most of them are spared after exposure to strong insults and diseases that kill other cells (Ferrante et al. 1985, 1987; Koh et al. 1986; Koh and Choi 1988a,b; Uemura et al. 1990; Hyman et al. 1992; Mufson and Brandabur 1994). Our results suggest that preexposure to sustained NO production may be responsible for the increased resistance of NOS-positive neurons to damage. Although apoptosis can be initiated by numerous different stimuli, both the control mechanisms and the execution mechanisms of programmed cell death are remarkably uniform for different settings and appear to involve a relatively limited set of genes. The ability of NO to induce an anti-apoptotic program and prevent programmed cell death after withdrawal of a growth factor may reflect its physiological function in the brain during disease. We propose that the unusual resistance of NOS-positive neurons in the brain is related both to the role of NO in maintaining growth arrest and to its ability to fortify the cellular anti-apoptotic defense, which may later protect the cells from insults.

NO REGULATES CELL PROLIFERATION DURING *DROSOPHILA* DEVELOPMENT

Our experiments with cultured cells suggested that the antiproliferative activity of NO is crucial for the transition from proliferation to differentiation during neuronal differentiation. To investigate whether NO has a similar role during normal development of organisms, we have used *Drosophila* as a model organism. 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). Disc cells divide rapidly throughout larval development and cease proliferating at the end of the third instar period (Fig. 2). In leg, wing, and haltere discs, progression through the cell cycle stops in G_2 phase 3–4 hours before puparium formation. It resumes 15–18 hours later and then stops again in a defined spatial pattern after 12–14 hours (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

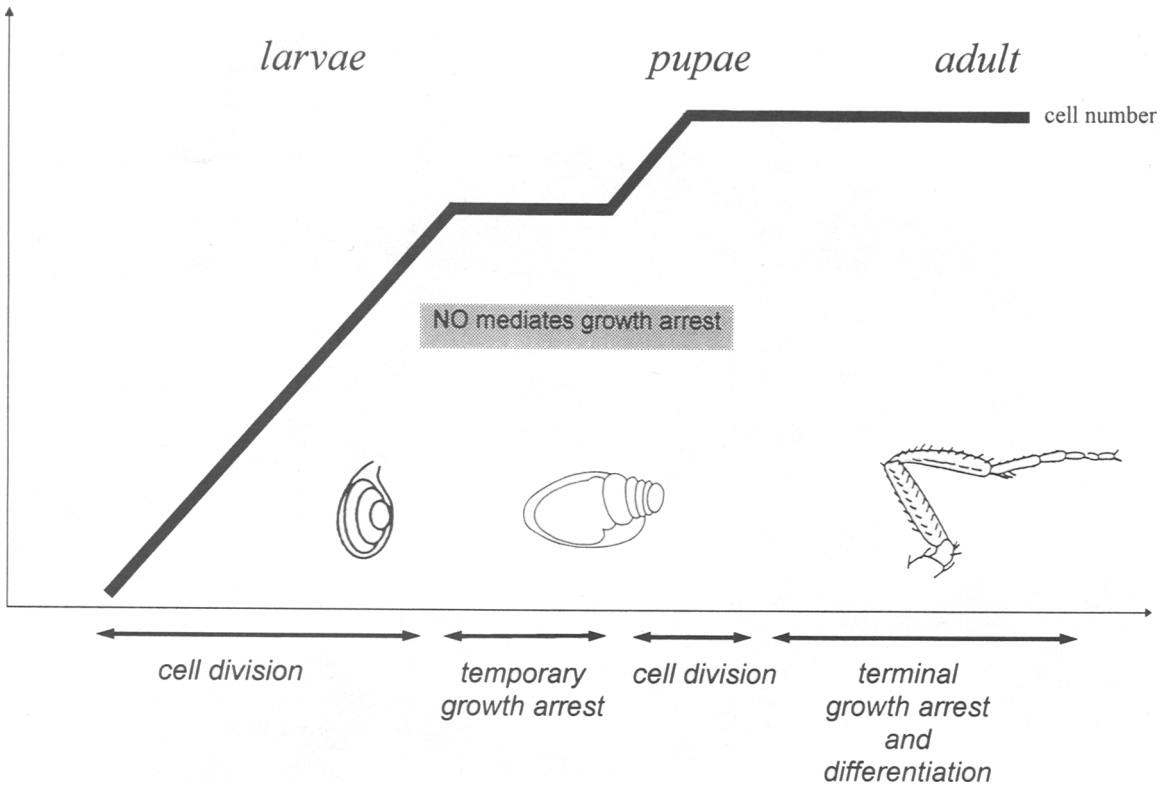

Figure 2. Schematic summary of cell proliferation in imaginal discs and organ development in *Drosophila*. Antiproliferative properties of NO mediate part of the temporary and, perhaps, terminal growth arrest during development of imaginal discs, and thus control the balance between cell proliferation and cell differentiation.

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. We tested whether NO is involved in the signaling pathways that control coordinated temporary growth arrest in larvae and pupae and subsequent terminal growth arrest in pupae and adults. We have found that NO acts as an antiproliferative agent during *Drosophila* development and controls the switch from cell proliferation to cell differentiation. Our data suggest that NO controls cell number and thus the size of anatomical structures in an intact animal. We propose that induction of NOS is a crucial step in *Drosophila* development and that NO may be a general regulator of cell proliferation and differentiation during organism development and morphogenesis.

NOS Is Expressed in Imaginal Discs during Larval Development

When imaginal discs of the third instar and early pupae were examined for the NADPH-diaphorase activity of NOS, 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, indicating accumulation of the NOS activity. In late third-instar larvae and early pupae, a highly specific and reproducible pattern of very intense staining was evident. Later in development, when the discs began to evert in the prepupae, diaphorase staining of the forming structures became less intense and a distinct characteristic staining pattern of individual segments became evident. Diaphorase staining further decreased in a specific spatial pattern during early pupal development. Thus, there is a gradual and specific accumulation of NOS in those developing imaginal structures, which undergo radical changes during metamorphosis before giving rise to adult organs. This staining reaches its highest levels at the time when progression through the cell cycle slows down.

Inhibition of NOS Results in Hypertrophy of Leg Segments

The strong antiproliferative properties of NO and the specific pattern of diaphorase staining seen in mature imaginal discs imply that NO may act as a growth arrest agent in these structures and be 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 various NOS inhibitors in the developing larvae at the end of the third instar, several hours before metamorphosis. The resulting adults exhibited dramatic enlargements of many structures: legs, wings, genital structures, tergites, and sternites, etc. The changes most often affected and were most profound in the legs of the adults, where the diameter of certain segments increased 3- to 4-fold. The number of bristles and the number of rows of bristles were 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. Identical changes were observed when structurally unrelated inhibitors of NOS were used, indicating that the observed effect resulted specifically from blocking NOS activity. To test whether changing the NO levels directly affects DNA synthesis in the imaginal discs, we labeled the nuclei with 5-bromo-deoxyuridine (BrdU) after inhibition of NOS activity. There were significantly more BrdU-labeled cells in the imaginal discs of flies in which NOS activity was blocked than in those of control flies. Thus, 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 and to a reduction in the size of organs of the adult fly. To increase NO production, we induced expression of *NOS* transgene in transformed larvae carrying the mouse *NOS2* cDNA gene under the control of the heat-shock promoter. NOS2 is a calcium-independent form of NOS that is capable of efficient constitutive NO production. Transgenic larvae were heat shocked within 1 hour after pupariation to induce ectopic expression of NOS before the final cell divisions take place. This resulted, among other changes, in a reduction in the size of the limbs of the fly. 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- to 2-fold and several segments were fused together with poorly defined boundaries. The most terminal structures of the appendage remained intact in these defective legs, suggesting that the observed reduction in size was due to incomplete growth of the developing appendage, rather than to complete loss of its distal structures. 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. In contrast to experiments with inhibition of NOS, when imaginal discs were labeled with BrdU after induction of ectopic NOS expression, there were markedly fewer labeled cells in imaginal discs from induced NOS-transformed flies than in uninduced controls. Thus, 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

For some *Drosophila* structures, for instance, the eye, we consistently detected an increase in the number of cells in S-phase in the imaginal disc after inhibition of NOS, but the resulting adult organ 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 in which apoptosis in the developing eye is largely prevented by expression of recombinant baculovirus p35 protein (Hay et al. 1994).

When NOS was inhibited in GMR-P35 larvae, the eyes of the adult flies showed numerous changes, reflecting the surplus 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 and untreated GMR-P35 flies to nearly 820 after NOS inhibition in GMR-P35 flies. In addition, different types of accessory cells in the ommatidia proliferated after NOS inhibition to levels higher than those achieved by blocking apoptosis by p35 (Hay et al. 1994). For instance, the number of secondary and tertiary pigment cells was increased from 12 per sample area in normal eyes to more than 35 in GMR-P35 flies after inhibition of NOS. This number exceeds the maximal number of pigment cells saved from programmed cell death in untreated GMR-P35 flies (~25 per sample area) 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. Furthermore, the number of bristles was increased in some areas of the eye in GMR-P35 flies after NOS inhibition, up to four to five per ommatidium, instead of the three seen in normal flies and untreated GMR-P35 flies. Similarly, the number of cone cells was increased from four in normal and in untreated GMR-P35 om-

matidia to five and six in many ommatidia of GMR-P35 flies after NOS inhibition. Thus, prevention of apoptosis in the developing *Drosophila* eyes revealed excessive proliferation of various cell types after NOS inhibition, which was otherwise masked by programmed cell death in the larvae and pupae.

Together, our experiments with *Drosophila* 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. In addition, 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 apoptosis, and it can be revealed by suppressing programmed cell death. Taken together, these results indicate that NO acts as an antiproliferative agent to control cell proliferation during *Drosophila* development (Fig. 2).

NO AND DEVELOPMENT

This study provides support for 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. It suggests a novel role for NO as a regulator of the balance between cell proliferation and cell differentiation during development. How general is the phenomenon of 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. For instance, in the brain, a strong elevation of NOS activity in the developing cerebral cortical plate and hippocampus at days 15–19 of embryonic development correlates with the time course of cessation of precursor cell proliferation, tight growth arrest, and cell differentiation; notably, NOS activity goes down after the proliferation of committed neuronal precursors is completed (Bredt and Snyder 1994b; Blottner et al. 1995). NOS levels are also transiently increased in developing lungs, bones, blood vessels, nervous system, and other tissues and organs (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 unregulated growth (Roscams 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 transition from proliferation to growth arrest and differentiation, thus contributing to the proper morphogenesis of the tissue and the organ.

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, their overlapping tissue distribution, and the possibility that NO can be transferred within the organism by hemoglobin (Jia et al. 1996), it is conceivable that any group of cells in the embryo and fetus can be exposed to NO action.

NO is easily diffusible, and it may therefore exert its antiproliferative properties not only in the cell that produces it, but in the adjacent cells as well. Thus, it is possible that NO can contribute to the coordinated development of a group of neighboring cells committed to form a particular structure and thus may participate in tissue and organ morphogenesis. An efficient source of readily diffusible transcellular messengers, such as NO, may induce synchronized changes in the adjacent cells within a limited volume of a tissue. Moreover, several adjacent cells producing easily diffusible antiproliferative 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. This way, by organizing groups of cells in functional clusters, NO may instruct the developing structures to terminate their growth when they attain the appropriate size and shape, at the same time coordinating their growth with the growth of the adjacent tissues.

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