Development 124, 3587-3595 (1997)
Printed in Great Britain © The Company of Biologists Limited 1997
DEV7564

Nitric oxide, an endogenous regulator of *Dictyostelium discoideum* differentiation

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SUMMARY

We have previously demonstrated that nitric oxide (NO)-generating compounds inhibit *D. discoideum* differentiation by preventing the initiation of cAMP pulses (Tao, Y., Howlett, A. and Klein, C. (1996) *Cell. Signal.* 8, 37-43). In the present study, we demonstrate that cells produce NO at a relatively constant rate during the initial phase of their developmental cycle. The addition of oxyhemoglobin, an NO scavenger, stimulates cell aggregation, suggesting that NO has a negative effect on the development of aggregation competence. Starvation of cells in the presence of glucose, which has been shown to prevent the initiation of cAMP pulses (Darmon, M. and Klein, C. (1978) *Dev. Biol.* 63, 377-389), results in an increased production of NO. The inhibition of cell aggregation by glucose treatment can be reversed by oxyhemoglobin. These findings indicate that NO is a signaling molecule for *D. discoideum* cells and that physiological or environmental conditions that enhance external NO levels will delay the initiation of cAMP pulses, which are essential for cell differentiation.

Key words: *D. discoideum*, interphase, nitric oxide, cell aggregation

INTRODUCTION

Nitric oxide (NO) has a variety of effects in different tissues or cells (for review see Lancaster, 1992; Stamler, 1994; Schmitdt and Walter, 1994). At low levels, it is believed to function as a mediator of cell communication in, for example, neuronal transmission and vascular smooth muscle relaxation. At higher concentrations, when it functions as an antitumor or antimicrobial agent, it can be cytostatic or cytotoxic. With increased recognition of the biological importance of NO, numerous laboratories have focused efforts toward determining its site(s) of action. The toxic effects of NO are associated with an inhibition of key enzymes in cell metabolism and/or growth. In general, the enzymes that are affected under these conditions contain iron-sulfur centers e.g. *cis*-aconitase. When NO functions as a signaling molecule, it has been shown to interact with iron-heme groups of proteins, as exemplified by its action on soluble guanylyl cyclase. It is the subsequent increase in cGMP levels that is currently believed to be the key to NO-induced vasodilation. Given that the activation of cytosolic guanylyl cyclase occurs almost universally in response to NO, changes in cGMP have received primary consideration as the mechanism by which NO acts. However, the diversity of the effects of NO cannot be explained solely by this mechanism and recently a number of actions of NO have been proposed that appear to be independent of guanylyl cyclase (Lancaster, 1992; Schmitdt and Walter, 1994). Unfortunately, for the most part, specific cellular components have been implicated as targets of NO action based on the ability of NO or NO-releasing compounds to alter their activities in vitro. Thus, whether these components are altered when intact cells encounter NO remains speculative.

Our approach to discerning how NO functions is to examine the effects of NO on intact cells. Previously, we demonstrated that the addition of NO-generating compounds inhibits the aggregation of *D. discoideum* amoebae (Tao et al., 1992a). This inhibition is not related to the NO-stimulated ADP-ribosylation of GAPDH which we have shown also to occur under these conditions (Tao et al., 1992b, 1994). In addition, unlike the situation seen in most mammalian tissues and cells, NO fails to activate the *D. discoideum* guanylyl cyclase or alter the cellular levels of cGMP (Tao et al., 1992a). The inhibitory action of NO donor compounds on cell aggregation is maximal when cells are exposed to NO during the first hours of nutritional starvation. Nutritional deprivation arrests cell growth and initiates the developmental program. During the initial hours of starvation, the pre-aggregative or interphase period, cells develop the components necessary for chemotactic signaling via extracellular cAMP pulses. Such components include the cell surface cAMP receptor, the adenylyl cyclase, and the G-protein(s) intermediate involved in receptor modulation of that activity. Although it is unclear how cAMP pulsing first begins, the signal relay response readily explains their maintenance. Signal relay reflects the binding of cAMP to the cell surface receptor, the resulting rapid and transient activation of adenylyl cyclase, and the secretion of a cAMP pulse into the medium. The secreted cAMP is rapidly hydrolyzed by both cell surface and extracellular phosphodiesterase (PDE;

reviewed by Van Haastert, 1991). In response to extracellular cAMP pulses, cells will advance in their differentiation to aggregation competence and chemotactically migrate toward central collection points (Darmon et al., 1975; Gerisch et al., 1975).

NO-releasing compounds inhibit the aggregation of *D. discoideum* by preventing the initiation of cAMP pulse production (Tao et al., 1996). The lack of cAMP synthesis does not reflect a failure of cells to respond to starvation with a developmental rise in adenylyl cyclase, cAMP receptors or PDE. Since NO-treated cells can transiently activate their adenylyl cyclase and produce a pulse of cAMP when stimulated with exogenously applied cAMP pulses, they are not defective in the receptor-mediated processes related to cAMP pulse production. Furthermore, NO-treated cells could be induced to aggregate when stimulated with exogenous cAMP pulses, indicating that the processes subsequent to cAMP receptor activation that regulate cell differentiation and aggregation are unaffected. These observations, and the fact that the effect of NO donors is completely reversible, indicate that the inhibition of aggregation is neither a general metabolic or toxic effect. More importantly, the data clearly demonstrate a cAMP receptor-independent mechanism for the regulation of adenylyl cyclase in this organism. The question arises as to the nature of the physiological mediator of that regulation and the possibility that it is NO itself. Here we show that *D. discoideum* produces NO and describe conditions under which that production can be regulated.

MATERIALS AND METHODS

Cells and culture conditions

Ax-2 amoebae were grown in HL-5 medium (Sussman, 1966). When nitrite production in growing cells was examined, amoebae that had reached a density of 2 x 10^6 cells/ml were washed twice with sterile 20 mM phosphate buffer and resuspended at the indicated densities in a suspension of 1.5×10^{10} heat-killed bacteria (Rathi and Clarke, 1992). To initiate development, exponentially growing cells were washed twice with 20 mM phosphate buffer, pH 6.4, and resuspended at 10^7 cells/ml in that buffer. Cells were starved as spinner suspensions (Beug et al., 1970) and their development was monitored microscopically by plating 10^6 cells onto 35×10 mm tissue culture dishes and observing their morphology after a 15 minute period (Juliani et al., 1981). Cells were considered to be aggregation competent when most (>75%) of the population displayed an elongated morphology and formed end-to-end contacts. When cells were elongated, formed polar contacts, and streamed toward central collection points, they were scored as fully aggregation competent. Loose aggregates are those that form in spinner suspension but from which some cells will dissociate when the mass is plated on a solid support. This contrasts with the more mature tight aggregates that form later, which are covered with a translucent sheath from which cells will not disperse when plated. Where indicated, cells were stimulated with 10^{-7} M cAMP pulses as described elsewhere (Darmon et al., 1975; Tao et al., 1996).

Treatment of cells with NO gas was accomplished by first making a saturated solution of NO in distilled water. The water had been sparged with helium for 60 minute before bubbling with NO gas for an additional 30 minutes. This results in an approximate 2 mM solution of NO. That solution was diluted directly with the buffer in which cells were starving to achieve the indicated final concentrations of NO. Control cells received distilled water that had been sparged with helium only. In these experiments, cell viability was monitored by plating serial dilutions of cells on bacterial lawns and comparing the number of colonies to those obtained from untreated cells. To examine the effects of oxyhemoglobin (oxyHb), 0.1 mM bovine methemoglobin (metHb) was treated with 1 mM sodium dithionite and then extensively dialyzed against distilled water. Solutions of oxyHb were freshly prepared for each experiment.

Measurement of nitrite production

At the indicated times, the medium was cleared of cells by centrifugation at 12,500 ***g*** for 5 minutes and then filtering through a NanoSpin filter with 10,000 M_r cutoff. Samples were maintained at -80 °C until assayed. Control samples consist of medium incubated under the same conditions but devoid of cells. Levels of accumulated nitrite in the medium were measured in duplicate using a spectrofluorometric assay described by Misko et al. (1993). Briefly, 2,3-diaminonaphthalene was reacted with nitrite under acidic conditions to form 1-H-naphthotriazole, a fluorescent product which was measured using a fluorescent plate reader (IDEXX laboratories, Westbrook, ME) with excitation at 365 nm and emission read at 450 nm. Duplicates agreed within 5%. In some experiments, nitrate in the medium was converted to nitrite by the action of nitrate reductase from *Aspergillus niger* (Misko et al., 1993).

Other assays

Cellular levels of gp80 were determined by western blot analysis (Tao et al., 1992a). Relative levels of gp80 were determined by scanning linear range autoradiographs on a Bio-Rad 620 densitometer and analyzing the data using the 1-D Analyst program.

Materials

L-N^5-iminoethyl ornithine hydrochloride (L-NIO) and diphenyleneiodonium chloride (DPI) were obtained from Cayman Chemical Company; NanoSpin centrifugal filters were from Gelman Sciences, and NO gas was purchased from Matheson. All other reagents were from Sigma.

RESULTS

D. discoideum produce NO

Using measurements of nitrite levels as an indicator of NO production, we observed that cells produce NO during the first phase of their developmental cycle (Fig. 1A). Growing cells were washed and resuspended in phosphate buffer. At the indicated times of starvation in spinner culture, aliquots were taken for measurement of nitrite levels in the medium. Nitrite levels reached readily detectable levels by 2.5 hours of starvation and continued to rise throughout the period of this experiment. By 7-8 hours of starvation, nitrite approached micromolar levels. In an oxygenated environment, NO can be oxidized to both nitrite and nitrate (Conner and Grisham, 1995). Therefore we treated media samples with nitrate reductase prior to measuring nitrite levels. We found that nitrate was present at levels roughly equal to that of nitrite and showed the same temporal increase (data not shown). When cells were starved in buffer supplemented with 10 mM arginine (arg), an approximate 10% increase in nitrite production was observed. This increase, although small, appeared specific for the presence of arg in that the addition of lysine did not alter nitrite production (data not shown).

To verify that the nitrite being measured in these experiments is derived from NO, we examined the effects of irreversible, noncompetitive NO synthase (NOS) inhibitors on nitrite production, L-NIO (L-N^5-iminoethyl ornithine) and DPI (diphenyleneiodonium chloride). The former compound is

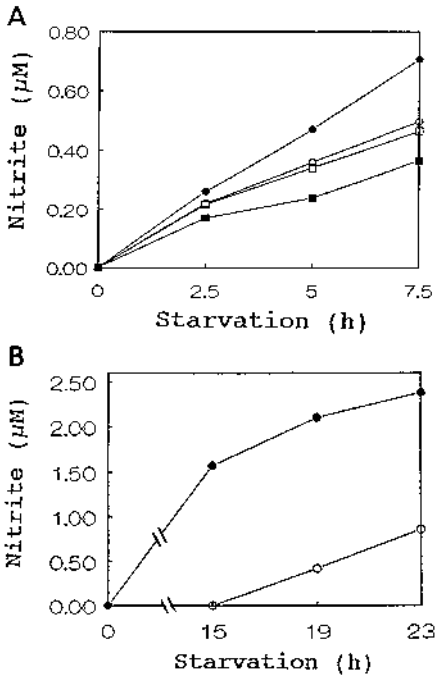

Fig. 1. Starved *D. discoideum* cells produce NO. (A) Cells were starved in spinner suspension. At time 0, DPI (10 μM) (-○-), DPI (100 μM) (-■-), L-NIO (5 mM) (-□-) or vehicle (-●-) was added. Vehicle refers to the solvent for DPI and L-NIO which was DMSO and water, respectively. The results were the same in both cases. At the indicated times, cell media were collected and nitrite levels were determined as described in Materials and Methods. The results are representative of four experiments. (B) Nitrite production in aggregated cells. Cells were starved for 15 hours, after which time they were washed and resuspended in fresh buffer (-○-). Control cells were not washed (-●-). Media were collected at the indicated times and nitrite was determined. The results are representative of three experiments.

specific for NOS while the latter inhibits flavin-requiring enzymes in general. As shown in Fig. 1A, the addition of 10 μM DPI resulted in 30-35% inhibition of nitrite production while 100 μM produced an approximate 50% inhibition by 7.5 hours of cell starvation. L-NIO resulted in an approximate 35% inhibition of nitrite production when present at 5 mM. At 0.5 mM, L-NIO showed a smaller effect on nitrite production, reducing medium levels by 11%. The relatively high concentrations of L-NIO necessary to inhibit NOS in our system most likely reflects its limited cellular uptake since *D. discoideum* lack a transport system for amino acids (Lee, 1972). The effective concentrations of DPI were more in the range of what is needed to inhibit NOS in mammalian systems but, in general, *D. discoideum* is more resistant to exogenous compounds. This is exemplified by the need to add 200-300 μg/ml of cycloheximide to the medium to arrest protein synthesis (Klein and Juliani, 1977). The addition of a competitive inhibitor of NOS, NMMA (N-monomethyl-L-arginine), had little effect on NO production (data not shown). This simulates the situation in rat peritoneal neutrophils and murine macrophages where NO production is not significantly inhibited by competitive NOS inhibitors while L-NIO is an effective inhibitor (McCall et al., 1991). In the case of *D. discoideum*, cells are grown on a rich medium of yeast extract and protease peptone digest to supply high levels of amino acids. The high endogenous *arg* pools are expected to severely restrict the effects of a competitive inhibitor of NOS. Indeed, some primary cell cultures may require several days of incubation in an arg-free medium before the effect of a competitive NOS inhibitor is optimal (Dawson et al., 1993). That NO production was only slightly increased when *D. discoideum* were starved in the presence of 10 mM arg indicates that the intracellular levels of arg are high and not limiting for NOS activity.

Cells continued to produce NO at a relatively constant rate when starved for longer times (Fig. 1B). Cells were starved for 15 hours and the accumulation of nitrite in the medium was monitored at that time and for an additional 8 hours. By 15 hours of starvation, cells had formed tight aggregates. They are unable to progress past that stage when starved in spinner suspension since further development requires an air-liquid interface. Cells that had been starved for 15 hours were also washed and resuspended in fresh buffer. Under those conditions, cells continued to produce nitrite at an apparent rate that paralleled the production seen in cells that had not been washed. Those data confirm that cells continue to produce NO at a relatively constant rate throughout the aggregation program. The data also indicate that the NO produced during aggregation does not feed-back regulate its production.

In the above experiments, cells were washed free of growth medium and resuspended in buffer to initiate starvation. Thus it was unclear if starvation induced NO production or if growing cells also synthesize NO. To examine this question, we grew cells in a bacterial suspension since the HL-5 medium already contains high levels of nitrite. Additionally, we needed to use heat-killed bacteria to eliminate interference by their production of nitrite. We verified that such heat-killed bacteria, when incubated in the absence of amoebae, did not produce any compounds that were detected as nitrite in our assay. Cells were innoculated at either 7.5×10^5 or 1.2×10^6 cells/ml bacterial suspension. At the indicated times, samples were taken to determine cell density and nitrite levels in the media. As shown in Fig. 2 in both cases nitrite levels continued to increase throughout the time course of the experiment, with the cells seeded at the lower density showing a proportionally lower accumulation of nitrite. For the cells seeded at lower density, cell growth was maintained throughout the experiment and by 24 hours they had reached a density of 5.8×10^6 cells/ml. For cells that had been seeded at the higher density, however, growth had plateaued by 19 hours at 1.5×10^7 cells/ml. It appears that growing cells produce low levels of nitrite in a manner proportional to the cell number. The data also indicate that nitrite production continues even when growth is arrested. As in the case of starved cells, we attempted to verify that the nitrite in the media reflected the production of NO by incubating cells in the presence of DPI. Since, as expected, cell incubation with DPI inhibited growth, we confined our assessment to cells that had reached high densities and were no longer growing but, as shown in Fig 2, still produce nitrite. Under those conditions we could observe an approximate 50% inhibition of nitrite production.

NO gas inhibits aggregation

We have previously shown that NO-generating compounds inhibit *D. discoideum* aggregation (Tao et al., 1992a). To

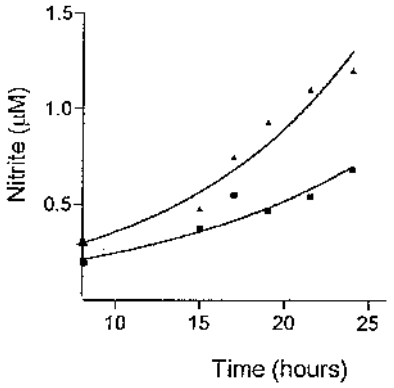

Fig. 2. Growing cells produce NO. Cells were seeded in a suspension of heat killed bacteria at either 7.5×10^5 cells/ml (squares) or 1.2×10^6 cells/ml (triangles). At the indicated times after seeding, the media were collected and nitrite levels were determined as described in Materials and Methods. The results are representative of three experiments.

confirm that the inhibition is a result of NO production by these donor compounds, and to gain a better indication of the concentrations of exogenous NO that are effective, we examined the effects of different concentrations of NO gas on cell development. Table 1 summarizes the results. The addition of 10 μM NO to cells at 2.5 hours of starvation inhibited their differentiation when monitored at 7.5 hours of starvation. Cell viability at this time, was greater than 95%. That the inhibition of aggregation by NO was not due to a toxic effect was also evidenced by its reversibility in that after an overnight incubation, cells had become aggregation competent and many formed streams when plated on tissue culture dishes. The addition of 10 μM NO to 4- to 5-hour starved cells was less effective, resulting only in a delay of aggregation as monitored at 7.5 hours of starvation. The single addition of lower concentrations of NO, in this case 1 μM, to 2.5-hour starved cells was ineffective. However, if 1 μM NO was repeatedly added to the medium at 10-15 minute intervals, an inhibition of aggregation resulted. At 7.5 hours of starvation, these cells showed no signs of aggregation competence. If at that time, NO treatment was discontinued, cells expressed aggregation competence after an additional 12-13 hours of starvation. The data confirm that NO effectively, and reversibly, inhibits cell aggregation and that it does so best when present before cAMP signaling occurs, the interphase period. It would also appear that repeated addition of NO enhances its effectiveness in inhibiting cell aggregation. This suggests that even lower concentrations of NO could regulate cell aggregation if NO were being constantly produced, e.g. as occurs in starved cells.

Glucose treatment stimulates NO production

Previously, we demonstrated that cells starved in the presence of glucose do not aggregate (Darmon and Klein, 1978). The inhibition appears related to the increased osmolarity of the medium since non-metabolizable sugars are also effective inhibitors of aggregation. Although glucose-treated cells progress in development to the point that cell lysates show high levels of adenylyl activity, intact cells do not make cAMP. The inhibition of aggregation by glucose can be overcome by stimulating cells with applied cAMP pulses to mimic the naturally occurring chemotactic signal. The addition of glucose to cells that have begun signaling is of limited effect. The similarities in the phenotype of these cells to that of NO-treated cells (Tao et al., 1992a, 1996), suggested that glucose might function by elevating NO levels. The data in Fig. 3A show the levels of nitrite produced by cells starved in the presence of glucose compared with control cells. In this particular experiment, the presence of 2% glucose resulted in an approximate 2- to 3-fold increase in the level of nitrite produced during a 7.5-hour incubation while the addition of 4% glucose resulted in a 4-fold increase. Measurements of nitrite levels in samples treated with nitrate reductase again indicated that both oxidation products of NO were present at equal levels. It should be noted that although the absolute levels of nitrite produced by cells varied in different experiments, the relative changes reported in this experiment were consistently observed. Each of the concentrations of glucose tested inhibited aggregation as determined microscopically at 6-8 hours of starvation. By that time, control cells were fully aggregation competent while glucose-treated cells remained undifferentiated. The inhibition of nitrite production in glucose-treated cells by NOS inhibitors was the same as seen in control cells. As an example, Fig. 3B shows that 10 μM DPI inhibited nitrite production by approximately 60% in cells starved for 8 hours in the presence of 2% glucose and by approximately 50% in control cells.

Since glucose-treated cells do not make cAMP, we next examined the possibility that the enhanced production of NO by these cells reflected the loss of a feedback inhibition of NOS via the cAMP receptor signaling pathway. Glucose-treated cells were starved in the presence of applied cAMP pulses and the levels of nitrite in the medium were monitored. Shown in Fig 3C are the data obtained when cells starved in the presence of 2% glucose were examined. The data are representative of the results seen with a range of glucose concentrations varying from 1% to 4%. Applied cAMP pulses had no effect on NO production. It would appear that the increase in NO seen in cells starved in the presence of glucose is not a consequence of inhibited cAMP signaling. It should also be recalled that, for both glucose-treated cells and cells treated with NO-releasing compounds, aggregation can be induced by exogenous cAMP pulses (Tao et al., 1992a, Darmon and Klein, 1978). That observation indicated that cAMP pulses can override the inhibition caused by NO. Our

Table 1. Effects of NO on cell development

	Starvation time (hours)	
Experimental condition	7. 5	20
1. No addition	+++	++++
2. 10 μM NO, once	–	+
3. 10 μM NO, once, at 5hours	++	++++
4. 1 μM NO, once	+++	++++
5. 1 μM NO, repeatedly	–	+/++

D. discoideum cells were starved in spinner suspension as described in Materials and Methods. NO was added at 2.5 hours of starvation, except in condition #3 where it was added at 5 hours of starvation. Repeatedly refers to the addition of NO every 10 minutes until cells had been starved for 7 hours. At the times indicated, cell development was monitored microscopically: – undifferentiated, + aggregation competent, ++ fully aggregation competent, +++ loose aggregates, ++++ tight aggregates. The data are representative of three experiments.

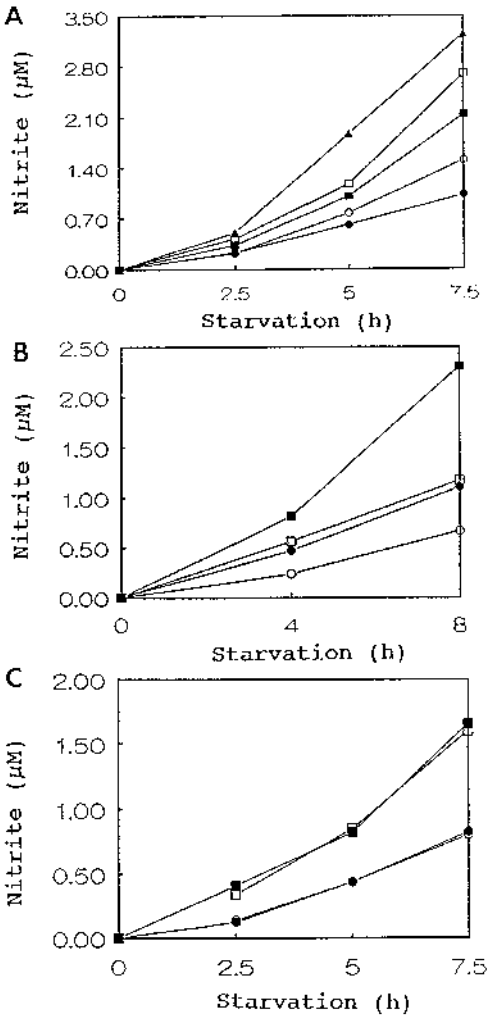

Fig. 3. Stimulatory effects of glucose on nitrite production in *D. discoideum.* (A) Concentration dependence of glucose stimulated production of nitrite. Glucose, at the concentrations of 0% (-●-), 1% (-○-), 2% (-■-), 3% (-□-), or 4% (-▲-), was added at the time cells were starved. At the indicated times of starvation, cell media were collected and nitrite levels determined. The results are representative of four experiments. (B) Effects of DPI on glucose stimulated production of nitrite. DPI (10 μM) (-○-), 2% glucose (-■-), or DPI plus glucose (-□-) were added at the time of starvation. Control cells (-●-) received no additions. At the indicated times, nitrite levels in the media were determined. The results are representative of three experiments. (C) Effects of cAMP pulses on glucose-stimulated production of nitrite. Cells were starved in the absence (●, ○) or presence (■, □) of 2% glucose. After 2 hours of starvation, some cells were stimulated with cAMP pulses as described in Materials and Methods (○, □). At the indicated times, nitrite levels were determined. The results are representative of three experiments (some performed using multiple concentrations of glucose).

current observations indicate that cAMP pulses do so without altering NO production. This is not unexpected in light of our findings, (a) that NO is less effective when added to cells that have begun signaling and (b) that NO inhibits cAMP production at a step distinct and proximal to those involved in cAMP receptor activation of the adenylate cyclase (Tao et al., 1996).

In general, the NOS isoforms that have been characterized

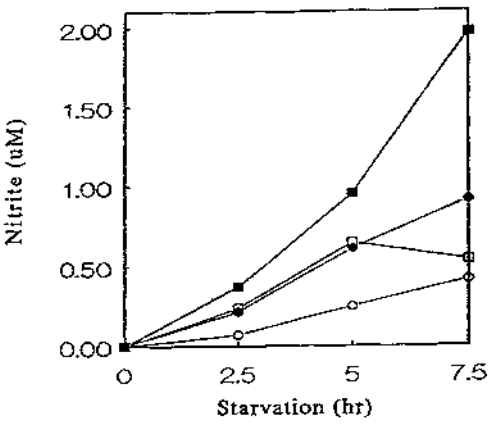

Fig. 4. Cells were starved in the presence of 2% glucose (-■-) or 2% glucose plus 300 μg/ml cycloheximide (-□-). Control cells were starved in the absence (-●-) or presence (-○-) of cycloheximide. At the indicated times, samples of the media were taken for nitrite determinations. The data are representative of two experiments.

in mammalian cells can be categorized into two groups, one that is strictly regulated by calmodulin binding in response to changes in intracellular Ca2+ (constitutive) and one that is Ca2+ independent (inducible). Changes in the latter activity reflect changes in gene expression and thus are dependent upon new protein synthesis (Nathan and Xie, 1994; Marletta, 1994). Although antisera against mammalian cNOS and iNOS isoforms are available, they have not been shown to cross-react with NOSs in lower eukaryotes. We therefore examined if the NO produced by our cells was dependent upon new protein synthesis (Fig. 4). Cells were starved in the absence or presence of cycloheximide and at the indicated times, nitrite levels in the media were determined. By 7.5 hours, cells starved in the presence of the drug produced approximately half the amount of nitrite as did control cells. A similar experiment using cells starved in the presence of glucose showed that glucose induction of nitrite production was inhibited 50-75% by cycloheximide. The requirement for protein synthesis would be consistent with the presence of an iNOS in our cells. However, we cannot rule out the possibility that the need for new protein synthesis reflects the turnover of a NOS co-factor. The premise that our cells contain an iNOS-like activity would be consistent with our observation that cells starved in media supplemented with 10^{-7} M A23187 plus or minus 2 mM Ca^{2+} did not alter NO production (data not shown) despite the fact that such treatments have been well documented to result in significant Ca^{2+} influxes in *D. discoideum* (Van Haastert, 1991).

Endogenous NO regulates aggregation

The regulatory role of NO in the aggregation process was examined by starving cells in the presence of oxyhemoglobin (oxyHb), an NO scavenger (Table 2). Cells starved for 6 hours in the absence of any additions were fully aggregation competent. When cells were starved in the presence of 6 μM oxyHb, their development was advanced such that by 6 hours cells had entered into loose aggregates. After an overnight incubation, both control and oxyHb-treated cells had formed tight aggregates that were indistinguishable from one another. A slight advancement of development was still observed when

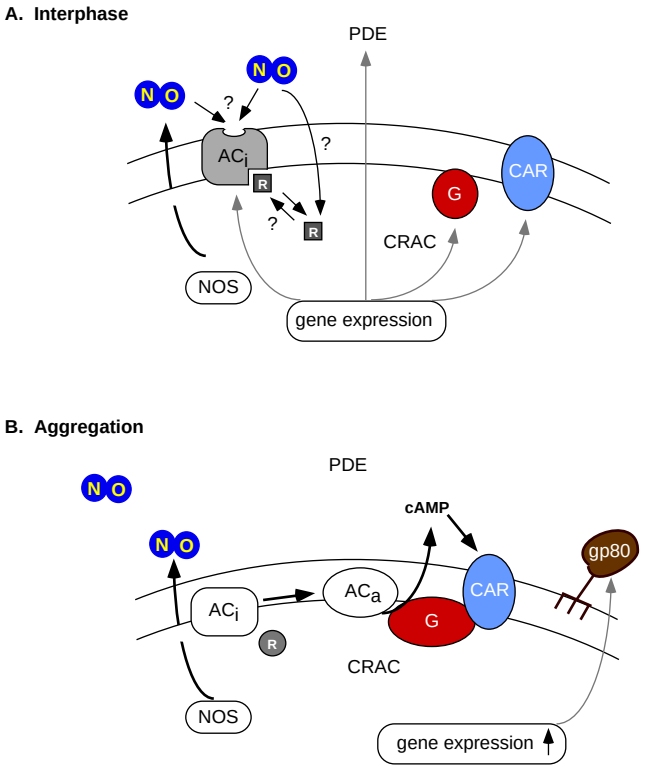

Fig. 5. A working model depicting the action of NO on *D. discodieum*. (A). During the interphase period, cells express the components necessary for chemotactic signaling. These include the adenylyl cyclase (AC), the cAMP receptor (CAR) and the components necessary for receptor mediated activation of the adenylyl cyclase e.g. the G protein, CRAC. NO is also produced during this period, preventing the adenylyl cyclase from producing cAMP. This could reflect a direct action of NO on a regulatory domain of the enzyme or on a distinct regulatory component (R). (B). Although NO is still produced, cells are refractory to its effects and adenylyl cyclase becomes active. cAMP is synthesized and released into the medium where it can promote CAR activation of adenylyl cyclase, maintain the signal relay and induce varied cellular responses involved in chemotactic migration including new gene expression e.g. gp80.

the concentration of oxyHb was reduced to 2 μM while no effect was seen with lower concentrations. MetHb (methemoglobin), which is not an effective scavenger of NO, did not advance development at any concentration. Higher concentrations of oxyHb (10 μM) could also stimulate aggregation but not as effectively as 6 μM. This probably reflects an adverse effect of protein (food) in the medium on the aggregation process since 10 μM metHb actually inhibited aggregation (data not shown). The effects of L-NIO and DPI on aggregation were also examined. Their effects were minor and variable, reflecting the fact that they are not efficient inhibitors of NO production in these cells.

Cells incubated with 1% glucose showed no signs of development during the first 6 hours of starvation. However, if oxyHb was also present, they displayed an elongated morphology and formed end-to-end contacts characteristic of aggregation competence at 6 hours of starvation. After an overnight incubation, glucose-treated cells were aggregation competent while cells

Table 2. Effects of glucose and oxyHb on cell development

Experimental condition	Starvation time (hours) 6	20
1. Control	++	++++
2. Control + oxyHb	+++	++++
3. 1% Glucose	–	+
4. 1% Glucose + oxyHb	+	+++
5. 2% Glucose	–	–/+
6. 2% Glucose + oxyHb	–/+	++
7. 3% Glucose	–	–
8. 3% Glucose + oxyHb	–/+	+/++

D. discoideum cells were starved in spinner suspension as described in Materials and Methods. Glucose and/or 6 μM oxyHb were added at the begining of starvation. Development of cells was monitored microscopically after 6 and 20 hours of starvation: – undifferentiated, + aggregation competent, ++ fully aggregation competant, +++ loose aggregates, ++++ tight aggregates. The data are representative of four experiments.

starved with 1% glucose plus oxyHb had formed loose aggregates. Cells starved for 6 hours in the presence of 2% or 3% glucose were also inhibited in their aggregation, showing no developmental signs in the first 6 hours of starvation. When starved overnight, cells in 2% glucose developed an elongated morphology but were unable to form end-to end contacts. Cells starved overnight in 3% glucose remained undifferentiated. When oxyHb was present, cells incubated with 2% or 3% glucose developed an elongated morphology and organized into territories after 6 hours of starvation, but they were as yet unable to form end-to-end contacts. After an overnight incubation, however, they were fully aggregation competent. The data indicate that the increase in extracellular NO when glucose is present inhibits cell differentiation.

The expression of gp80 as a biochemical marker of the differentiated state of the cells was also monitored. gp80 is a cell adhesion molecule whose expression is regulated by cAMP pulses: the protein is absent from vegetative cells but is present to increasing degrees as cells express aggregation competence and form aggregates (Darmon et al., 1975; Gerisch et al., 1975). The expression of gp80 is clearly cAMP-dependent and recent experiments have identified specific sequences upstream of the gp80 coding region that confer this dependence (Desbarats et al., 1992). The relative levels of the protein in cells starved for 4 and 7.5 hours in the absence or presence of 3% glucose, with or without oxyHb, are reported in Table 3. Consistent with the morphological data, control cells starved in the presence of oxyHb showed slightly advanced expression of gp80. This was more obvious at 4 hours of starvation when gp80 expression had not yet reached maximum levels. Glucose-treated cells did not express gp80 at any time during the experiment unless oxyHb had been added to the medium. Under those latter conditions, gp80 levels increased with time, albeit to lower levels than control cells. This was expected since the differentiation of these cells was delayed compared to control cells. Cells starved overnight in the presence of glucose and oxyHb showed levels of gp80 similar with those of control cells that had been starved for 7.5 hours.

DISCUSSION

Criteria used to confirm the production of NO by cells or tissue

Table 3. Effects of glucose and oxyHb on gp80 expression

Starvation		% of
time	additions	gp80
4 hours	none	30
	oxyHb	40
	glucose	0
	glucose + oxyHb	0
8 hours	none	92
	oxyHb	100
	glucose	0
	glucose + oxyHb	18
16 hours	glucose	0
	glucose + oxyHb	38

Cells were starved for the indicated times in the presence or absence of 6 mM oxyHb, 3% glucose, or both. Levels of gp80 were determined as described in Materials and Methods. The 100% value was assigned to the highest level of the protein observed. The data are representative of three experiments.

are the production of nitrite, an inhibition of that production by a NOS inhibitor(s), and/or the loss of a biological effect upon addition of an NO scavenger (Lancaster, 1992; Stamler, 1994; Schmidt and Walter, 1994). We have provided the first demonstration that *D. discoideum* makes NO. Cells produced NO during the initial phase of their developmental cycle in a manner proportional to their time of starvation. NO production was inhibited by two different irreversible, non-competitive inhibitors of NOS. Consistent with the production of NO, both its oxidative products, nitrite and nitrate, were produced and were present in roughly equal amounts. Additional evidence that *D. discoideum* produce NO was obtained in experiments in which cells were incubated with oxyHb. NO released by cells is scavenged by oxyHb. In so doing, this treatment advanced cell differentiation.

Physiological roles for NO have been proposed based either on the effects of NO donor compounds or on the consequences of modulating the endogenous levels of NO (Lancaster, 1992; Stamler, 1994; Schmitdt and Walter, 1994). We have employed both approaches to examine this question in *D. discoideum.* During the initial period of cell starvation, interphase, cells arrest growth and express the genes necessary for chemotactic signaling via cAMP pulses (Van Haastert, 1991). In response to those pulses, cells develop aggregation competence and migrate toward central collection points to form a multicellular aggregate. The addition of NO to interphase cells, either in the form of dissolved gas (this work) or as released from donor compounds (Tao et al., 1992a), inhibited cell differentiation to aggregation competence. The reversibility of this inhibition was demonstrated by removal of the donor compounds, or in the case of NO gas, allowing for normal oxidation of the NO to render it ineffective. The effects of NO were selective for interphase cells: cells were resistant to NO if treatment was initiated after they had became aggregation competent.

Using NO gas, we demonstrated that a single addition of 10 μM NO effectively inhibited cell differentiation. Since NO has a half-life within seconds in an oxygenated environment (Wink and Ford, 1995), this would imply that the cellular event(s) elicited by NO do not require its continued presence to inhibit cell differentiation. In light of the short half-life of NO, it was not surprising that the required concentration of NO could be reduced if NO was reapplied. 1 μM NO added at 10-15 minute intervals was even more effective than the single addition of 10 μM NO. Micromolar concentrations are within an order of magnitude of those produced endogenously. Clearly, cell differentiation is regulated by even lower concentrations of endogenously produced NO since oxyHb can accelerate that process. The ability of low endogenous levels of NO to affect development likely reflects the fact that cells maintain a continued production of NO and that endogenously produced NO is immediately available to the microenvironment of the neighboring cells before being oxidized.

To modulate endogenous levels of NO, we starved cells in the presence of oxyHb. Those experiments indicated that endogenously produced NO is an extracellular regulator of cell differentiation. The presence of oxyHb stimulated differentiation as evidenced both by morphological criteria and by the more rapid expression of gp80, a gene whose transcription is developmentally regulated. OxyHb advanced the differentiation of cells starved under control conditions and of cells starved in media of increased osmolarity, i.e. in the presence of glucose. In the case of control cells, starvation conditions have been optimized for rapid cell differentiation (Beug et al., 1970) and thus represent conditions under which the inhibitory effects of endogenous NO are not optimal. However, we could still stimulate differentiation with oxyHb. Under such conditions, other factors e.g. the time required to express the necessary genes, determine when pulsing can begin. In its normal environment, *D. discoideum* is unlikely to encounter optimal starvation conditions, thus the role of NO would be more significant. Indeed, by raising the osmolarity of the medium, we demonstrated a much more dramatic effect of oxyHb. Cells starved in the presence of glucose do not make cAMP pulses and thus do not develop aggregation competence (Darmon and Klein, 1978). This was the first demonstration that the A-cyclase is a component of the osmotic stress response. We have shown here that another component of that response is NO. Cells starved in the presence of glucose produced enhanced levels of NO compared with untreated cells, levels which prevented cAMP production: when oxyHb was added to capture the NO produced, cells were then able to make cAMP pulses. Chemotactic signaling was evidenced by their differentiation, monitored morphologically and by their expression of gp80.

The mechanism by which NO inhibits cAMP signaling is currently not clear. We have previously shown that neither the soluble guanylyl cyclase (GC) nor cGMP levels are affected by NO in our cells (Tao et al., 1992a), ruling out the more commonly-cited mechanism of NO action. In other systems, NO has also been shown to inhibit enzymes involved in carbohydrate metabolism e.g. GAPDH and *cis*-aconitase, leading to the hypothesis that the subsequent decrease in ATP levels is responsible for the effects of NO (Lancaster, 1992; Stamler, 1994; Schmitdt and Walter, 1994). We have shown in *D. discoideum* that GAPDH activity is inhibited when cells are treated with NO donors but that the inhibition is so rapidly reversed (within 10 minute) that this would not account for the effects of NO on cell differentiation (Tao et al., 1992b, 1994). The fact that NO-treated cells undergo normal development when stimulated with applied cAMP pulses rules out a general

metabolic effect. That observation also rules out an effect of NO on the cAMP signal transduction and desensitization mechanisms. Thus components like the cAMP receptor (CAR), G2 (the G protein coupled to the receptor), CRAC (a cytosolic component), PDE, and CAR-kinase, which are believed to be involved in receptor-mediated adenylyl cyclase activation and adaptation (Van Haastert, 1991; Meier and Klein, 1988) are not the targets of NO action. Our biochemical analyses have verified that NO-treated cells show normal biogenesis of such components, normal receptor-mediated stimulation of adenylyl cyclase activity, and normal cAMP pulse production when stimulated with a pulse of cAMP (Tao et al., 1996). Keeping in mind that cells are resistant to the effects of external NO when it is applied to cells after signaling has begun, the sum of the data indicate that NO functions selectively to prevent the initiation of cAMP pulses.

Fig. 4 presents a working model to describe the action of NO in starved *D. discoideum*. The figure does not include all of the events involved in chemotactic signaling in our cells, but rather depicts representative components of that system. Before cAMP signaling can be initiated, cells must express adenylyl cyclase and the other components fundamental to that process. The developmental expression of those components occurs during interphase and this is not regulated by NO (this work and Tao et al., 1996). Other factors, e.g. pre-starvation factor (Rathi and Clarke, 1992) have been linked to the expression of genes involved in chemotactic signaling. To explain the action of NO on the initiation of cAMP pulses, we have depicted the adenylyl cyclase as having two possible states, one active and another inactive. Since NO-treated cells can synthesize cAMP in response to exogenous stimulation, we conclude that the catalytic site of the adenylyl cyclase is not the target of NO action. This focuses attention to a possible regulatory domain of the adenylyl cyclase or a distinct regulatory component (R) as the target of NO action, the end result of which would be to promote an inactive enzyme. Since growth arrest is not synchronous, this action of NO could ensure sufficient differentiation of the cell population, such that once begun, cAMP synthesis would result in productive signaling. We have shown that cells produce NO throughout growth. More importantly cells continue to produce NO when they enter the stationary phase of growth, allowing for relatively high levels of NO to be present during the pre-aggregative phase to function during the transition from growth to development.

That NO is a determinant of the pre-aggregative phase represents a novel but fundamental addition to our concept of how chemotactic signaling begins. Current models of adenylyl cyclase regulation involve the transient activation of the cell surface cAMP receptor, leading to the activation of adenylyl cyclase. The release of the resulting cAMP pulse into the medium allows for another round of receptor activation (Van Haastert, 1991). That the activity of the adenylyl cyclase is dependent upon cAMP pulsing, however, ignores the very basic problem of how pulse production is first initiated. Our data argue that NO suppresses adenylyl cyclase activity and that it is the release from this negative influence that allows the enzyme to first become functional. Continued experiments into the mechanism by which NO does so should identify its target as well as indicate how cells eventually escape the inhibitory effects of endogenous NO to begin signaling and enter the aggregation phase of their developmental cycle.

Having demonstrated that, as part of their developmental cycle, *D. discoideum* produce NO and that it functions to regulate the interphase period, our current efforts are directed toward characterizing the enzyme responsible for NO production. The inhibition of NO production by DPI and L-NIO indicate that, like the mammalian NOS, the enzyme in *D. discoideum* is flavin and arginine requiring. The sustained production of NO by *D. discoideum* during aggregation, a production that could be induced by changes in the osmolarity of the medium, the inhibition by cycloheximide and the lack of an effect of A23187/Ca^{2+} treatment of cells, are consistent with the presence of an inducible isoform of NOS in our cells. Relative to inducible NO synthesis in most mammalian cells, the levels of NO produced by *D. discoideum* during aggregation appear low. A possible exception being human macrophages which, when induced with a combination of IL-4 and CD23 antibody, produce NO at a rate similar to *D. discoideum* (Vouldoukis et al., 1995). We emphasize, however, that since most of the information concerning NO and the enzymes responsible for its production is derived from studies in mammalian cells/systems (Nathan and Kie, 1994; Marletta, 1994), it is not clear to what extent current generalities are universally applicable. Continued studies in *D. discoideum* are expected not only to expand our understanding of the enzymes responsible for NO production, but may reveal some currently unappreciated regulatory mechanisms, in particular, as they function in a developmental context. In that regard, it is also noteworthy that *D. discoideum* continued to produce NO even after the aggregation program was completed in spinner suspension, allowing for the possibility that cells use NO to regulate additional stages of morphogenesis. However, since further development requires that cells are starved on a solid support to provide an air-liquid interface, simple determinations of nitrite production are not feasible. Thus, insights into such questions are also expected as we progress in our biochemical characterization of the NOS activity present in *D. discoideum* and its regulation throughout development.

This work was supported by research funds from the NIH (A. H.), AHA (Y. P. T.), and NSF (C. K.). The authors thank M. Klevorn for the typing of this manuscript.

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(Accepted 19 June 1997)