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Nitric oxide regulates the proliferation of chick embryo retina cells by a cyclic GMP-independent mechanism

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Abstract

Nitric oxide (NO) is an intercellular messenger involved in many physiological and pathological processes of vertebrate and invertebrate animal tissues. In the embryonic chick retina, nitric oxide synthase (NOS) activity and a system for L-arginine transport between neurons and glial cells were described, supporting the idea that nitric oxide is a critical molecule during retinal development. In the present work we show that nitric oxide is a modulator of cell proliferation in chick embryo retina. Mixed cultures of retinal neurons and glial cells were submitted to [^{3}H]-thymidine incorporation after drug treatment. Incubation for 24 h with the NO donors *S*-nitroso-*N*-acetyl-penicillamine (SNAP) or Spermine nitric oxide (SpNO) complex promoted a decrease of approximately 70% of [^{3}H]-thymidine incorporation in a dose-dependent manner. SNAP did not increase Lactate dehydrogenase release and its effect was not mimicked by 8-bromo cyclic GMP, or blocked by the guanylate cyclase inhibitor 1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one (ODQ), indicating that the effect was not due to cell death or mediated by increases of cyclic GMP levels. The inhibition was completely prevented by dithiotreitol (DTT), strongly indicating the participation of an *S*-nitrosylation mechanism. SNAP blocked the increase of [^{3}H]-thymidine incorporation induced by ATP. Using purified cultures of glial cells we showed that the NO donor SNAP produced an inhibition of 50% in cell proliferation and did stimulate ERK1/2 phosphorylation, indicating that the inhibition of this pathway was not involved in its cytostatic effect. [^{3}H]-Thymidine autoradiography of mixed cultures showed labeling of oval nuclei of glial flat cells. The injection of eggs with SNAP also did promote an intense inhibition of [^{3}H]-thymidine incorporation in retinas from 9-day-old embryos. These data suggest that nitric oxide affects the proliferation of chick embryo retina glial cells in culture or ''in vivo'' through cyclic GMP and ERK-independent pathways.

Keywords: Development; Purified glial cell cultures; Autoradiography; Extracellular-regulated kinases; Nitrosylation

1. Introduction

Nitric oxide (NO) is produced as a by-product of the conversion of L-arginine to L-citruline catalyzed by nitric oxide synthases (NOS), which are present in many cell types in different isoforms characterized by structural and biochemical features and tissue localization (for review see Yun et al., 1996). Endothelial and neuronal NOS, respectively, found in endothelial cells and neurons, are constitutively expressed, and produce NO in small amounts in a calcium-dependent manner (Bredt and Snyder, 1990). Immune system-related NOS is an inducible and calcium-independent enzyme which produces NO in large scale causing deleterious effects to several pathogenic agents (Simmons and Murphy, 1992).

NO interacts with several target proteins and generates physiological changes in cells. Activation of soluble guanylyl cyclase by NO and the consequent increase in the production of cGMP is a relatively well known mechanism. However, many studies show cGMP-independent effects of NO mediated by its reaction with sulphydril residues of proteins in a process named

Abbreviations: 8Br-cGMP, 8-bromoguanosine 3′,5′-cyclic monophosphate; BME, Basal Medium of Eagle; CMF, calcium and magnesium-free balanced salt solution; DFMO, alpha-difluoromethylornithine; DTT, dithiotreitol; ERK, extracellular-regulated kinase; HEPES, *N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; LDH, Lactate dehydrogenase; NARG, N_ω-nitro-L-arginine; NO, nitric oxide; ODC, ornithine decarboxylase; ODQ, 1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one; SDS-PAGE, sodium dodecyl sulphate poliacrylamide gel electrophoresis; SNAP, *S*-nitroso-*N*-acetyl-penicillamine; SpNO, Spermine nitric oxide complex; ZAP, zaprinast

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S-nitrosylation (Ignarro et al., 2002; Gardner et al., 1997; Zhang and Snyder, 1992). Moreover, NO-derived nitrogen reactive species such as peroxynitrite interact with tyrosine residues of proteins in a process called nitration (Li and Whorton, 2002; Minetti et al., 2002).

Previous work has suggested putative roles of NO in the mature and developing nervous system (Yamauchi et al., 2003; Wu et al., 2001), including the retina (Ientile et al., 1996a; Goureau et al., 1999). Cell proliferation, differentiation, migration, survival and death are events observed during development of the nervous system which are ultimately responsible for cell number, physical structure and final size of the brain. Cell proliferation is normally restricted to a limited period during CNS development. However, glial cells, the non-neuronal components of the nervous system, are able to proliferate and regenerate even in mature life whereas most neurons are not able to divide after the embryonic period (Mey and Thanos, 2000).

In the chick retina, neuronal elements are born between embryonic Day 2 (E2) and shortly after E12 (Prada et al., 1991). Previous studies have shown the presence of high NOS activity at early embryonic stages from E8 up to E14, when the retinal layers are already structurally defined (Ientile et al., 1996a; Paes-de-Carvalho et al., 1996; Paes-de-Carvalho and Mattos, 1996). Furthermore, a high affinity transport system for L-arginine and NOS-containing cells were detected in retinal cultures obtained from E8 embryos (Goureau et al., 1999; Cossenza and Paes-de-Carvalho, 2000). In the retina, NADPH diaphorase or NOS-containing cells include photoreceptors and amacrine cells (Kim et al., 1999; Haberecht et al., 1998; Rios et al., 2000; Paes-de-Carvalho et al., 1996).

Monolayer cultures of chick retina cells reproduce features of the tissue in vivo, including the development of several neurotransmitter systems (Paes-de-Carvalho, 1990; de Mello et al., 1990). In the present work we show that NO has an anti-proliferative role in these cultures. Using mixed neuronal-glial or purified glial cultures we show that NO donors inhibit [^{3}H]-thymidine incorporation by a cyclic GMP-independent mechanism that does not appear to involve the MAP kinase pathway or inhibition of ornithine decarboxylase (ODC). Autoradiographic data corroborate the finding that retinal glial cells are the targets of NO-induced anti-proliferative effect. Injection of eggs with *S*-nitroso-*N*-acetyl-penicillamine (SNAP) was also able to decrease [^{3}H]-thymidine incorporation in the retina.

2. Experimental procedures

2.1. Materials

Fertilized White Leghorn chicken eggs were obtained from a local hatchery. Hepes, SNAP, Spermine nitric oxide complex (SpNO) were purchased from Sigma/RBI Chem.Co. (St. Louis, MO, USA). Glutaraldehyde 25% was from Fluka Chemie (Steinheim-Switzerland). [^{3}H]-Thymidine (5 Ci/mmol) was purchased from Amersham Biosciences. Trypsin and Basal Medium of Eagle (BME) were from Gibco (Grand Island, NY, USA). Anti-phospho ERK antibody was from Cell Signaling. ECL and HRP-conjugated secondary anti-rabbit antibody were from Amersham. All other reagents used were of analytical grade.

2.2. Preparation of mixed primary cultures of retina cells

Monolayer cultures of chick retina cells were prepared as previously described (de Mello, 1978). Briefly, retinas from E8 chick embryos were dissected from other ocular tissues, including the pigmented epithelium, and digested with 0.1% trypsin, in Ca^{2+} and Mg^{2+}-free Hanks' balanced salt solution (CMF) for 20 min at 37 °C. Then, cells were suspended in BME supplemented with 5% heat-inactivated fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml), and seeded in 35 mm tissue culture plastic dishes or 16 mm wells in a density of 2×10^4 cells/mm^2. Cells were maintained at 37 °C in a humidified incubator with 95% air and 5% CO_2.

2.3. Preparation of purified glial cell cultures

Retinas from E11 chick embryos were dissected and cells dissociated as described for the mixed cultures. Cells were seeded in 16 mm multiwell plates in a density of 2×10^3 cells/mm^2 and cultured in BME for 21 days. At this time, glial cells were confluent and virtually no neurons were present, as determined by morphological criteria. The medium was changed every 3 days.

2.4. [^{3}H]-Thymidine Incorporation

Different drugs were added to mixed cultures 2 h after seeding the cells. Purified glial cell cultures were incubated for 20 days and then the drugs were added. After 24 h, media from both types of cultures were removed, the cultures incubated in saline with [^{3}H]-thymidine (1 μCi/ml) for 1 h at 37 °C, washed and lysed with 0.4 M NaOH for 15 min at 4 °C. Cells were scrapped off from the dishes and transfered to tubes containing trichloroacetic acid (TCA, 10% final concentration). After further incubation for 30 min at 4 °C, the material was filtered using GFB glass fiber filters. The radioactivity was determined by liquid scintillation counting.

2.5. Injection of eggs and incubation of retinas

Eggs with E8 embryos were injected in the air chamber with 10 μl SNAP 120 mM dissolved in DMSO, sealed with a tape and returned to the incubator. Control eggs were injected with the same volume of DMSO. Twenty four hours later, the embryos were removed, the retinas dissected in CMF and incubated for 1 h with [^{3}H]-thymidine. The procedure was the same as described above for the cultures. The protein content of each retina was determined by the method of Lowry et al. (1951). The results of these experiments were expressed as % of total incorporation after correction of each value in cpm by the protein content and the input radioactivity.

2.6. Autoradiography

Mixed cultures at C1 were incubated with [^{3}H]-thymidine (5 μCi/ml) for 1 h at 37 °C, washed and fixed with 2.5% glutaraldehyde in 0.2 M phosphate buffer (v/v) for 1 h. Dishes were dehydrated in ethanol series (50–100%), covered with autoradiographic emulsion 50% (v/v) in water (Amersham, Hypercoat EM 1) and incubated in the dark for 25 days at 4 °C. After this period, the cultures were coverslipped and analysed in a Zeiss Axioskop microscope.

2.7. LDH measurement

Cell survival in cultures after exposure to NO donors was assessed by determining the extracellular LDH activity in culture media, measured by spectrophotometric assays using the cytotoxicity detection kit from Promega (Cyto Tox 96® Non-Radioactive Citotoxicity Assay).

2.8. Western blot analysis

For detection of ERK phosphorylation, purified glial cell cultures were treated with SNAP for 24 h, washed, the cells scraped off from the dishes in sample buffer and the material boiled for 10 min. Samples containing 60 μg protein were submitted to SDS-PAGE and the proteins transferred to PVDF

membranes which were incubated overnight with anti-phospho-ERK 1/2 antibody (1:2,000), washed, incubated with peroxydase-conjugated secondary antibody (1:4,000) and revealed by the ECL chemiluminescence. Total amount of protein in each sample was determined using the Bradford reagent, with bovine serum albumin as standard.

2.9. Statistical analysis

Statistical analysis was performed using analysis of variance (ANOVA) followed by Bonferroni test, or by an unpaired *T*-test using the software Graph Pad Prism.

3. Results

3.1. [^{3}H]-Thymidine incorporation

We have used the protocol of [^{3}H]-thymidine incorporation to assess the rates of DNA synthesis of retinal cells in culture that correspond to cell proliferation. Treatment of mixed cultures with the NO donors SNAP or SpNO (1 mM) for 24 h decreased [^{3}H]-thymidine incorporation by 70% (Fig. 1(A)). The effect of SNAP was not accompanied by increases in LDH release (Fig. 1(B)), although a slight, but not significant citotoxic effect was observed using the same concentration of SpNO (Fig. 1(B)). N_ω-nitro-L-arginine (NARG), an L-arginine analog, which was used to test the hypothesis that endogenously generated NO could be inhibiting cell proliferation, had no effect on the levels of [^{3}H]-thymidine incorporation (Fig. 1(A)). *N*-acetyl penicillamine (NAP), the resulting metabolite of SNAP after NO release, also had no effect, excluding the possibility that this compound affected the incorporation of [^{3}H]-thymidine (Fig. 1(A)).

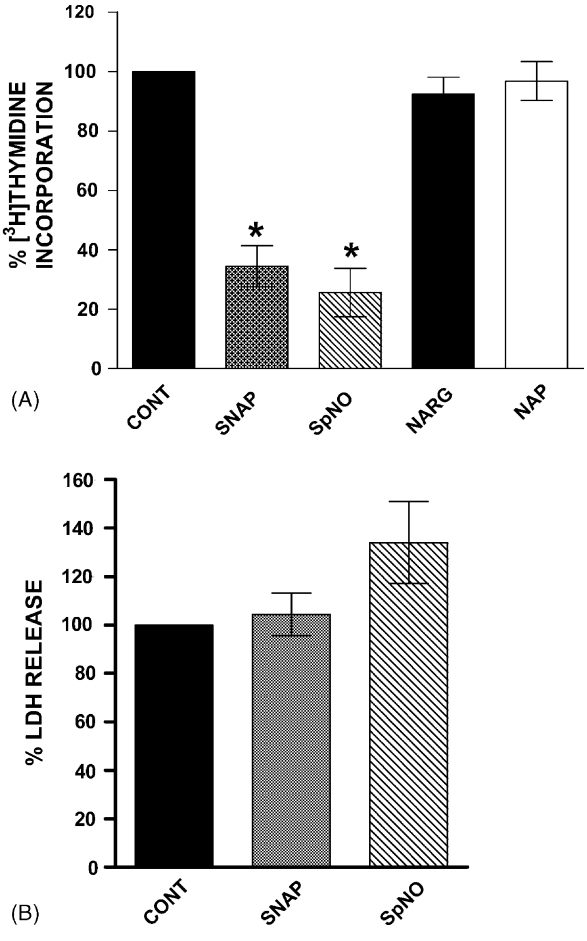

Fig. 1. Effect of NO donors and NOS inhibitor on [^{3}H]-thymidine incorporation and LDH release by retinal cells in mixed culture. (A) Cultures of retinal cells from E8 embryos were treated for 24 h with 1 mM SNAP or SpNO, 200 μM NARG or 1 mM NAP, washed and processed for [^{3}H]-thymidine incorporation assay. (B) LDH release after 24 h treatment with SNAP or SpNO (1 mM). Results are the means ± S.E.M. of at least three experiments performed in duplicate. The asterisks indicate $P < 0.001$.

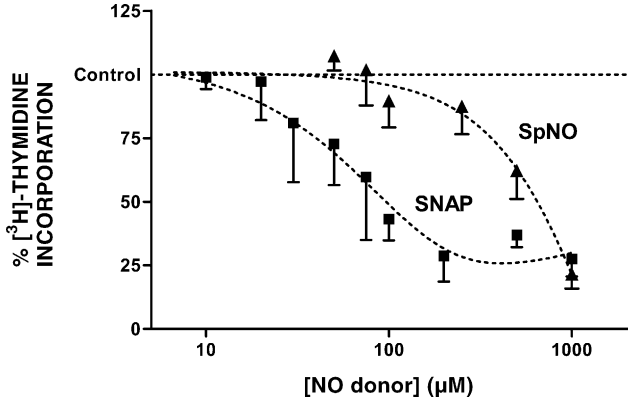

Fig. 2. Inhibition of [^{3}H]-thymidine incorporation by different concentrations of NO donors in cultured retinal cells. Mixed cultures were treated for 24 h with NO donors in concentrations from 10 μM to 1 mM (SNAP), and 50 μM to 1 mM (SpNO). After treatment, cultures were incubated for 1 h with 1 μCi/ml [^{3}H]-thymidine. The inhibition of [^{3}H]-thymidine incorporation was dose-dependent, with EC_{50} of approximately 75 and 250 μM for SNAP and SpNO, respectively. Each point represents the mean ± S.E.M. of at least three separated experiments performed in triplicates.

In order to better characterize the effects of NO donors, we performed concentration curves for SNAP and SpNO using concentrations between 10 μM and 1 mM (Fig. 2). The maximal inhibitory effect of SNAP was obtained at the concentration of 200 μM and an IC_{50} of 75 μM was calculated for this compound. SpNO exhibited an IC_{50} of 250 μM and maximal inhibition was achieved with 1 mM.

3.2. Effect of cyclic GMP analog or inhibition of guanylate cyclase

To verify whether the effects of NO were mediated by changes in cellular cGMP levels, we investigated the effect of 8Br-cGMP (500 μM), a permeable cGMP analog. No effect of this compound on DNA synthesis rate of mixed cultures was observed (Fig. 3). Moreover, the inhibitor of soluble guanylate cyclase 1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one (ODQ) (5 μM) did not affect [^{3}H]-thymidine incorporation or the inhibitory effect of SNAP. Zaprinast (10 μM), a cGMP phosphodiesterase inhibitor, also had no significative effect (Fig. 3).

3.3. Effect of dithiotreitol (DTT) and an ornithine decarboxylase inhibitor

Many effects of NO on cells are explained by its ability to *S*-nitrosylate proteins and these effects are invariably blocked by

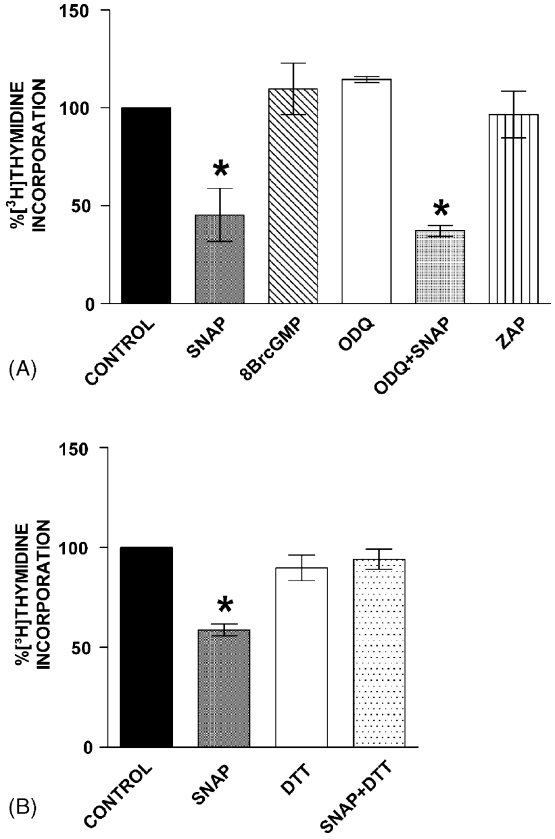

Fig. 3. Effect of cyclic GMP-related drugs and DTT on [^{3}H]-thymidine incorporation in retinal cells in cultures. Cultures were incubated for 24 h with SNAP (1 mM), 8Br-cGMP (0.5 mM), ODQ (5 μM), SNAP + ODQ, or zaprinast (ZAP, 10 μM) in (A) and SNAP (100 μM), dithiotreitol (DTT, 100 μM) or SNAP + DTT in (B) and then washed and incubated with 1 μCi of [^{3}H]-thymidine for 1 h at 37 °C. Data represent the mean ± S.E.M. of three to four separate experiments performed in duplicate or triplicate. The asterisks indicate $P < 0.001$. Control values in cpm/well were 4065.2 ± 1036.2 (A) and 1465.9 ± 74.4 (B).

dithiotreitol (Park et al., 2000; Garbán et al., 2005). In order to verify if the observed inhibition of cell proliferation by SNAP was mediated by *S*-nitrosylation of proteins, we incubated cultures with SNAP in the absence or presence of DTT. As observed in Fig. 3(B), while DTT alone had no effect on cell proliferation this treatment completely prevented the inhibition of [^{3}H]-thymidine incorporation by SNAP.

An event involved in cell cycle arrest of smooth vascular muscle is the inhibition of the enzyme ornithine decarboxylase by NO (Ignarro et al., 2001, 2002). ODC catalyses the synthesis of putrescine, a polyamine which, as spermine and spermidine, is involved in cell proliferation (Wei et al., 2001). In order to investigate if the reduction of retinal cell proliferation induced by NO occurred by mechanisms involving ODC, we incubated the cultures with difluoromethylornitine (DFMO), a blocker of ODC. Both DFMO (1 mM) or putrescine (100 μM) did not have detectable effect on [^{3}H]-thymidine incorporation or on the inhibition mediated by SNAP (data not shown). These results discard the participation of this metabolic pathway in the regulation of cell proliferation in our model.

3.4. [^{3}H]-Thymidine autoradiography

In order to characterize the cell type proliferating in mixed cultures, we performed experiments using [^{3}H]-thymidine autoradiography. As shown in Fig. 4(A, B and E), [^{3}H]-thymidine corresponding autoradiographic grains could be observed over large oval nuclei of flat cells localized under neurons, suggesting that those labeled cells represented the glial population in the cultures. Cultures treated with SNAP have shown a complete absence of autoradiographic labeling (Fig. 4(C and D)). In agreement with these observations, incubation of purified cultures of glial cells with 100 μM SNAP promoted a decrease of [^{3}H]-thymidine incorporation (Fig. 5(A)), corroborating the idea that glial Müller cells are the targets of the anti-proliferative effects of NO.

3.5. Effect of SNAP on ERK phosphorylation

The MAP kinase cascade is important for cell proliferation, diferentiation and apoptosis (Oh-Hashi et al., 1999). In order to verify the effect of SNAP on ERK 1/2 activity, we performed experiments measuring the phosphorylation of ERK 1/2 in purified cultures of glial cells treated with the NO donor. The results shown in Fig. 5(B) demonstrate that SNAP increases the phosphorylation of ERK1/2 in our cultures, indicating that inhibition of ERK 1/2 phosphorylation is not directly involved in the anti-proliferative effects of NO. Moreover, previous work have shown that ATP stimulates cell proliferation in chick retinal cultures through a MAP kinase-dependent pathway (Sanches et al., 2002). As shown in Fig. 6, SNAP (1 mM) abolished the increase of [^{3}H]-thymidine incorporation induced by ATP (100 μM).

3.6. Inhibition of [^{3}H]-thymidine incorporation by SNAP "in vivo"

To verify whether the effect of SNAP on cell proliferation is also present "in vivo", we performed experiments injecting this NO donor into the air chamber of eggs containing E8 embryos. Twenty four hours later E9 embryos were removed, their retinas dissected, incubated with [^{3}H]-thymidine and processed for measurement of incorporation. As observed in Fig. 7, SNAP produced a decrease of approximately 50% of [^{3}H]-thymidine incorporation.

4. Discussion

In the present work we show that NO is a modulator of cell proliferation in the embryonic chick retina. The NO donors SNAP or SpNO were able to inhibit [^{3}H]-thymidine incorporation at the intact retina or in cultures of retinal cells, in a dose-dependent fashion. SNAP was more potent than SpNO, the latter showing a small toxic effect at high concentrations. These effects probably reflect distinct chemical features of NO donors such as kinetics of NO release or NO-reactive species generated, since they belong to the distinct nitrosothyol or NONOate classes of NO donors, respectively.

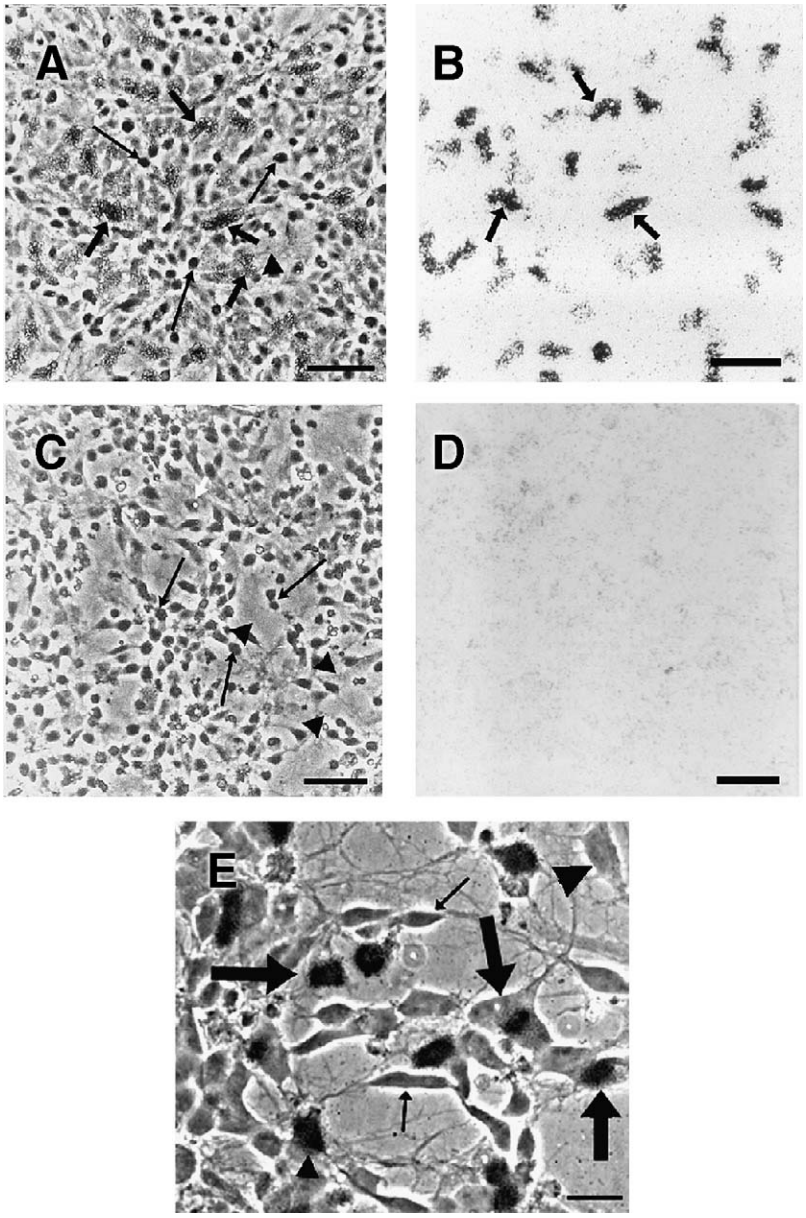

Fig. 4. Autoradiography of [^{3}H]-thymidine incorporation in mixed chick retina cells. (A–B) Phase contrast and correspondent bright field micrographs showing a dark stain on flat cells (short arrows). Unlabeled flat cells (arrowhead) and unlabeled neurons (long arrows) can also be observed. (C–D) Phase contrast and correspondent bright field micrographs of SNAP-treated cultures showing the virtual absence of autoradiographic grains. Arrow heads point to flat cells and long arrows to neurons. A white arrow in figure (C) point to a photoreceptor. (E) High power photomicrograph showing details of extensive labeling of flat glial cells (large arrows) and virtual absence of labeling on neurons and photoreceptors. Scale bars = 100 μm in (A–D) and 20 μm in (E).

The use of different donors such as SIN-1 or sodium nitroprussiate promoted cell death in retinal cultures (not shown) probably due to the generation of peroxynitrite or cyanide by these compounds (see Khan et al., 1997; Holm et al., 1998). Endogenous NO seems not to be generated in sufficient amounts in the cultures at the developmental stages studied, since treatment of mixed cultures with NARG, a NOS inhibitor, had no effect on cell proliferation.

4.1. Effect of NO is cGMP-independent

One possible mechanism of action of NO would be the elevation of cGMP levels after stimulation of endogenous guanylil cyclase activity. However, no change in [^{3}H]-thymidine incorporation was observed when the permeable cGMP analog 8Br-cGMP or the guanylil cyclase inhibitor ODQ were added to the cultures. Furthermore, a small but yet not significative decrease was observed after incubation of cells with the specific cGMP phosphodiesterase inhibitor zaprinast, but this effect was accompanied by an increase of LDH release (data not shown). These results corroborate the idea that the effect of NO was not due to increase of cGMP levels. The NO donor SNAP also modulates the release of amino acids from the chick embryo retina by a cyclic GMP-independent mechanism (Ientile et al., 1996b).

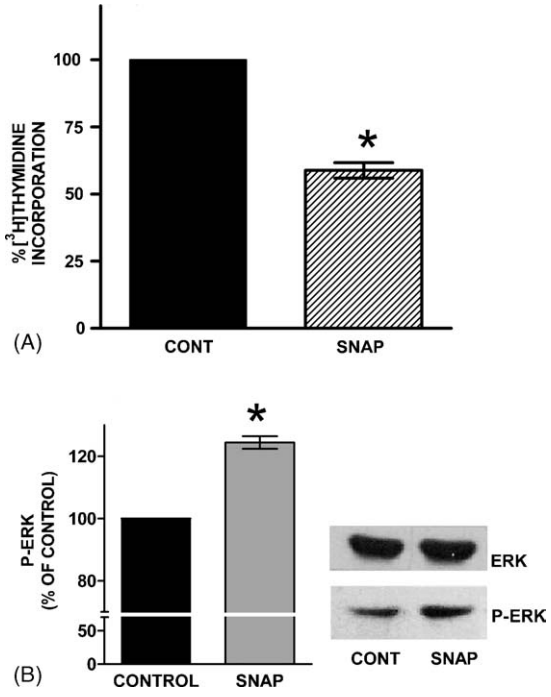

Fig. 5. Inhibition of [^{3}H]-thymidine incorporation and increase of ERK phosphorylation by SNAP in purified glial cultures. (A) Purified glial cell cultures were incubated for 24 h with SNAP (100 μM) and submitted to [^{3}H]-thymidine incorporation as described in the experimental procedures. Data are expressed as means ± S.E.M. of three experiments performed in duplicate. (B) Western blot staining of phospho-ERK 1/2 in purified glial cell cultures incubated for 24 h in the absence or presence of 100 μM SNAP. Left panel shows the densitometric analysis of blots obtained form three separate experiments. The asterisks indicate $P < 0.01$.

4.2. Effect of NO does not involve the ERK1/2 pathway

The blockade of the MAP kinase cascade is known to inhibit cell proliferation in some cell populations (Horne and Guadagno, 2003). Previous work have shown that ATP increases cell proliferation in chick retinal cultures and that this effect is related to stimulation of ERK 1/2 phosphorylation

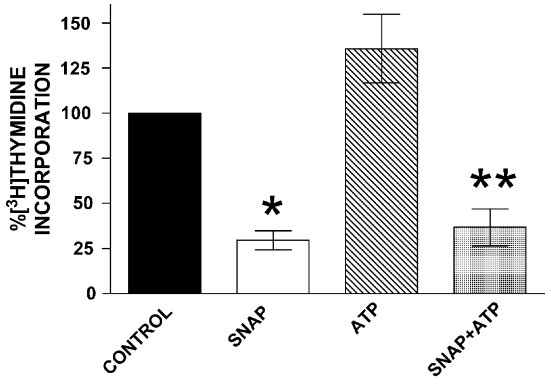

Fig. 6. Effect of SNAP on the increase in [^{3}H]-thymidine incorporation promoted by ATP in mixed cultures. Cultures were treated with SNAP (1 mM), ATP (100 μM) or SNAP + ATP for 24 h and incubated with 1 μCi [^{3}H]-thymidine for 1 h at 37 °C. Data are expressed as mean ± S.E.M. from three separate experiments. Asterisks indicate statistical significance: $^*P < 0.01$ compared to control, $^{**}P < 0.001$ when ATP was compared with ATP + SNAP.

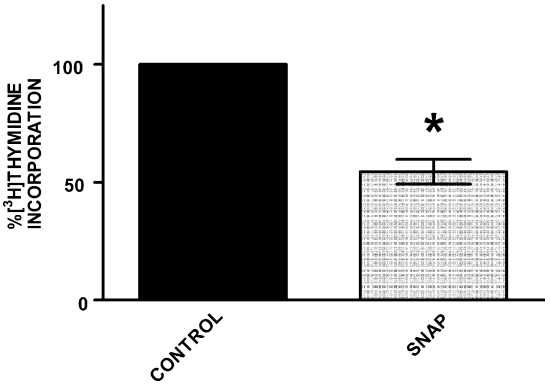

Fig. 7. Effect of SNAP on [^{3}H]-thymidine incorporation in vivo. Chicken eggs with E8 embryos were injected in the air chamber with 100 μM SNAP or vehicle (control eggs). After 24 h (E9), retinas were dissected and used for the assay of [^{3}H]-thymidine incorporation. Data were normalized for the protein content of each retina and the amount of input radioactivity. The results are expressed as percent of control ± S.E.M. from three separate experiments. The asterisk indicates $P < 0.05$.

(Sanches et al., 2002). The present work shows that NO promotes a decrease of both basal and ATP-induced proliferation of cells in these cultures. A similar effect was previously observed with the MEK inhibitor PD98059 (Sanches et al., 2002). However, the effect on retinal cell proliferation does not seem to involve inhibition of this pathway, since NO increases ERK activation in purified cultures of glial cells. One possibility is that NO-mediated activation of MAP kinase cascade is involved in other physiological events such as survival and differentiation (Hardingham et al., 2001; Lee et al., 2002). Indeed, our preliminary results show that the effect of SNAP on ERK activation is cGMP-dependent since it is mimicked by zaprinast and blocked by ODQ (data not shown). Other recent observations indicate that the effect of NO on cell survival in purified neuronal cultures involves a cyclic GMP-PKG-ERK cascade (Mejía-García, T.A., and Paes-de-Carvalho, R. unpublished results).

4.3. Possible involvement of S-nitrosylation of proteins

Several lines of evidence indicate that NO is also able to promote post-translational modifications in proteins such as *S*-nitrosylation and nitration (Hess et al., 2005). Another interesting modification of proteins by NO occurs after the intracellular formation of *S*-nitrosoglutathione and *S*-glutathionylation of cysteine residues of proteins as for example, glyceraldeyde-3-phosphate dehydrogenase (Mohr et al., 1999). Among the proteins modified by *S*-nitrosylation are important transcription factors as NFkB (Marshall et al., 2004), calcium channels (Ma et al., 1999), or the enzyme ODC. The inhibition of ODC was observed in smooth muscle cells (Wei et al., 2001) decreasing the levels of polyamines, as well as cell proliferation, an event that is reverted by the addition of putrescine. This alternative was ruled out by the finding that DFMO, an inhibitor of ODC, was not able to inhibit cell proliferation in our cultures. However, addition of DTT completely blocked [^{3}H]-thymidine

incorporation in the cultures, strongly suggesting that the effect of NO donors are mediated by *S*-nitrosylation. If the anti-proliferative effect observed in our experiments involves *S*-nitrosylation of some specific proteins and activation or inhibition of different transduction pathways deserves further investigation.

4.4. Glial cells are the target for the anti-proliferative effect of NO

At E8, the chick retina has few neuronal elements within the mitotic cycle, but the Müller glia cells are proliferating and differentiating (Prada et al., 1991; Mey and Thanos, 2000). This cell type grows in mixed cultures as flat cells and adhere to the dish surface in order to form a substrate for neurons, which are small and branched cells. [^{3}H]-Thymidine autoradiography showed the presence of grains over the nucleus of these flat cells and no grains were observed in cultures treated with SNAP. These data, summed to previous knowledge, indicate that the target of NO in the cultures is the population of Müller glial cells characterized by morphological criteria. This conclusion is corroborated by the experiments showing that SNAP promotes inhibition of [^{3}H]-thymidine incorporation in purified cultures of glial cells.

4.5. Development and the regulation of cell proliferation

Cell proliferation is a fine-tuning controlled event during development of the nervous system. However, at a given stage of development, cells undergo asymmetrical division, start to differentiate in several cell types and migrate to their final positions in the tissue. After these events, neuronal precursor cells cease division (for review see Sommer and Rao, 2002). One possibility is that NO generated at early embryonic stages is able to inhibit the proliferation of specific cell populations, with the aim to restrict cell number and favor cell differentiation. Indeed, neuritogenesis is stimulated by NO in PC12 cells after cell cycle arrest (Peunova and Enikolopov, 1995) and an increase of cell number and tissue growth is found after inhibition of NOS activity in Drosophila embryos (Kuzin et al., 1996). A recent study have shown a correlation between the localization of NO-forming cells and decrease in the number of mitotic cells in neighboring areas of the developing nervous tissue (Peunova et al., 2001). In the present work we did not show any evidence that NO regulates the proliferation of neuronal precursor cells, since our studies were restricted to cells obtained from E8 embryos. At this stage, neurons are essentially postmitotic and the only cells to divide in culture are the glial cells. However, our results showing that injection of eggs with SNAP decreases [^{3}H]-thymidine incorporation "in vivo" raise the possibility that this effect is also present in the intact developing retina. Further experiments are necessary to check if the target cell population in the retina is also composed of glial cells. Since NOS activity is high in E8 retinas (Ientile et al., 1996a; Paes-de-Carvalho and Mattos, 1996), it would also be interesting to see if NOS inhibitors could interfere with glial cell proliferation "in vivo".

Many studies show that glial cells are important partners during central nervous system development (Hertz et al., 2001; Fischer and Reh, 2002), and a physiological balance between neuronal and glial metabolism is essential for the functioning of important neurotransmitter systems (Taylor et al., 2003). Glial–neuronal interactions are also necessary for NO metabolism, since L-arginine provided by glia is taken up by neurons and used for NO synthesis (Cossenza and Paes-de-Carvalho, 2000; Grima et al., 2001). Our results have shown that inhibition of NOS with NARG was not able to promote significant effects on [^{3}H]-thymidine incorporation, meaning that NO produced by neurons is not synthesized in sufficient amounts to inhibit proliferation of glial cells in our mixed cultures at the period studied. However, another interesting possibility is that NO regulates glial cell proliferation after CNS trauma or neurodegenerative diseases (Garg et al., 1992). The induction of iNOS in glial cells after citokine or lipopolysacaride exposure promotes the synthesis of large amounts of NO that in turn regulates proliferation of the same or neighbour cells. Preliminary experiments in our laboratory show that lipopolysaccharide also induces cell division arrest in retinal cultures (Coutinho, R.N., Cossenza, M. and Paes-de-Carvalho, R., unpublished observations).

In conclusion, the present study showed that NO is a potent regulator of mitosis of cultured retinal glial cells. Together with previous studies showing that NADPH diaphorase, which corresponds to NOS activity in the retina (de Faria et al., 1995), is present at high levels at early embryonic stages of chick retinal development (Ientile et al., 1996a; Paes-de-Carvalho et al., 1996), and that a high affinity transport system for L-arginine and neuronal NOS are found in embryonic retina cells in culture (Cossenza and Paes-de-Carvalho, 2000), the present results support an important role for NO as a signaling molecule during retinal development.

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