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Int. J. Devl Neuroscience 24 (2006) 53–60

INTERNATIONAL JOURNAL of DEVELOPMENTAL NEUROSCIENCE

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

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Received 28 July 2005; received in revised form 21 October 2005; accepted 26 October 2005

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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doi:10.1016/j.ijdevneu.2005.10.004

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