AJRI 2000; 43:70–77
Printed in Ireland - all rights reserved.

American Journal of Reproductive Immunology
ISSN 8755-8920

Localization of Nitric Oxide Synthase in Human Trophoblast Cells: Role of Nitric Oxide in Trophoblast Proliferation and Differentiation

MRINMOY SANYAL, TAPAS CHANDRA NAG, AND CHANDANA DAS

Sanyal M, Nag TC, Das C. Localization of nitric oxide synthase in human trophoblast cells: Role of nitric oxide in trophoblast proliferation and differentiation. AJRI 2000; 43:70–77 © Munksgaard, Copenhagen

PROBLEM: There are conflicting reports about the isoform of nitric oxide synthase (NOS) present in trophoblast cells. In this study, we have examined the presence of different NOS isoforms in trophoblast cells. In addition, the role of nitric oxide (NO) in trophoblast function has also been studied by investigating the possible role of nitric oxide in trophoblast proliferation and differentiation.

METHOD OF STUDY: NOS isoforms in primary-term trophoblast and JEG-3 cells were identified by immunocytochemistry. The intracellular localization of this enzyme was determined by confocal laser scanning microscopy. Trophoblast proliferation was studied by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasolium bromide (MTT) conversion assay and cellular differentiation was monitored by human chorionic gonodotropin (hCG) and progesterone secretion, measured by radioimmunoassay.

RESULTS: The immunoreactive NOS was present in human trophoblast cells of normal term placenta and JEG-3 cells (a choriocarcinoma cell line) maintained in culture. Nicotinamide adenine dinucleotide phosphate (NADPH)-dependent diaphorase activity overlapped with the immunostaining of NOS. Specific antibodies against the different isoforms of NOS detected the presence of neuronal-type NOS (nNOS) only. The other two isoforms, i.e., eNOS (endothelial) and iNOS (macrophage specific) were completely absent. The nNOS was localized in cell cytoplasm. In culture, JEG-3 cells normally undergo proliferation and cytotrophoblast cells in primary culture differentiate to form hormone-secreting syncytial cells. Sodium nitroprusside (SNP), a nitric oxide donor, when added to the culture, significantly increased proliferation of JEG-3 cells and inhibited the differentiation of cytotrophoblast cells. The arrest by SNP in the formation of syncytial cells was further evidenced by the low secretion profile of hCG and progesterone.

CONCLUSIONS: Our findings suggest for the first time the presence of nNOS in the human trophoblast cells and a previously unrecognized role of NO in trophoblast proliferation and differentiation.

Key words:
cell proliferation, differentiation, hCG, nitric oxide, nitric oxide synthase, trophoblast

CHANDANA DAS
MRINMOY SANYAL
Department of Biochemistry, All India Institute of Medical Sciences, New Delhi, India

TAPAS CHANDRA NAG
Department of Anatomy, All India Institute of Medical Sciences, New Delhi, India

Address reprint requests to Prof. Chandana Das, Head, Department of Biochemistry, All India Institute of Medical Sciences, New Delhi-110029, India.

E-mail:
chandana@medinst.ernet.in

Submitted January 17, 1999; accepted April 28, 1999.

INTRODUCTION

Nitric oxide (NO) is a messenger molecule that in recent years has been implicated in a variety of cellular functions in neuronal and non-neuronal tissues. The substrates of NO synthesis, L-arginine and molecular oxygen, are converted by the enzyme nitric oxide synthase (NOS) to citrulline, and NO, an extremely labile and freely diffusible compound across the cell membrane, which acts in a paracrine manner on the adjacent structures. NOS and its product NO are shown to have modulatory roles on myoblast differentiation and acquisition of properties of skeletal muscle.[1] It has also been shown to amplify fibroblast growth factor-induced mitogenesis of the aortic smooth muscle cells.[2] Nerve Growth Factor (NGF) has been demonstrated to act through NOS-NO system to induce terminal differentiation of neuronal cells.[3] Recent studies show that NO can play multiple roles affecting bone marrow cell growth and differentiation. It also mediates increased blood flow to the marrow under haemopoeitic stress.[4]

Several isoforms of NOS have been characterized, notably nNOS (from brain), iNOS (from macrophages), and eNOS (from endothelium). Conrad et al.[5] have reported the presence of calcium-calmodulin sensitive NOS located mainly in the microsomal cell fraction of human term placental villi.[5] By *in situ* hybridization using a riboprobe generated from human endothelial NOS cDNA, they have further observed expression of NOS in the syncytiotrophoblast layer of the term villous placenta. The immunocytochemical studies conducted by Eis et al.[6] on term syncytiotrophoblasts formed in culture by differentiation of a pure population of cytotrophoblast cells, however, did not show immunostaining for eNOS in majority of term syncytial aggregates, but were positive in syncytial cells from early placenta. The main objectives of the present work, therefore, were to identify the NOS isoform that is expressed in the pure population of term trophoblast cells as well as JEG-3 cells, a human trophoblast-derived choriocarcinoma cell line maintained in culture, and to examine the effect of NO on trophoblast proliferation and differentiation.

MATERIALS AND METHODS

Cell Culture

Human term placenta obtained from spontaneous vaginal delivery of healthy women were collected in normal saline and processed aseptically. Villous tissues were sampled from several cotyledons and rinsed extensively in normal saline to remove excess blood. Cells were isolated by differential trypsinization (0.125 g % of trypsin 1:250 [Life Technologies, Grand Island, New York, USA] and 0.01% DNase [Sigma, St Louis, MO, USA]) for 3×30 min in calcium, magnesium free Hank's balanced salt solution containing penicillin (100 IU/mL) and streptomycin (100 μg/mL). The heterogeneous cell population was subjected to discontinuous Percoll (Pharmacia, Uppsala, Sweden) gradient (5–70%) centrifugation. The band corresponding to the density of 1.048–1.058 was 95% pure trophoblast cells.[7] These cells were plated onto 16-mm, 24-well tissue culture plates (Costar, Acton, MA, USA) at a density of 1×10^6 cells/well/mL in Dulbecco's Modified Eagle's Medium-High Glucose (Life Technologies, USA) containing 10% fetal bovine serum and the antibiotics. The plates were maintained at 37°C in sterile humid atmosphere for 4 days with a daily change of medium. For cytochemical, immunocytochemical, and confocal studies, cells were cultured on 12-mm coverslips.

JEG-3 cell line was purchased from American Type Culture Collection (Parksville, MA, USA). They were expanded in tissue culture flask in Ham's nutrient mixture F12 (Life Technologies, USA) with 15% fetal calf serum (FCS) and the antibiotic/antimycotic solution. Cells were harvested by trypsinization (0.25 g % trypsin, 0.03 g % ethylenediaminetetraacetic acid in phosphate-buffered saline [PBS]), counted and plated onto 16-mm, 24-well tissue culture plates (Costar) at an initial density of 5×10^4 cells/mL/well for quantitating their hormone secretion profile and 5×10^3 cells in 96-well plates for proliferation assay.

Diaphorase Staining

Cells grown on coverslips were fixed in 0.02% glutaraldehyde, washed twice in PBS, and then incubated in solution consisting of 2 mM β-nicotinamide adenine dinucleotide phosphate (NADPH), 0.02% nitroblue tetrazolium, and 0.3% Triton X-100 in 0.1 mM Tris buffer, pH 7.2, for 1 hr at 37°C.[3] In control incubation, β-NADPH was omitted. After incubation, coverslips were washed in PBS, dehydrated, cleared, and mounted in DPX.

Immunocytochemistry

Cells grown in monolayer on coverslips were fixed in 4% paraformaldehyde in PBS for 10 min. The cells were initially blocked with goat normal serum (for anti-nNOS staining) or horse normal serum (in case of anti-eNOS and anti-iNOS staining) for 1 hr and then treated with primary antibodies against nNOS (1:1000 dilution; rabbit polyclonal; Transduction Laboratories, Lexington, KY, USA), eNOS (1:2000 dilution; mouse monoclonal; Transduction Laboratories), and

iNOS (1:500 dilution; mouse monoclonal; Transduction Laboratories) for 4 hr at room temperature. The antibody dilutions were made in 0.01 M PBS, pH 7.4, containing 0.5% Triton X-100 and 10% normal serum. After washing, the cells were incubated with secondary antibody (goat anti-rabbit immunogloublin (Ig) G for nNOS, 1:200 dilution, and horse anti-mouse IgG for both eNOS and iNOS, 1:200 dilution; Vector Laboratories Burlingame, CA, USA) containing 5% normal serum for 2 hr at room temperature. Following this, the cells were incubated in avidin-biotin peroxidase complex (1:100 dilution; Vector Laboratories, Burlingame, CA, USA) in PBS for 1 hr at room temperature. For visualization of antigen-antibody complex, the cells were treated with 0.03% diaminobenzidine tetrahydrochloride (Sigma, St Louis, MO, USA), 0.5% nickel sulphate, and 0.3% hydrogen peroxide in acetate-imidazole buffer (pH 7.4) for 30 s. Finally, the coverslips were rinsed in phosphate buffer, dehydrated in ethyl alcohol, cleared, and mounted onto slides in Dibutyl Phythalate Polystyrene Xylene (DPX).

Confocal Microscopy

Cells cultured on coverslips were fixed and processed as described in Immunocytochemistry. The antigen-antibody complex formed were visualized by using fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (1:200 dilution; Reagent Bank, National Institute of Immunology, New Delhi). The coverslips were finally mounted in glycerol. For confocal laser scanning microscopy, a BioRad MRC-1024 laser scanning confocal imaging apparatus equipped with argon/krypton laser coupled to a Zeiss Axiophot microscope was used. Images were stored as BioRad Lasersharp*.PIC file and photographed in Pan-Ilford 100 b/w film using BioRad Imagecorder. Images of 0.5-μ thick serial sections were acquired using blue line.

Cell Proliferation Assay

Cells in 96-well plates were challenged with increasing concentrations (0.625–40 μM) of freshly prepared sodium nitroprusside (SNP) dissolved in medium such that the total culture volume was only 100 μL. After 48 hr of challenge, 10μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasolium bromide (MTT) reagent (Cell proliferation Kit I; Boehringer Mannheim, Germany) was added according to manufacturer's instruction. Four hours after addition of MTT, 100 μL of solubilizer was added to each well. Plates were kept overnight in humidified chamber with 5% CO_2. Absorbance was read by microtiter plate-reader (Anthos HT-II) at 550 nm taking 690 nm as reference. Data were expressed in terms of percent of control.

Bromodeoxy Uridine (BrdU) Incorporation and Flow Cytometry

Cells grown on 25 mm^2 tissue culture flasks were challenged with 10 μM SNP. After 48 hr of challenge, the medium was removed from the flask, washed once with medium, replaced with fresh medium, and incubated with 30 μg/mL BrdU for 1 hr at 37°C in humidified atmosphere with 5% CO_2. Finally, cells were harvested by trypsinization and dispersed thoroughly by repeated pipetting. The cell suspension was transferred to 1.5 mL microfuge tube and briefly centrifuged. The supernatant containing trypsin solution was discarded. The cell pellet was washed twice with 0.1 M PBS and fixed with 4% paraformaldehyde in 0.1 M PBS for 1 hr at 4°C. Non-specific sites were blocked with 10% normal horse serum containing 0.5% Triton X-100 in 0.1 M PBS for 30 min, following which, the cells were incubated with 100 μL of undiluted anti-BrdU antibody for 1 hr at room temperature according to the manufacturer's instruction. At the end of the incubation with the primary antibody, cells were washed thrice with PBS and incubated with FITC-conjugated horse anti-mouse IgG at 1:200 dilution for 30 min at room temperature. After washing, cells were incubated with 10 μg/mL propidium iodide containing 100 U RNase A for 30 min at room temperature. They were then washed three times with PBS and resuspended in 1 mL PBS. The cell-associated fluoresence was acquired in a Coulter FACS® apparatus. A total of 10,000 cells were acquired. Data analysis and graphics generation was done using WinMDI software. Data was expressed in terms of mean peak value.

Assay of Human Chorionic Gonadotropin and Progesterone

Term trophoblast cells as well as JEG-3 cells maintained in culture in 24-well plates were challenged with SNP (10 μM) for 24 hr in serum free medium. The collected spent media were assayed for progesterone and human chorionic gonodotropin (hCG) by radioimmunoassay following the WHO protocol and using the National Matched Assay Reagents of the Indian Council of Medical Research, New Delhi. Results of the assay were calculated by a special software 'RiaCalc RM, version 2.69 by Wallac Inc.' obtained from Pharmacia, Sweden. Secretion levels of hCG and progesterone were expressed in terms of total cellular protein level.[8]

RESULTS

Diaphorase Staining

Cytotrophoblast, syncytiotrophoblast, and JEG-3

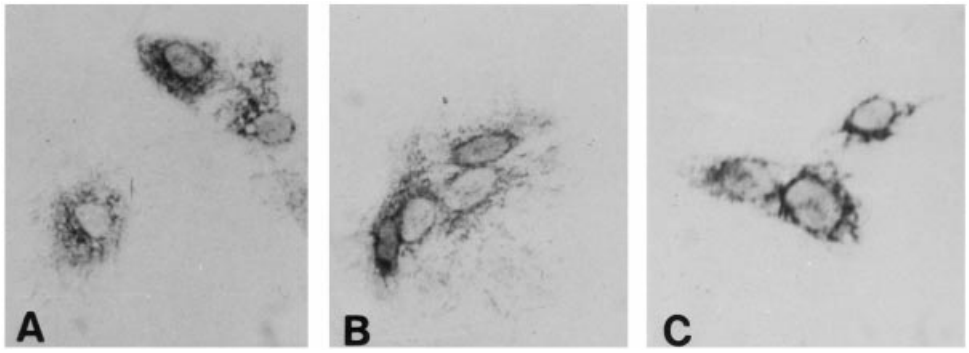

Fig. 1. Diaphorase staining of cultured trophoblast cells. **A**, cytotrophoblast; **B**, syncitiotrophoblast; **C**, JEG-3 cell.

cells exhibited intense diaphorase staining (Fig. 1A–C). Control experiments demonstrated that this reaction was NADPH dependent. Diaphorase staining remained unchanged when the cells received 10% FCS in addition to the incubation mixture (data not shown).

Detection of NOS Isoforms in Trophoblast Cells

Immunostaining for different isoforms of NOS revealed the presence of nNOS in cytotrophoblast cells (Fig. 2A) at 24 hr of culture as well as in syncytiotrophoblast cells (Fig. 2B) at 96 hr of culture, and also in JEG-3 cells (Fig. 2C). Anti-eNOS (Fig. 2E, F) and

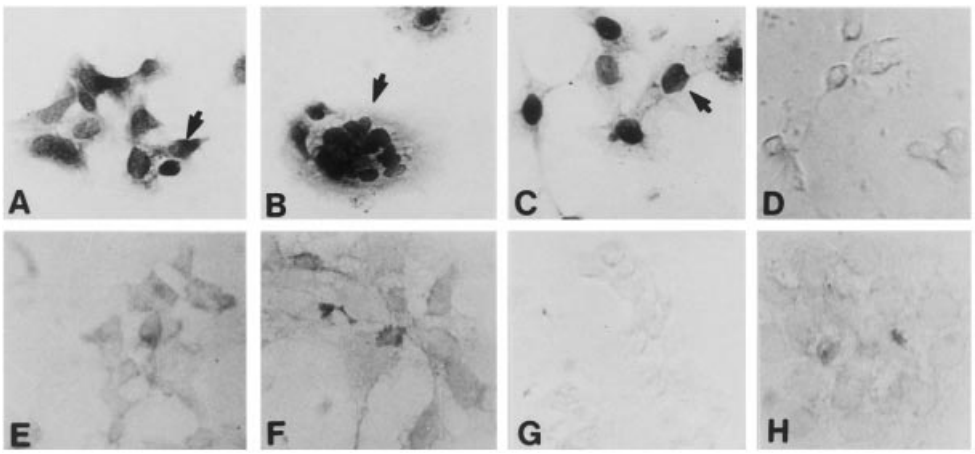

Fig. 2. Immunocytochemical localization of NOS isoforms expressed in cultured trophoblast cells (**A**–**C**) showing nNOS immunoreactivity in cytotrophoblast, syncitiotrophoblast and JEG-3 cells, respectively. **D**, control staining in cytotrophoblast cells omitted with anti-nNOS primary antibody. **E** and **F,** showing eNOS immunoreactivity in cytotrophoblast and JEG-3 cells, respectively. **G** and **H**, showing iNOS reactivity in cytotrophoblast and JEG-3 cells. *Arrow* indicates strong immunostaining.

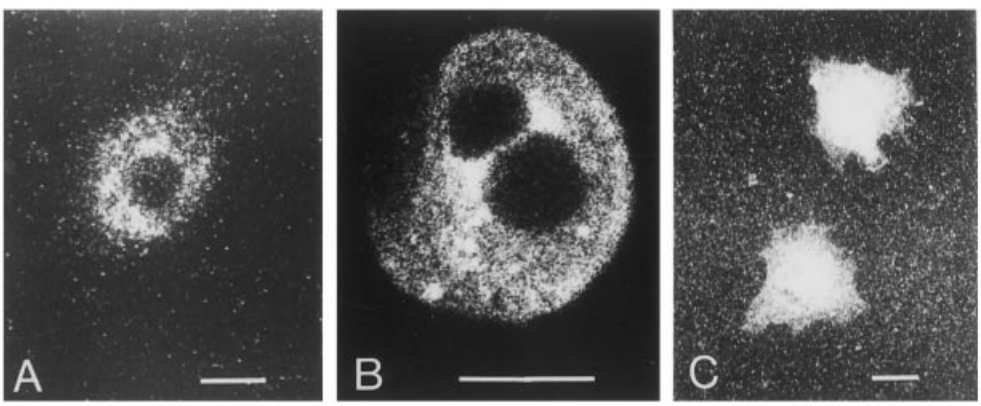

Fig. 3. Confocal images of cells stained with anti-nNOS antibody. **A**, cytotrophoblast; **B**, syncytiotrophoblast; and **C**, JEG-3 cells. The horizontal bar represents 10 μM. Note that in all cells nNOS is localized in cytoplasm.

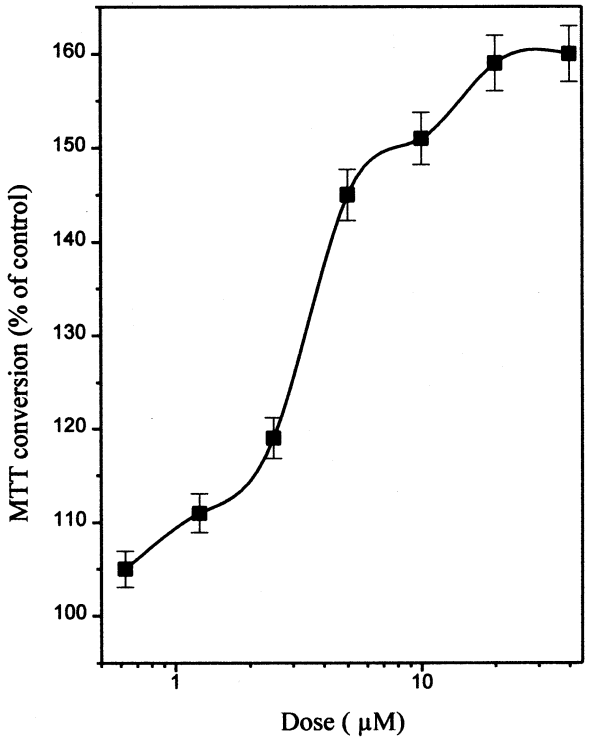

Fig. 4. Effect of SNP on proliferation of JEG-3 cell line. In culture, increasing concentration of SNP causes increased JEG-3 cell proliferation. Note that even at a dose of 40 μM SNP induces JEG-3 cell proliferation.

anti-iNOS (Fig. 2G, H) did not stain these cells. Confocal microscopic studies further revealed cytoplasmic localization of nNOS in cytotrophoblast (Fig. 3A), syncytiotrophoblast (Fig. 3B), and also in JEG-3 cells (Fig. 3C).

Effect of SNP on Trophoblast Proliferation and Differentiation

SNP enhanced proliferation of JEG-3 cells in a dose-dependent manner as observed by MTT conversion assay (Fig. 4). Challenge of JEG-3 cells with SNP increased BrdU labeled cells as compared to control cells (Fig. 5). The mean fluoresence of control cells was 26.41 ± 2.63, which increased to the level of 68.87 ± 4.33 after challenge with 10 μM SNP. The difference in mean was significant at a P value of 0.01.

SNP also inhibited the normal aggregation and fusion of cytotrophoblasts to form syncytial cells (Fig. 6A, B). The inhibitory effect of SNP on the differentiation of cytotrophoblast cells in culture was further evident from the low secretion levels of hCG and progesterone. The decrease was highly significant ($*P \leq 0.001$) at 72, 96, and 120 hr in primary culture and 72 and 96 hr in JEG-3 culture (Fig. 7A, B). Similar inhibition was observed in the progesterone secretion (Fig. 8A, B).

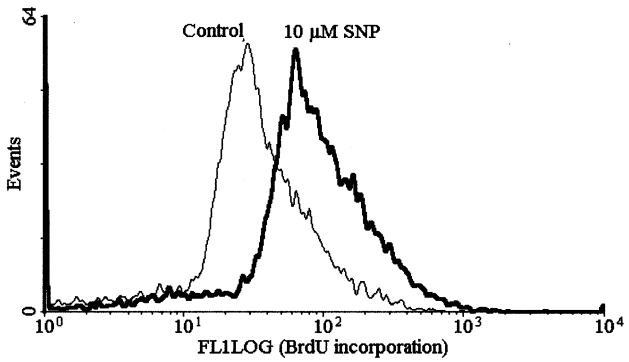

Fig. 5. Flow cytometric analysis of the effect of SNP on BrdU incorporation by JEG-3 cells. Challenge with 10 μM SNP shifted the mean fluorescence intensity from the control level of 26.41 ± 0.31 to 68.87 ± 4.33.

DISCUSSION

The data presented in this paper are intended to provide evidence for the presence of NOS isoform in a pure population of full-term trophoblast cells maintained under culture conditions and the role of NO in trophoblast proliferation and differentiation. Since NOS bears sequence homology to NADPH diaphorase, in preliminary experiments we have used NADPH diaphorase staining to localize NOS in trophoblast cells. Both undifferentiated cytotrophoblast cells and differentiated syncytiotrophoblast cells, as well as JEG-3 choriocarcinoma cells, stained for diaphorase activity (Fig. 1). The fact that the activity was not altered by the addition of serum suggests the presence of a constitutive form of NADPH-dependent reductase in the trophoblast cells. Although discrepancies in the staining pattern of diaphorase and NOS

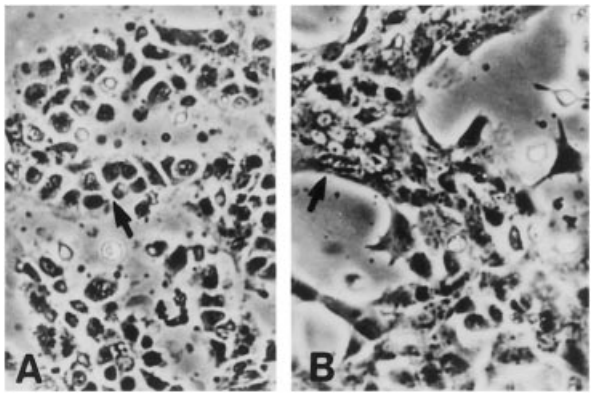

Fig. 6. Effect of SNP on trophoblast differentiation. Trophoblast cells were challenged with SNP (10 μM) at 48 hr of culture and phase contrast photomicrographs were taken at 72 hr of culture. **A**, cells challenged with SNP; **B**, cells without SNP challenge. Note that addition of SNP in culture stopped aggregation and subsequent fusion (*arrow* in **A**) of trophoblast cells. *Arrow* in **B** indicates fusion of cells.

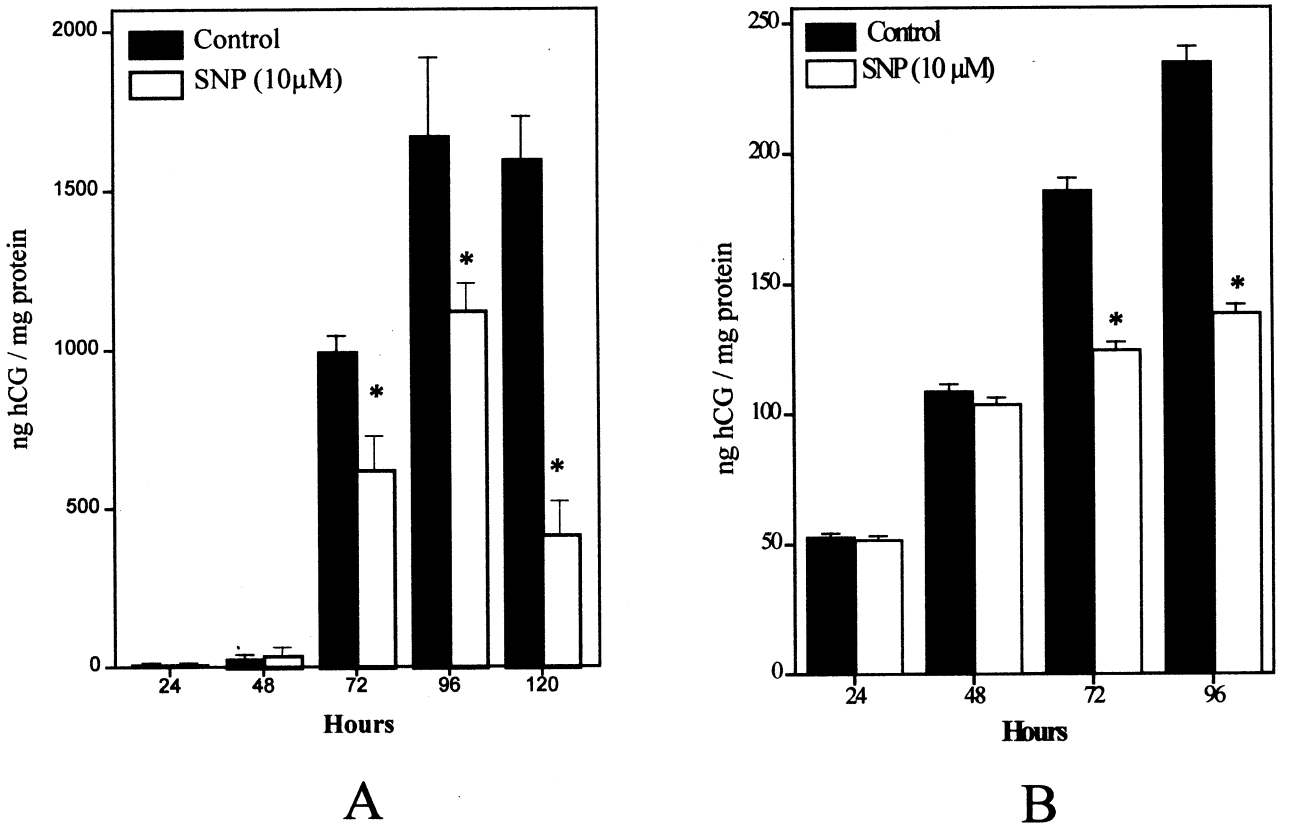

Fig. 7. Effect of SNP on hCG secretion by trophoblast cells. **A**, effect of SNP on hCG secretion by primary trophoblast cells. *Solid bar* indicates control (without addition of SNP) and *hollow bar* indicates hCG secretion after addition of SNP (10 μM). Note that SNP significantly (*$P \leq 0.001$) reduced hCG secretion at 72, 96, and 120 hr of culture. **B**, effect of SNP on hCG secretion by JEG-3 cells. *Solid bar* indicates control and *hollow bar* indicates hCG secretion after addition of SNP (10 μM). As in **A**, a similar kind of effect is observed at 72 and 96 hr of JEG-3 culture where SNP significantly (* $P \leq 0.001$) reduced hCG secretion (**B**).

has been reported,[9] a direct link between the two has also been suggested by others.[10,11] Our data show that the NOS immunoreactivity and NADPH-diaphorase staining are colocalized in the term trophoblast cells and JEG-3 choriocarcinoma cells maintained under culture conditions (Figs. 1 and 2). We have further tried to identify the isoforms of NOS present in these cells. Strong immunoreactivity was observed only with the neuronal type of NOS (Fig. 2A–C). Antibodies specific to eNOS and iNOS did not show any immunoreactivity, indicating their absence in the trophoblast cells (Fig. 2E–H). The two antibodies, however, showed positive immunostaining in the endothelial cells and macrophages, respectively (data not shown). It may be possible that as these cells were a pure population of trophoblast cells without any contamination of endothelial cells or macrophages; immunoreactivity for eNOS was not observed, as has been reported by others, using placental tissue sections.[5,9] In a pure population of cytotrophoblast cells from early placenta, Eis et al.[6] did not observe any immunoreactivity for eNOS. Diffused immunostaining for eNOS was observed by them in some but not in all syncytial cells formed in culture from the cytotrophoblast cells of early placenta, but the term syncytial aggregates formed in culture were negative for eNOS. Constitutive nNOS as well as iNOS are cytosolic enzymes,[12–16] whereas eNOS is present in the particulate fraction[17] associated with membranes.[18] The cytosolic localization of nNOS observed in our studies with confocal microscopy further substantiates the presence of the particular isoform in the trophoblast cells. The physiological significance of the differential subcellular localization of the isoforms is, however, not yet fully understood.

In the placental tissue, trophoblast cells are surrounded by endothelial cells as well as macrophages, all of which express different isoforms of NOS leading to the local generation of NO. It has been proposed that placenta-derived NO plays a role in the regulation of feto-maternal blood flow.[20] In placenta, NO has been postulated to contribute in surveillance against maternal immune insult.[21] NO also modulates cellular growth. Inhibition of cellular proliferation by

NO was observed in several kinds of cells types, including fibroblasts,[22] hepatocytes,[23] bone marrow,[24] retinal,[25] and mesangial cells.[26] However, in endothelial and glial cells, the effects obtained were not only inhibitory,[27,28] but also stimulating.[29,30] It had also been demonstrated that NO-induced cellular proliferation is apparently important for angiogenesis associated with tissue healing.[29,31] In brief, nitric oxide exerts both inhibitory as well stimulatory effects on cellular proliferation. NO also influences differentiation of skeletal muscle cell,[1] neuronal cell,[2] and bone marrow cells.[4] This poses a question of what effect NO may exert on trophoblast cells. NO-NOS system may be involved in trophoblast proliferation as in enterocytes[29] or glial cells.[30] To address the role of NO on trophoblast function, we have further studied the effect of NO using SNP as the NO donor on trophoblast cell proliferation and differentiation. NO released from SNP significantly stimulated the proliferation of JEG-3 cells over and above their normal rate of proliferation in culture (Fig. 4). To further correlate this observation with increase in DNA content in the cell during proliferation, BrdU labeling experiments followed by flow cytometry was performed. It was observed that SNP also increased BrdU incorporation into the DNA (Fig. 5), which further substantiates the results obtained by MTT conversion assay. Morphological observations of primary trophoblasts indicate an arrest in the formation of syncytial cells in presence of SNP (Fig. 6). This inhibition was also reflected in the low level of secretion of hCG and progesterone (Figs. 7 and 8). The possibility of any cytotoxic effect of SNP, even at the highest concentration of 40 μM, was ruled out by the observation of MTT assay (Fig. 4) and Trypan Blue exclusion by the trophoblast cells (data not shown).

Under *in vivo* conditions, cytotrophoblast cells undergo proliferation as well as differentiation to form multinucleated syncytiotrophoblast cells. These cellular events occur sequentially and are precisely coordinated in the utero-placental context. Cytotrophoblasts isolated from the placenta and maintained under culture condition, differentiate terminally and follow a temporal pattern of syncytia formation reproducing the *in vivo* process.[19] The regulatory mechanisms controlling proliferation and differentiation of trophoblast cells are largely unknown. Our studies indicate that NO induces proliferation and reduces

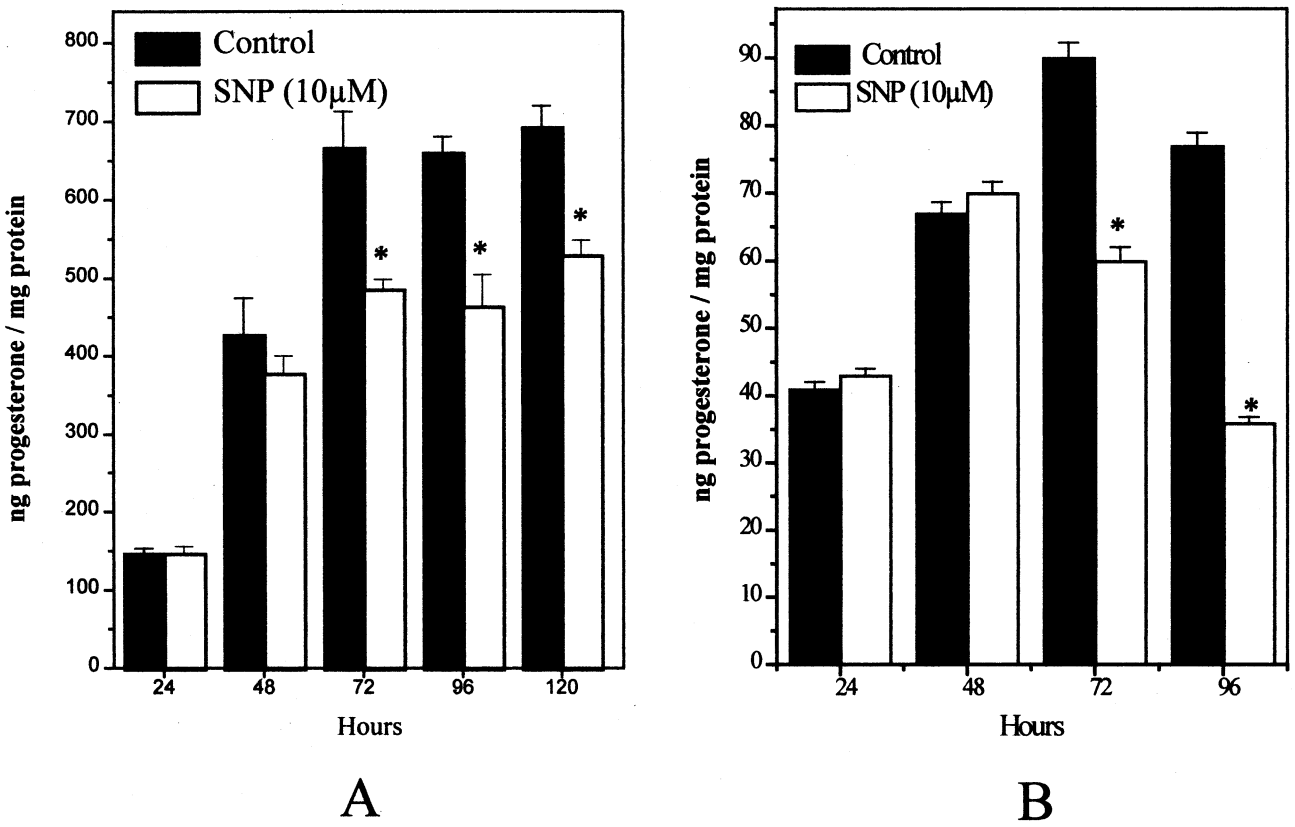

Fig. 8. Effect of SNP on progesterone secretion by trophoblast cells. **A**, progesterone secretion by primary trophoblast cells. Solid bar indicates control and hollow bar indicates SNP (10 μM) challenge. Note that SNP significantly ($*P \leq 0.001$) reduced progesterone secretion at 72, 96, and 120 hr culture. **B**, progesterone secretion by JEG-3 cells. *Solid bar* indicates control and *hollow bar* indicates SNP (10 μM) challenge. As in primary, SNP also significantly ($*P \leq 0.001$) reduced secretion of progesterone by JEG-3 cells at 72 and 96 hr of culture.

differentiation of trophoblast cells, thereby synchronizing placental growth and development. The mechanism by which such a control may be exerted needs to be further elucidated.

Acknowledgments

The work was supported by grants from the Indian Council of Medical Research, New Delhi and the Indo-French Centre for the Promotion of Advanced Research, New Delhi. Mrinmoy Sanyal is a recipient of Dr. K.S. Krisrnan Research fellowship of the Department of Atomic Energy, Government of India.

REFERENCES

1. Lee KH, Baek MY, Moon KY, Song WK, Chung CH, Ha DB, Kang M: Nitric oxide as a messenger molecule for myoblast fusion. J Biol Chem 1994; 269:14371–14374.
2. Hassid A, Arabshahi H, Bourcier T, Dhanussi GS, Mathews C: nitric oxide selectively amplifies FGF-2-induced mitogenesis in primary rat aortic smooth muscle cells. Am J Physiol 1994; 267:H1040–H1048.
3. Peunova N, Enikolopov G: Nitric oxide triggers a switch to growth arrest during differentiation of neuronal cells. Nature 1995; 375:68–73.
4. Shami PJ, Weinberg JB: Differential effects of nitric oxide on erythroid and myeloid colony growth from $CD34^+$ human bone marrow cells. Blood 1996; 87:977–982.
5. Conrad KP, Vill M, McGuire PG, Dail WG, Davis AK: Expression of nitric oxide synthase by syncytiotrophoblast in human placental villi. FASEB J 1993; 7:1269–1276.
6. Eis AL, Brockman DE, Pollack JS, Myatt L: Immunohistochemical localization of endothelial nitric oxide synthase in human villous and extravillous trophoblast populations and expression during syncytiotrophoblast formation *in vitro*. Placenta 1995; 16:113–126.
7. Kliman HJ, Nestler JE, Sermasi E, Sanger JM, Strauss JF III: Purification, charecterization, and *in vivo* differentiation of cytotrophoblasts from human term placentae. Endocrinology 1986; 118:1567–1582.
8. Lowry OH, Rosenbrough NJ, Farr AL, Raneall RJ: Protein measurement with the Folin phenol reagent. J Biol Chem 1951; 193:265–275.
9. Kristofferson EK, Ulvestad E: Nitric oxide synthase in human placenta. APMIS 1994; 102:509–513.
10. Dawson TM, Bredt DS, Fotuhi M, Hwang PM, Snyder SH: Nitric oxide synthase and neuronal NADPH diaphorase are identical in brain and peripheral tissue. Proc Natl Acad Sci USA 1991; 88:7797–7801.
11. Hope BT, Michael GF, Knigge KM, Vincent SR: Neuronal NADPH diaphorase is a nitric oxide synthase. Proc Natl Acad Sci USA 1991; 88:2811–2814.
12. Bredt DS, Snyder SH: Isolation of nitric oxide synthase, a calmodulin-requiring enzyme. Proc Natl Acad Sci USA 1990; 87:682–685.
13. Schmidt HH, Pollock JS, Nakane M, Gorosky LD, Forstermann U, Murad F: Purification of a soluble isoform of guanylyl cyclase-activating factor synthase. Proc Natl Acad Sci USA 1991; 88:365–369.
14. Mayer B, John M, Bohme K: Purification of a Ca^{2+}/calmodulin-dependent nitric oxide synthase from porcine cerebellum. Cofactor-role of tetrahydrobiopterin. FEBS Lett 1990; 277:215–219.
15. Hevel JM, White KA, Marletta MA: Purification of the inducible murine macrophage nitric oxide synthase. J Biol Chem 1991; 266:22789–22791.
16. Stuehr DJ, Cho HJ, Kwon NS, Weise MF, Nathan CF: Purification and characterization of cytokine-induced macrophage nitric oxide synthase: An Fad- and FMN-containing flavoprotein. Proc Natl Acad Sci USA 1991; 88:7773–7777.
17. Boje KM, Fung H: Endothelial nitric oxide generating enzyme(s) in the bovine aorta: Subcellular location and metabolic characterization. J Pharmacol Exp Ther 1990; 253:20–26.
18. Marletta MA: Nitric oxide synthase structure and mechanism. J Biol Chem 1993; 268:12231–12234.
19. Bhattacharya S, Chaudhary J, Das C: Antibodies to hCG inhibit progesterone production from human syncytiotrophoblast cells. Placenta 1992; 13:135–139.
20. Buttery LD, McCarthy A, Springall DR, Sullivan MH, Elder MG, Michel T, Polak JM: Endothelial nitric oxide synthase in the human placenta: Regional distribution and proposed regulatory role at the fetomaternal interface. Placenta 1994; 15:257–265.
21. Myatt L, Eis ALW, Brockman DE, Kossen Jans W, Greer I, Lyal F: Inducible (type II) nitric oxide synthase in human placental villous tissue of normotensive, pre-eclamptic and intrauterine growth-restricted pregnancies. Placenta 1997; 18:261–268.
22. Garg UC, Hassid A: Nitric oxide-generating vasodilators inhibit mitogenesis and proliferation of BALB/C 3T3 fibroblasts by a cGMP dependent mechanism. Biochem Biophys Res Commun 1990; 171:474–479.
23. Miyazaki M, Wahid S, Bal L, Namba M: Effects of intracellular cyclic Amp and cyclic GMP lavels on DNA synthesis of young adult rat hepatocytesin primary culture. Exp Cell Res 1992; 200:404–409.
24. Punjabi CJ, Laskin DL, Heck DL, Laskin JD: Production of nitric oxide by murine bone marrow cells. Inverse corelation with cellular proliferation. J Immunol 1992; 149:2148–2179.
25. Goureau O, Lepoivre M, Becquet F, Curtouis Y: Differential regulation of inducible nitric oxide synthase by fibroblast growth factor and transforming growth factor-β in bovine retinal pigmented epithelial cells: Inverse correlation with cellular proliferation. Proc Natl Acad Sci USA 1993; 90:4276–4280.
26. Garg UC, Hassid A: Inhibition of rat mesangial cell mitogenesis by nitric oxide-generating vasodilators. Am J Physiol 1989; 257:F60–F66.
27. Garg UC, Devi L, Turndorf H, Goldfrank LR, Bansinath M: Effect of nitric oxide on mitogenesis and proliferation of cerebellar glial cells. Brain Res 1992; 592:208–212.
28. Yang W, Ando J, Korenaga R, Toyo-oka T, Kamiya A: Exogenous nitric oxide inhibits proliferation of cultured vascular endothelial cells. Biochem Biophys Res Commun 1994; 203:1160–1167.
29. Konturek SJ, Brzozowski T, Majka J, Pytko-Polonczyk J, Stachura J: Inhibition of nitric oxide synthase delays healing of chronic gastric ulcers. Eur J Pharmacol 1993; 239:215–217.
30. Munoz-Fernandez MA, Fresno M: Involvement of nitric oxide in cytokine-induced growth of glial cells. Biochem Biophys Res Commun 1993; 194:319–325.
31. Ziche M, Morbidelli L, Masini E, Amerini S, Granger HJ, Maggi CA, Geppetti P, Ledda F: Nitric oxide mediates angiogenesis *in vivo* and endothelial cell growth and migration *in vivo* promoted by substance P. J Clin Invest 1994; 94:2036–2044.