Vol. 198, No. 1, 1994
January 14, 1994
BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 120-126

HUMAN RETINAL PIGMENTED EPITHELIAL CELLS PRODUCE NITRIC OXIDE IN RESPONSE TO CYTOKINES

O. Goureau[1], D. Hicks[2] and Y. Courtois [1]

[1]Unité de Recherches Gérontologiques, INSERM U 118, Asssociation Claude Bernard, 29 rue Wilhem, 75016 Paris, France

[2] INSERM, CJF 92-02, Laboratoire Laveran, Hôpital civil, B.P. 426, 67091 Strasbourg cedex, France

Received November 22, 1993

The present study demonstrates that human retinal pigmented epithelial cells produce nitric oxide (NO) upon co-treatment with interferon γ (IFNγ) and interleukin-1β (IL-1β). The biosynthesis of NO, which was measured by the accumulation of the stable end-product nitrite, requires an induction period of approximately 12 hours and continues for at least three days. The synthesis was abolished by the stereoselective inhibitors of NO synthase (NOS), N^G-monomethyl-L-arginine, Nω-nitro-L-arginine and Nω-nitro-L-arginine methyl ester and by cycloheximide. Transforming growth factor β suppressed cytokine-induced NOS. The results indicate that cytokines such as IFNγ and IL-1β are capable of inducing NOS, while TGFβ prevents this induction, in subcultured pigmented epithelial cells from the human retina.

Nitric oxide (NO) is a free radical, synthesized from L-arginine by NO synthase (for review see 1). The existence of at least two different isoforms of NO synthase (NOS) has been demonstrated : i) one form of NOS is calcium/calmodulin dependent and is constitutively expressed in endothelial cells (2) and brain (3) ; ii) a second form is induced by endotoxins and/or cytokines in some mammalian cells (1). Induction of NO synthesis was first demonstrated in rodent macrophages (4) and has since been extended to other cells including smooth muscle cells (5), chondrocytes (6), renal mesengial cells (7), endothelial cells (8) and hepatocytes (9). Although NO is a cytotoxic and cytostatic effector molecule in host defense against tumor cells, and parasites in macrophages (10), its role in all other cells and tissues is not clearly understood. Furthermore, the expression of inducible NOS in human cells has been difficult to demonstrate. Evidence for inducible human NOS has been shown in patients receiving interleukin 2 cancer therapy (11) and during sepsis (12). In vitro studies demonstrate the capacity of some cells to release nitrite after cytokine stimulation, such as hepatocytes (13), smooth muscle cells (14), mesangial cells (15) and chondrocytes (16). More recently, the cloning of inducible NOS from human hepatocytes was reported (17).

In the retina, we have demonstrated that bovine retinal pigmented epithelial (RPE) cells, cultured in presence of interferon γ (IFNγ) with lipopolysaccharide (LPS) or tumor necrosis factor α (TNFα), released nitrite, the stable end product of NO, into the culture supernatant (18). Furthermore, we have shown that some growth factors are able to modulate

0006-291X/94 $5.00

the biosynthesis of NO in these cells (19). In this paper, we report that nitrite are also produced by subcultured human RPE cells, demonstrating that a human retinal cell type can also express an inducible NOS.

MATERIALS AND METHODS

Cell cultures : Primary human RPE cell cultures were established from eyes that had been enucleated for melanoma. In the authors opinion, methods for securing human tissue were humane, included proper consent and approval, and complied with the tenets of the Declaration of Helsinki. Cells were isolated from areas not invaded by the tumor, using a modification of published techniques (20). Briefly, the posterior eye cup was rinsed in calcium, magnesium free phosphate buffer saline (PBS) and incubated for 30-45 min in PBS containing 0.1% trypsin and 0.1mM EDTA at 37°C. The solution was then gently triturated, cells aspirated and centrifugated, then seeded into 60 mm dishes in nutritive Ham F-10 medium containing 10% fetal calf serum (FCS), 2mM glutamine and 0.1% Penicillin-Streptomycin-Fungizone, the contents of one eye plated in 2 dishes. Cultures were maintained at 37°C, and upon reaching confluence the RPE monolayer was trypsinized (0.1% trypsin in PBS). In the present study, cells were used between the 4^{th} and 6^{th} passages.

Chemicals and cytokines : N^G-monomethyl-L-arginine was from Calbiochem corp. (France Biochem., Meudon). LPS from *Salmonella typhymurium*, cycloheximide, N_ω-nitro-L-arginine and N_ω-nitro-L-arginine-methyl ester were purchased from Sigma France. Human recombinant IFNγ was generously provided by Dr M. Lepoivre (CNRS, URA 1131, Orsay, France). Human recombinant interleukin-1β (IL-1β) and human recombinant TNFα were obtained from Genzyme (Tebu, France). Human recombinant bFGF was prepared by Carlo Erba (Italy). Transforming growth factor beta (TGFβ) was purchased from Realef France.

Formation of nitrite : Retinal pigmented epithelial cells were seeded at a density of 5.10^4/ml in 12-well culture plates (Falcon Laboratories) in nutritive Ham F-10 medium. At confluency, cells were treated with lipopolysaccharide (LPS) and/or other cytokines, in fresh medium. After incubation for the time indicated in each specific experiment, nitrite concentration was determined in cell-free culture supernatants using the spectrophotometric method based on the Griess reaction (21). Briefly, samples were reacted with 1% sulfanilamide, 0,1% naphtyl-ethylenediamine at room temperature for 10 minutes and the nitrite concentration was determined by absorbance at 540 nm in comparison with standard solutions of sodium nitrite prepared in nutritive Ham F-10 medium.

RESULTS

To determine whether NO production is inducible in human RPE cells, confluent cell cultures were exposed to various stimuli for 48 hours. Mediators (LPS, IFNγ, TNFα and IL-1β) were chosen for their capacity to induce NO biosynthesis in bovine RPE cells (17). NO generation was measured as the accumulation of the stable end-product nitrite in the culture medium. When added separately, none of the above-mentioned stimulants could induce a

Table I : Nitrite level in supernatants of human RPE cells stimulated with various cytokines and LPS

Addition	Nitrite (μM)
None	0.4 ± 0.2
LPS	0.3 ± 0.1
IFNγ	0.6 ± 0.2
TNFα	0.5 ± 0.1
IL-1β	0.8 ± 0.2
IFNγ + IL-1β	9.9 ± 1.1*
LPS + IFNγ + TNFα	0.7 ± 0.3
IFNγ + IL-1β + TNFα	11.2 ± 2.4*
LPS + IFNγ + IL-1β + TNFα	11.5 ± 1.4*

RPE cells were incubated with IFNγ (100U/ml), IL-1β (100U/ml), LPS (1μg/ml) or TNFα (100U/ml) alone or in combination. After 48h, Nitrite level was measured in the culture supernatants using the Griess reagent. Values are means ± S.E.M for four independent cultures, each done in duplicate. *$p<0.001$ when compared with untreated cultures.

significant production of nitrite by RPE cells (Table I). Various combinations of stimulatory agents were tested in order to evaluate their effects on NO production (Table I). In these conditions, a significant increase in NO biosynthesis was found when IFNγ was associated with IL-1β. Further addition of TNFα resulted in maximal nitrite release, greater than 10μM / 48 hours, while addition of LPS did not increase nitrite release (Table I).

The response to IFNγ, when added in conjuction with IL-1β (100U/ml), was dose-dependent, with 50% of maximal nitrite accumulation observed at 10 U/ml (Fig.1A). Maximal accumulation was observed at IFNγ concentrations of > 100U/ml. Furthermore, treatment of the cells with 100U/ml of IFNγ in combination with different concentrations of IL-1β induced RPE cells to produce nitrite in a dose-dependent manner (Fig.1B). Optimal co-stimulation was achieved at 100 U/ml of IL-1β. Fig.2 shows the time course of nitrite formation upon stimulation with IFNγ, IL-1β and TNFα. Nitrite production was first detected in RPE cells after 12 hours treatment, and nitrite biosynthesis reached a plateau at 60 hours.

Data given in Table II demonstrate that cytokine-induced nitrite release was markedly decreased by co-incubation of cells with L-arginine analogues, namely N^G-monomethyl-L-arginine (L-NMMA), Nω-nitro-L-arginine (L-NA) and Nω-nitro-L-arginine methyl ester (L-NAME). Cycloheximide prevented nitrite accumulation by LPS/IFNγ by 80% (Table II), demonstrating that protein synthesis is required for the induction of NOS. Co-incubation of RPE cells with transforming growth factor β (TGFβ) and NOS inducers (IL-1β/IFNγ/TNFα) for 48 hours, markedly reduced nitrite production, while addition of basic fibroblast growth factor (bFGF) did not modify cytokine-induced nitrite release (Fig.3). The inhibitory effect of TGFβ was dose-dependent, with half maximal inhibition at 0.1ng/ml. When TGFβ was added

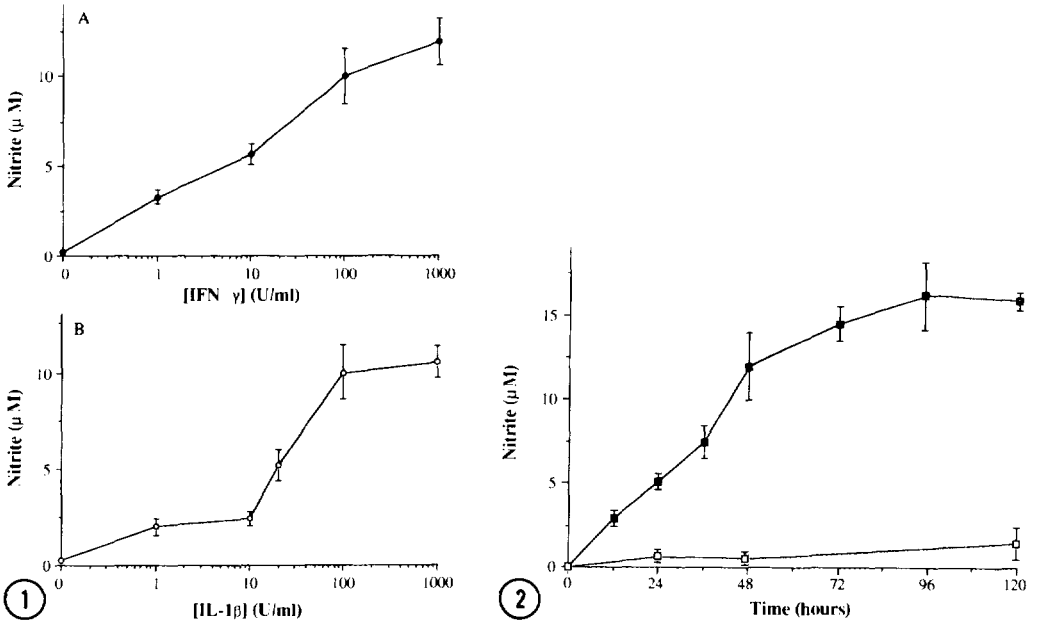

Figure 1. **Effect of increasing cytokines concentrations on nitrite release from RPE cells.** Cells were incubated with (A) 100U/ml IFNγ and different concentrations of IL-1β ; (B) 100U/ml IL-1β and different concentrations of IFNγ. After 48 h of incubation, the nitrite level was evaluated in the supernatants, as described in materials and methods.Values are means ± S.E.M for three independent cultures, each done in triplicate.

Figure 2. **Time course of cytokine-induced nitrite production in RPE cells.** Cells were incubated without (open square) or with (closed square) IL-1β (100U/ml) plus TNFα (100U/ml) and IFNγ (100U/ml) in DMEM. Supernatants were harvested at indicated time points, and nitrite was determined using the Griess reagent, as described in materials and methods. Values are means ± S.E.M for two independent cultures, each done in triplicate.

to the culture medium, 12 hours after the addition of cytokine, the inhibition of nitrite release was significantly reduced (data not shown), demonstrating an inhibition of NOS induction by this growth factor.

Table II : Inhibition of cytokine-induced nitrite formation by L-arginine analogues and cycloheximide

Addition	Nitrite (μM)
Control	10.7 ± 0.7
L-NMMA (0.1mM)	2.4 ± 0.8
L-NA (0.1mM)	1.7 ± 0.1
L-NAME (0.1mM)	1.3 ± 0.4
Cycloheximide (0.25μg/ml)	3.8 ± 0.5

Cells were treated with IFNγ, IL-1β and TNFα all at 100U/ml, without (control) or with the indicated agents for 48 h. After this time, the nitrite release in the supernatants was performed as decribed in materials and methods. Values are means ± S.E.M for three independent cultures, each done in duplicate.

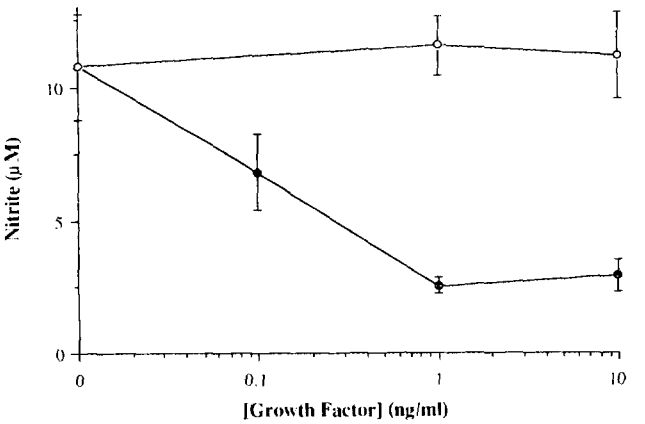

Figure 3 . **Effect of increasing concentrations of TGFβ and bFGF on nitrite release from cytokine-activated RPE cells.** Cells were co-stimulated with IFNγ (100U/ml), IL-1β (100U/ml), TNFα (100U/ml) and indicated concentrations of TGFβ (closed circle) or bFGF (open circle). After 48 h, culture media were assayed for nitrite. Values are means ± S.E for three different experiments each done in duplicate.

DISCUSSION

In this report, we demonstrate that human RPE cells are able to produce nitrite upon stimulation with IFNγ in the presence of IL-1β. Maximal nitrite production was observed with the combination IL-1β, IFNγ and TNFα. The inhibition of cytokine-induced nitrite release by L-arginine analogues, L-NMMA, L-NA and L-NABE, demonstrate that nitrite results from the oxidation of L-arginine via the NOS pathway. The lag period of 12 hours for detecting nitrite production, as well as the inhibitory effects of cycloheximide indicate that IFNγ and IL-1β induce the synthesis of a protein that is required for the production of NO by the RPE cells. The present study indicates that human RPE cells differ from bovine RPE cells with respect to the mediators responsible for the induction of NO biosynthesis. In contrast to bovine RPE cells, where IFNγ combined with either LPS or TNFα leads to NO generation (17), human RPE cells required both IL-1β and IFNγ for NO biosynthesis. Furthermore, LPS did not increase nitrite production when added with IL-1β and IFNγ. However, human RPE cells resemble other human cell types, in which IL-1β is often required for NOS induction (13-16). In addition, we demonstrate that regulation of NOS induction by growth factors in human RPE cells was different from that in bovine RPE cells. Indeed, bFGF did not modify IFNγ/TNFα/IL-1β-induced nitrite release in human cells, while it markedly prevented LPS/IFNγ-induced nitrite production in bovine RPE cells (19). On the other hand, TGFβ markedly inhibited NO production in human RPE cells, whereas it slightly increased this production in bovine RPE cells (19). This differential regulation could be explained by the different types of mediators responsible for NOS induction in each cellular species. The inhibitory effect of TGFβ on IL-1β-induced nitrite release has been previously described in human smooth muscle cells (14). The results from our study indicate that the inducible NOS pathway in subcultured human RPE cells is regulated differently from that of bovine cells.

Recent data support important roles for cytokines, including IL-1β, TNFα and IFNγ, in the ocular inflammatory response. Indeed, intravitreal injections of IL-1β and TNFα cause intraocular inflammation (22) and high levels of IFNγ have been detected in the eye during inflammation (23, 24), such as in the vitreous humor from AIDS patients with retinitis (24). Furthermore, human RPE cells can express class II antigens in response to *in vitro* stimulation with lymphokine or IFNγ (25). Since ocular inflammation involves an increase in production of these cytokines in the retina, this may induce NOS in RPE cells. The subsequently high production of NO may account for the tissue damage which is observed in some retinal inflammatory diseases, such as retinitis. NO could directly exert a cytotoxic activity on the RPE cells themselves and for the neighbouring photoreceptors. We demonstrate here that TGFβ inhibits NO production by cytokine-stimulated RPE cells. Since TGFβ was described as an inhibitor of inflammatory development in the posterior pole of the eye (26), we propose that the inhibition of NO production by TGFβ in RPE cells could be of major importance in the regulation of ocular inflammation. Further research into the possibilities of inhibiting NO production by blocking NOS induction with growth factors or inhibiting NOS activity with NOS inhibitors, may lead to new therapeutic approaches being applied to retinal inflammatory diseases.

REFERENCES

1- Nathan C.F. (1992) Faseb J., 6 : 3051-3064.
2- Bredt D.S and Snyder S.H. (1992) Neuron, 8 : 8-11.
3- Palmer R.M.J., Ferridge A.G. and Moncada S. (1987) Nature, 327: 524-526.
4- Stuehr D.J. and Marletta M.A. (1987) J. Immunol., 139 : 519-525.
5- Busse R. and Mülsch A. (1990) FEBS Letter, 275: 87-90.
6- Stadler J., Stefanovic-Racic M., Billiar T.R., Curran R.D., McIntyre L.A., Georgescu H.I., Simmons R.L. and Evans C.H. (1990) J. Immunol., 147 : 3915-3920.
7- Pfeilschifter J. and Schwarzenbach H. (1990) FEBS Letter, 273: 185-187.
8- Radomski M.W., Palmer R.M.J. and Moncada S. (1990) Proc. Natl. Acad. Sci., 87: 10043-10047.
9- Curran R.D., Billiar T.R., Stuehr D.J., Hoffman K. and Simmons R.L. (1989) J. Exp. Med., 170: 1769-1774.
10- Nathan C.F., and Hibbs J.B. Jr (1991) Curr. Opinion Immunol., 3 : 65-70.
11- Hibbs J.B. Jr, Westenfelder C., Taintor R., Vavrin Z., Kablitz C., Baranowski R.L., Ward J.H., Menlove R.L., McMurry M.P., Kushner J.P. and Samlowski W.E. (1992) J. Clin. Invest., 89 : 867-877.
12- Ochoa J.B., Udekwu A.O., Billiar T.R., Curran R.D., Cerra F.B., Simmons R.L. and Peitzman A.B. (1991) Ann. Surg., 214 : 621-626.
13- Nussler A.K., DiSilvio M.D., Billiar T.R., Hoffman R.A., Geller D.A., Selby R., Madariaga J.and Simmons R.L. (1992) J. Exp. Med., 176 : 261-264.
14- Junquero D.C., Scott-Burden T., Schini V.B. and Vanhoutte P.M. (1992) J. Physiol. London, 454 : 451-465.
15- Nicolson A.G., Haites N.E., McKay N.G., Wilson H.M., MacLeod A.M. and Benjamin N. (1993) Biochem. Biophys. Res. Comm., 193 : 1269-1274.
16- Palmer R.M.J., Hickery M.S., Charles I.G., Moncada S., and Bayliss M.T. (1993) Biochem. Biophys. Res. Comm., 193 : 398-405.
17- Geller D.A., Lowenstein C.J., Shapiro R.A., Nussler A.K., Di Silvio M., Wang S.C., Nakayama D.K., Simmons R.L., Snyder S.H., and Billiar T.R. (1993) Proc. Natl. Acad. Sci. USA, 90: 3491-3495.
18- Goureau O., Lepoivre M., and Courtois Y. (1992) Biophys. Res. Comm., 186: 854-859.
19- Goureau O., Lepoivre M., Becquet F., and Courtois Y. (1993) Proc Natl. Acad. Sci. USA, 90: 4276-4280.

20- DelMonte M.A. and Maumenee I.H. (1980) Birth Defects, 16: 327.
21- Green L.C., Wagner D.A., Glogowski J., Skipper P.L., Wishnok J.S. and Tannenbaum S.R. (1982) Anal. Biochem., 126: 131-138.
22- Fleischer L.N., Ferrell J.B. and McGahan M.C. (1992) Invest. Ophthalmol. Vis. Sci., 33: 2120-2127.
23- Hooks J.J., Chan C.C. and Detrick B. (1988) Invest. Ophthalmol. Vis. Sci., 29: 1444-1451.
24- Mondino B.J., Sidikaro Y., Mayer F.J. and Sumner H.L. (1990) Invest. Ophthalmol. Vis. Sci., 31: 798-804.
25- Liversidge J.M., Sewell H.F. and Forrester J.V. (1988) Clin. Exp. Immunol., 73: 489-494.
26- Cousins S.W., McCabe M.M., Danielpour D. and Streilen J.W. (1991) Invest. Ophthalmol. Vis. Sci., 32: 2201-2211.