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The effect of nitric oxide on cytokine-induced release of PGE_2 by human cultured astroglial cells

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1 The role of the L-arginine-nitric oxide (NO) pathway on the formation of prostaglandin E_2 (PGE_2) by human cultured astroglial cells incubated with interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α) was investigated.

2 Incubation of T 67 astroglial cell line with IL-β (10 ng ml^{-1}) and TNF-α (500 u ml^{-1}) produced a significant ($P<0.05$) increase of both nitrite (the breakdown product of NO), cyclic GMP and PGE_2 levels in cell supernatants. N^{ω}-nitro-L-arginine methyl ester (L-NAME; 20–300 μM), an inhibitor of NO synthase (NOS), inhibited the increase of cyclic GMP and nitrite levels found in supernatants of cytokine-treated astroglial cells and reduced the release of PGE_2. The latter effect showed that the enhanced arachidonic acid (AA) metabolism subsequent to stimulation of astroglial cells with IL-1β and TNF-α was, at least in part, induced by NO. This occurred also when sodium nitroprusside (SNP; 120 μM), an NO donor, was incubated with astroglial cells, an effect antagonized by oxyhaemoglobin (OxyHb; 10 μM).

3 The inhibition elicited by L-NAME on PGE_2-release by cytokine-treated astroglial cells was reversed by adding AA (40 μM), showing that the effect of NO on cytokine-dependent PGE_2 release occurred at the cyclo-oxygenase (COX) level. Furthermore, the release of PGE_2 in cytokine-treated astroglial cells was inhibited by indomethacin (10 μM), a COX inhibitor as well as by preincubating cells with dexamethasone (20 μM), an inhibitor of inducible enzymes, showing that the inducible isoform of COX (COX-2) was involved.

4 On the other hand, pretreating astroglial cells with methylene blue (MB; 10 μM), an inhibitor of NO biological activity acting at the guanylate cyclase level, failed to affect PGE_2 release in cytokine-treated astroglial cells, leading to the conclusion that cyclic GMP changes related to NO formation are not involved in the generation of AA metabolites.

5 The present experiments demonstrated that the release of PGE_2 by astroglial cells pretreated with IL-1β and TNF-α is due to enhanced COX-2 activity via activation of the L-arginine-NO pathway, and this may be relevant to the understanding of the pathophysiological mechanisms underlying neuroimmune disorders.

Keywords: Nitric oxide; cytokines; prostaglandins; cyclo-oxygenase; neuroimmune disorders

Introduction

Evidence exists that astroglial cells may play a role in the communications occurring between the neuroendocrine and the immune system. Indeed, because of their position between cerebral blood vessels and neurones, they represent an important site of modulation of chemical signals coming from peripheral tissues to the brain (Fontana *et al*., 1984). Indeed, astroglial cells are able to respond to different neurochemical stimuli including several neurotransmitters and neuromodulators (Pearce & Murphy, 1988; Agullo' & Garcia, 1993). In addition, they are able to secrete cytokines as well as being a target for cytokine activity. In particular, astroglial cells produce interleukin 1 (IL-1; Fontana *et al*., 1982; 1984; Nieto Sampedro & Berman, 1987), tumour necrosis factor (TNF; Chung & Beneviste, 1990), IL-6 (Beneviste *et al*., 1990) and prostaglandin E_2 (PGE_2; Lauro *et al*., 1986). However, the intracellular mechanisms through which astrocytes exert their activity in neuroimmune responses and their pathophysiological role in neuroimmune disorders is still unclear.

In 1990 we showed, for the first time, that cultured astroglial cells release nitric oxide (NO; Mollace *et al*., 1990; Murphy *et al*., 1990; 1993; Moncada *et al*., 1991) a nitrogen free radical which is released within the brain and in peripheral tissues through the bioconversion of L-arginine into citrulline by NO synthase (NOS), a Ca^{2+}/calmodulin-dependent constitutive enzyme (see Moncada *et al*., 1991), thus playing a role in some physiological mechanisms in the brain including arousal (Baggetta *et al*., 1993) and neuronal plasticity (Garthwaite, 1991). In addition, an abnormal release of NO has been shown to occur in some cerebral disorders including epilepsy and neurodegeneration (Mollace *et al*., 1991; Baggetta *et al*., 1993).

Astrocytes are also able to express the inducible, Ca^{2+}/calmudulin-independent isoform of NOS (Mollace *et al*., 1993a) which is stimulated by incubating astroglial cells with *Escherichia coli* lipopolysaccharide (LPS), interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) and γ-interferon (IFN).

The possible role of the cytokine-induced activation of NOS on astroglial cells has recently been widely explored. In particular, evidence has been collected indicating that NO formation, via cytokine network, may contribute to the

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immunocompetent activity of astroglial cells. (Colasanti *et al*., 1993; Mollace *et al*., 1994a). Indeed, we found that major histocompatibility complex II class antigens (MHCII), a cytokine-dependent glycoprotein family expressed on astroglial cell surface during neuroimmune disorders such as multiple sclerosis and Alzheimer disease (Wong *et al*., 1984; Fierz *et al*., 1985; Beneviste, 1989) is modulated by inducible NOS and is blocked by NOS inhibitors such as N^{ω}-nitro-L-arginine methyl ester (L-NAME; Colasanti *et al*., 1993). However, the correlation between cytokines and L-arginine-NO pathway in astroglial cells as well as their role in the mechanism(s) underlying neuronal cell death remains to be elucidated.

Activation of NOS in astroglial cells is accompanied by activation of enzymathic pathways other than L-arginine-NO (Mollace *et al*., 1994b). Indeed, preincubation of human cultured astrocytoma cells with the HIV coating gp 120 glycoprotein has been shown to mediate a significant elevation of PGE_2 levels in cell supernatant, an effect which seems, at least in part, to underlie NO formation (Mollace *et al*., 1994b). Since NO has been found to stimulate cyclo-oxygenase(s) (COX) enzymes via possible activation of the eme-centre of the enzyme, thus leading to enhanced metabolism of arachidonic acid into prostanoids (Salvemini *et al*., 1993), it is likely that gp 120 may release PGE_2 via enhanced formation of NO (Mollace & Nistico', 1995).

Thus, it seemed of interest to verify whether cross-talk between NOS(s) and COX(s) occurs in astroglial cells and the involvement of some neuroimmune disorders where cytokines could play a role. Indeed, both NO and arachidonic acid metabolites possess neurotoxic activity (Mollace *et al*., 1991). In addition, astroglial cells release many cytokines in the brain and may well represent the site where different neurotoxins, such as HIV-derived soluble antigens, may interfere with brain tissues via cytokine-derived intercellular mediators.

The present experiments have been performed in order to verify whether incubation of human culture astroglial cells with IL-1β and TNF-α, at concentrations able to activate the release of NO, enhance the release of PGE_2 in cell supernatant. In addition, the possible role of NO in activating the inducible isoform of cyclo-oxygenase (COX-2) has also been explored.

Methods

Preparation of astrocytoma cells

Human astrocytoma cells (T67 cell line) were obtained from explant of a III WHO gemistocytic astrocytoma (55th–60th passage in culture) and were characterized by means of monoclonal and polyclonal antibodies directed against Glial Fibrillary Acidic Protein (GFAP), S100 protein, fibronectin, factor VIII and vimentin. T67 cells were grown in monolayers within 200 μl multiwell plate (NUNC, Denmark) containing Ham's-F10 (Gibco, U.K.) supplemented with 10% foetal calf serum (FCS, Seralab, U.K.) and 40 μg ml^{-1} gentamycin (Hazleton, K.S., U.S.A.). When the cells were confluent, IL-1β or TNF-α alone or in the presence of L-NAME, indomethacin, dexamethasone or methylene blue, was added and the cells incubated for 24 h. On the day of the experiments, supernatants were aspirated and then assayed for PGE_2, nitrite and guanosine 3′ : 5′-cyclic monophosphate (cyclic GMP). When required, sodium nitroprusside (SNP) and arachidonic acid (AA) alone or in the presence of oxyHb and indomethacin, respectively, were added to untreated or pretreated cells. Cell viability in the presence or absence of IL-1β, TNF-α, methylene blue, L-NAME and SNP was more than 95% as assessed by trypan blue uptake.

All experiments were performed in endotoxin-free media.

Measurement of PGE_2 in supernatant of astrocytoma cells

PGE_2 was assayed by using a specific enzyme immunoassay (EIA) system (Amersham, U.K.). Briefly, supernatant of astrocytoma cells or standard PGE_2 were incubated for 3 h with specific anti-PGE_2 reagent into pre-packed 96 well plate containing a goat anti-mouse solid phase. Peroxidase labelled PGE_2 was then added to each well and incubated for a further 1 h. Unlabelled PGE_2 (standard or unknown) and PGE_2-peroxidase complex competed for a limited number of binding sites of PGE_2 specific antibodies and the amount of peroxidase labelled ligand was inversely proportional to the concentration of added standard or unknown. After washing 3 times with washing buffer, tetramethylbenzidine (TMB)/hydrogen peroxide single pot substrate was added and incubated for 30 min. Addition of acid solution stopped the reaction and the resultant colour was read at 450 nm in a microtitre plate photometer. The concentration of unlabelled PGE_2 in a sample was determined by interpolation from a standard curve and data were expressed as pg labelled PGE_2 for each well.

Measurement of cyclic GMP in supernatant of astrocytoma cells

An enzyme immunoassay similar to that described for PGE_2 measurement was used for assessing cyclic GMP levels in astrocytoma cell supernatant. Standard or unknown (sample) were acetylated by using an acetylation reagent obtained by mixture of 1 volume of acetic anhydride with 2 volumes of triethylamine before addition to wells. Rabbit-cyclic GMP reagent and anti-cyclic GMP peroxidase were added to samples into each pre-packed well containing a monkey anti-rabbit solid phase; TMB represented the peroxidase substrate. Changes of absorbency at 450 nm were calculated by using a microtitre plate photometer and a standard curve ranging from 2 to 512 fmol/well was used for calculation of unlabelled cyclic GMP into each well. Data have been expressed as fmol cyclic GMP/0.5×10^5 cells for each well.

Nitrite analysis

Nitrite (NO_2^-) in astrocytoma cell supernatant was measured by the Griess reaction. Aliquots of the cell supernatants were mixed with an equal volume of Griess reagent (1% sulphanilamide/0.1% naphlethylendiamine dihydrochloride/2.5% H_3PO_4). The absorbency was measured at 546 nm and nitrite concentration was determined with sodium nitrite as a standard. Results are expressed as nmol NO_2^- ml^{-1}.

Materials

Sodium nitroprusside, dexamethasone, arachidonic acid, methylene blue, sodium hydrosulphite, haemoglobin (from bovine blood), sodium nitrite, sulphanilamide, naphthylethylendiamine dihydrochloride, indomethacin, N^{ω}-nitro-L-arginine methyl ester were obtained from Sigma (Milan). Oxyhaemoglobin was prepared by reduction of bovine haemoglobin with sodium hydrosulphite. Interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) were obtained from Biogen SA (Geneva, Switzerland).

Statistics

Results are expressed as mean ± s.e.mean for (n) experiments and Student's unpaired t test was used to determine the significant difference between means, and a P value of <0.05 was taken as significant.

Results

Effect of IL-1β, TNF-α and SNP on nitrite and cyclic GMP levels in supernatant of astrocytoma cells

IL-1β (10 ng ml^{-1}; $n=4$) or TNF-α (500 u ml^{-1}; $n=4$), incubated for 24 h with T67 astrocytoma cells, dose-dependently increased nitrite levels in cell supernatant when compared to control cells (Figure 1). This effect was inhibited when incubation of cells with cytokines was carried out in the presence of L-NAME (300 μM; $n=4$), an inhibitor of NO-synthase (Figure 1). The rise of nitrite in supernatant of astrocytoma cells incubated with IL-1β and TNF-α was accompanied by a significant ($P<0.05$) increase of cyclic GMP levels on astroglial cells (from 0.5 ± 0.2 and 0.7 ± 0.2 to 8 ± 1.5 and 9 ± 1.8 fmol/0.5×10^5 cells, respectively; $n=4$), an effect antagonized by methylene blue (MB; 20 μM; $n=4$), an inhibitor of NO biological activity acting at the guanylate cyclase level (from 8 ± 1.5 and 9 ± 1.8 to 2.5 ± 0.6 and 1.9 ± 0.4 fmol/0.5×10^5 cells, respectively). Neither indomethacin (10 μM; $n=4$) nor arachidonic acid (40 μM; $n=8$) affected basal release of nitrite in cell supernatant or IL-1β and TNF-α-dependent increase of nitrite and cyclic GMP levels (not shown) seen in pretreated astroglial cells. Sodium nitroprusside (SNP 120 μM; $n=4$), incubated with T67 astroglial cells significantly increased nitrite levels in cell supernatant and this was inhibited by oxyHb (10 μM; $n=4$), a trapping agent for NO (Figure 1).

Effect of L-NAME and dexamethasone on IL-1β- and TNF-α-dependent release of PGE_2 by cultured astroglial cells

Incubation of T67 human cultured astroglial cells for 24 h with IL-1β or TNF-α (10 ng ml^{-1} and 500 u ml^{-1}, respectively; $n=4$ for each compound), significantly ($P<0.05$) increased PGE_2 levels in cell supernatant, an effect antagonized by indomethacin (10 μM; $n=4$; Figure 2). Pretreating cells with dexamethasone (20 μM; $n=4$) significantly ($P<0.05$) blocked PGE_2 elevation subsequent to IL-1β and TNF-α activation of astroglial cells, indicating that inducible COX-2 is responsible for cytokine-derived enhancement of PGE_2 biosynthesis (Figure 2). Furthermore, coincubation of cells with L-NAME (20, 100, 300 μM; $n=4$ for each dose), reduced IL-1β and TNF-α-dependent elevation of PGE_2 level in cell supernatants (Figure 3), showing that the formation of NO contributed to the release of PGE_2 induced by cytokines via activation of COX-2. Removal of either cytokines and L-NAME from cell culture and resuspending cells in AA (40 μM; $n=4$)-rich fresh medium, restored PGE_2-release (Figure 3). Methylene blue (20 μM) failed to affect the increase of PGE_2 levels in IL-1β- and TNF-α-pretreated astroglial cell supernatant (Figure 3), showing that the increase in cyclic GMP produced by IL-1β and TNF-α is not involved in PGE_2-release.

SNP (120 μM; $n=4$) stimulated the release of PGE_2 by astrocytoma cells (Figure 2), this effect being antagonized by

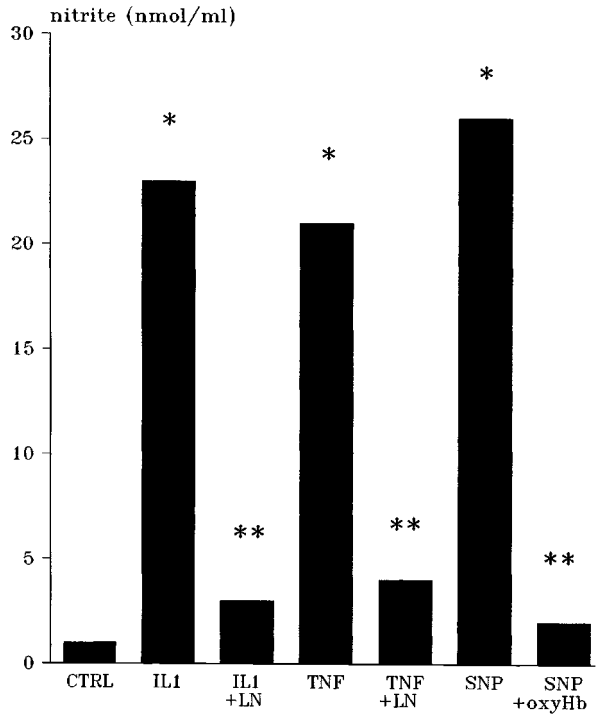

Figure 1 IL-1β (10 ng ml^{-1}), TNF-α (500 u ml^{-1}) and SNP (120 μM) induced an increased release of nitrite when incubated with cultured astroglial cells. L-NAME (LN; 300 μM) and oxyhaemoglobin (oxyHb; 10 μM) reversed this effect. Each column represents the mean of 4 experiments. *$P<0.05$, control vs IL-1β-, TNF-α- and SNP-treated astrocytoma cells. **$P<0.05$, IL-1β, TNF-α and SNP-treated astrocytoma cells vs L-NAME and oxyHb, respectively.

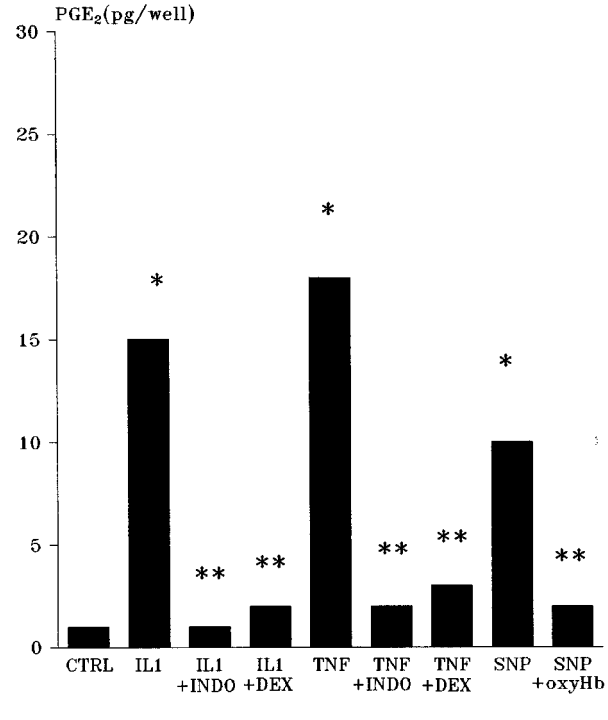

Figure 2 IL-1β (10 ng ml^{-1}), TNF-α (500 u ml^{-1}) and SNP (120 μM) induced an increased release of PGE_2 in cell supernatant when incubated with astroglial cells. Indomethacin (INDO; 10 μM) dexamethasone (DEX; 20 μM) and oxyHb (10 μM), respectively, inhibited this effect. Each column represents the mean of 4 experiments. *$P<0.05$, control *vs* IL-1β-, TNF-α- and SNP-treated astroglial cells. **$P<0.05$, IL-1β-, TNF-α- and SNP-treated astroglial cells *vs* INDO, DEX and oxyHb, respectively.

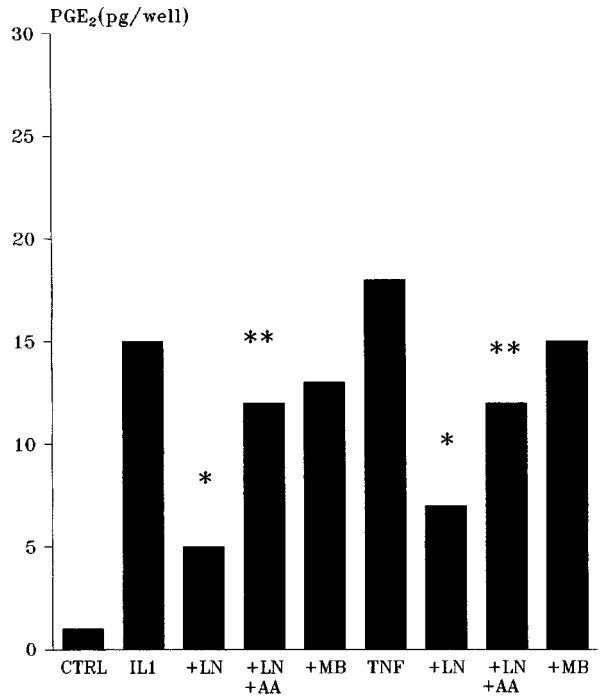

Figure 3 The elevation of PGE_2 level in supernatant of astroglial cells treated with IL-1β (10 ng ml^{-1}) and TNF-α (500 u ml^{-1}) was reduced by the NOS inhibitor L-NAME (LN; 300 μM), an effect which was reversed by arachidonic acid (AA; 30 μM). Methylene blue (MB) failed to affect IL-1β- and TNFα-dependent PGE_2 release. Each column represents the mean of 4 experiments. $^*P<0.05$, IL-1β- and TNFα-treated cells *vs* LN. $^{**}P<0.05$, LN alone *vs* LN + AA.

oxyhaemoglobin (10 μM; $n=4$; Figure 2), which shows that the effect of SNP on PGE_2-release is due to NO formation by astroglial cells.

Discussion

Cytokines represent an heterogeneous group of polypeptide mediators released by immunocompetent cells that have classically been associated with activation of the immune system and with inflammatory responses. They can be produced within the brain and are implicated as mediators of pathologies of the central nervous system (CNS; for a review, see Brenner *et al*., 1994; Hopkins & Rothwell, 1995; Faggioni *et al*., 1995).

Apart from evidence that cytokines are involved as mediators in neuroimmune disorders, the mechanism underlying the role of astroglial cell-derived release of cytokines in neuronal cell death has not been fully resolved. It is known that stimulation of cytokine-release by astroglial cells, as well as activation of receptors for many cytokines located on the astroglial cell membrane, is followed by important biochemical changes including the formation of free radicals and prostanoids (Mollace *et al*., 1993a; 1994a,b; Mollace & Nistico', 1995).

Our data demonstrate that IL-1β and TNF-α, two cytokines that contribute to the immunocompetent activity of astrocytes, stimulate the coupled release of NO and PGE_2 via activation of inducible NOS and COX (iNOS and COX-2). In addition, the present experiments clearly show that IL-1β- and TNF-α-dependent elevation of PGE_2 level in supernatant of astroglial cells is modulated by NO. This was also confirmed by the finding that SNP, an NO-donor, was able to enhance PGE_2 generation in astroglial cells. Our data are in agreement with evidence that NO interacts with emoproteins, such as constitutive and inducible COX (COX-1 and COX-2) (see Salvemini *et al*., 1993), in many cells including astroglial cells, leading to enhanced formation of PGE_2. This effect was shown to be totally independent of an elevation of cyclic GMP depending upon NO-release. In particular, this has also been shown to occur in astrocytes pretreated with NMDA and gp 120 glycoprotein (Mollace *et al*., 1994b; 1995), as well as in some pathophysiological conditions such as in hypoxic astrocytes (Mollace *et al*., 1998).

Thus, NO released following different neurochemical stimuli by astroglial cells, interacts with COX in astroglial cells leading to enhanced formation of AA metabolites. This is important since both NO and prostanoids exert many modulatory functions in astrocytes as well as in neurones. Indeed, activation of iNOS by LPS or cytokines leads to modulation of constitutive release of NO, as can be found after stimulation of NMDA receptors, indicating a feed-back regulatory mechanism involving astroglial cells, directed against an exaggerated excitotoxic neurotransmission (Colasanti *et al*., 1997). On the other hand, PGE_2 not only affects many glial immune functions, including antigen presenting cell activity, but also contributes to immune-mediated neurodegenerative responses, as demonstrated by the beneficial effect exerted by non-steroidal anti-inflammatory drugs, such as indomethacin, on experimental encephalomyelitis (Reder *et al*., 1994). In addition, both AA and its metabolites, such as PGE_2, regulate the turnover of glutamate at the amino acidergic nerve endings and interfere with NMDA receptors, thus playing a modulatory role in excitotoxic mechanisms within the CNS (see Mollace & Nistico', 1995).

In conclusion, our data provide further evidence that astroglial cells represent an important site of cooperation between constitutive and inducible NOS and COX, thereby exerting an important modulatory role under physiological as well as pathological conditions. In addition, interactions between NO and prostanoids, mainly after stimulation of astroglial cells with exogenous as well as endogenous cytokines, may represent one of the most important mechanisms through which many neurotoxins produce neurodegenerative effects and this sheds new light onto the pharmacological approach to neuroimmune disorders.

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