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HIV COATING gp 120 GLYCOPROTEIN-DEPENDENT PROSTAGLANDIN E_2 RELEASE BY HUMAN CULTURED ASTROCYTOMA CELLS IS REGULATED BY NITRIC OXIDE FORMATION

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SUMMARY: The role of the L-arginine-NO pathway on the formation of PGE_2 by cultured astroglial cells incubated with the HIV coating glycoprotein gp120 was investigated. Preincubation of human cultured T 67 astrocytoma cells with gp 120 (100-500 nM) produced a significant increase of nitrite (the breakdown product of NO) and PGE_2 in cell supernatants. The effect of gp 120 on both nitrite and PGE_2 production was antagonized by inhibition of NO synthase by L-NAME (20-300 μM). The inhibition of gp120-induced PGE_2 production by L-NAME was reverted by addition of arachidonic acid (30 μM), an effect antagonized by the cyclo-oxygenase inhibitor indomethacin (10 μM). Methylen bleu, an inhibitor of the biological activity of NO acting at the guanylate cyclase level failed to affect gp 120-mediated PGE_2 release showing that the increase of cGMP subsequent to NO production was not involved in the modulatory activity of NO on arachidonic acid cascade. On the basis of present experiments we conclude that gp-120-induced release of PGE_2 by astroglial cells is driven by NO, thereby contributing in the involvement of glial cells in HIV-related cerebral disorders

The HIV coating gp120 glycoprotein has recently been shown to mediate some of neuropathological modifications that can be associated to AIDS (see 1). Indeed, direct microinfusion of gp 120 in mice as well as its incubation with cortical neurones in culture is able to produce neurodegenerative effect (see 1). On the other hand, microinfusion of this glycoprotein in rats failed to produce brain damage though the mechanism of gp120-mediated neurotoxicity remains to be elucidated (see 2, 3). The release of neurotoxins by astroglial cells stimulated with gp 120 seems to be required in mediating neurodegenerative effect.

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Abbreviations: NO: nitric oxide; L-NAME: N^{ω}nitro-L-arginine methyl ester; SNP: sodium nitroprusside; AA: arachidonic acid.

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In fact, neurotoxicity produced by gp120 increases by culturing neurons in the presence of astroglial cells (4) . In addition, a significant release of cytokins and arachidonic acid metabolites, both endoved of neurodegenerative properties, may occur when incubating astroglial cells with gp 120 (4). On the other hand, incubation of human cultured astrocytoma cells with gp 120 stimulates the release of NO (5), a nitrogen free radical species whose generation has been demonstrated to be involved in gp 120-related neurodegeneration (6) as well as in other models of neurotoxicity (7, 8). Therefore, it is likely that the interaction of different neurochemical stimuli in astroglial cells may represent an important step in gp120-induced neurotoxicity. The present experiments have been carried out in order to ascertein whether gp120 enhances the release of PGE_2 by human cultured astrocytoma cells and whether this effect is related to the formation of NO.

MATERIALS AND METHODS

Materials. Sodium nitroprusside, arachidonic acid, methylen bleu, sodium hydrosulphyte, hemoglobin (from bovine blood), sodium nitrite, sulphanilamide, naphtylethylendiamine dihydrochloride, indomethacin, N^{ω}-nitro-L-arginine methyl ester were obtained from Sigma (Milan). Oxyhemoglobin was prepared by reduction of bovine hemoglobin with sodium hydrosulphite as previously described (9). Gp120 (Recombinant HIV-1 gp120 from Baclovirus expression system; 90°/o pure following immunoaffinity chromatography; binding to CD4 detected using ABT's CD4/gp120 Capture ELISA assay) was a generous gift of Dr. Holmes (NIBSAC, South Mimms, U.K.).
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), S 100 protein, Fibronectin, Factor VIII and Vimentin, as previously described (10). T67 cells were grown in monolayers within 200 µl multiwell plate (NUNC, Denmark) containing Ham's-F 10 (Gibco, U.K.) supplemented with 10°/o Foetal Calf Serum (FCS, Seralab, U.K.) and 40 $\mu g\ ml^{-1}$ gentamycin (Hazleton, K.S., U.S.A.). When confluent, gp120 alone or in the presence of L-NAME, indomethacin, methylen bleu were added and incubated for 12h. The day of the experiments, supernatants were aspirated and then assayed for PGE_2 and nitrite.When required, SNP and 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 gp120, methylen bleu, L-NAME and SNP was more than 95°/o as assessed by trypan blue uptake.

Measurement of PGE_2 in supernatant of astrocytoma cells. PGE_2 was assayed by using a specifc enzymeimmunoassay (EIA) system (Amersham, U.K.). Briefly, supernatant of astrocytoma cells or standard PGE_2 were incubated for 3h with specifc 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 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 rection and the resultant colour was read at 450 nm in a microtitre plate fotometer. The concentration of unlabelled PGE_2 in a sample was determined by interpolation from a standard curve and data expresses as pg PGE_2 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°/o sulphanilamide/0.1°/o naphthylethylendiamine dihydrochloride/ 2.5°/o H_3PO_4). The absorbency was measured at 546 nm, and nitrite concentration was determined using sodium nitrite as a standard. Results were expressed as nmol NO_2ml^{-1}.

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

RESULTS

The effect of gp120 and SNP on nitrite levels in supernatant of astrocytoma cells. Gp120 (100-500 nM; n=4 for each dose), incubated for 12h with T67 astrocytoma cells, dose-dependently increased nitrite levels in cell supernatant (Fig.1 A). This effect was inhibited by incubation of gp 120 (500 nM)-pretreated cells with L-NAME (20- 500 μM; n=4 for eqach dose), a selective inhibitor of NO-synthase (Fig.1B). Neither indomethacin (10 μM; n=4) nor arachidonic acid (AA; 30 μM; n=4) affected basal as well as gp 120-stimulated release of nitrite in cell supernatant (Fig. 1B).

Sodium nitroprusside (SNP; 120 μM; n=4), incubated with T67 astrocytoma cells, significantly increased nitrite levels in cell supernatant and this was inhibited by oxyHb (10 μM; n=4), a trapping agent for NO (Fig. 1A).

Effect of L-NAME on gp 120-dependent release of PGE_2 by astrocytoma cells. Incubation of T67 human cultured astrocytoma cells with gp120 (100, 500 nM; n=4 for each dose), dose-dependently increased PGE_2 levels in cell supernatant, an effect antagonized by indomethacin (10 μM; n=4; Fig. 2A). When coincubated with gp120 (100 nM; n=4), L-NAME (20, 100, 300 μM; n=4 for each dose), dose-dependently inhibited gp 120-dependent elevation of PGE_2 levels in cell supernatant (Fig. 2B).

Removal of either gp120 and L-NAME from cell culture and resuspending cells in AA (30 μM; n=4)-rich fresh medium, restored PGE_2 release, an effect blocked by indomethacin (10 μM; n=4) (Fig. 2B). MB (20 μM) failed to affect the increase of PGE_2 level in the supernatant of gp 120-pretreated astrocytoma cells (Fig. 2B), showing that the biological activity of NO at guanylate cyclase level is not involved in PGE_2 release. SNP (120 μM; n=4) stimulated the release of PGE_2 by astrocytoma cells (Fig. 2A), being this effect antagonized by oxyhemoglobin (10 μM; n=4; Fig.2A), and this shows that the effect of SNP on PGE_2 release was related to NO-formation by astrocytoma cells.

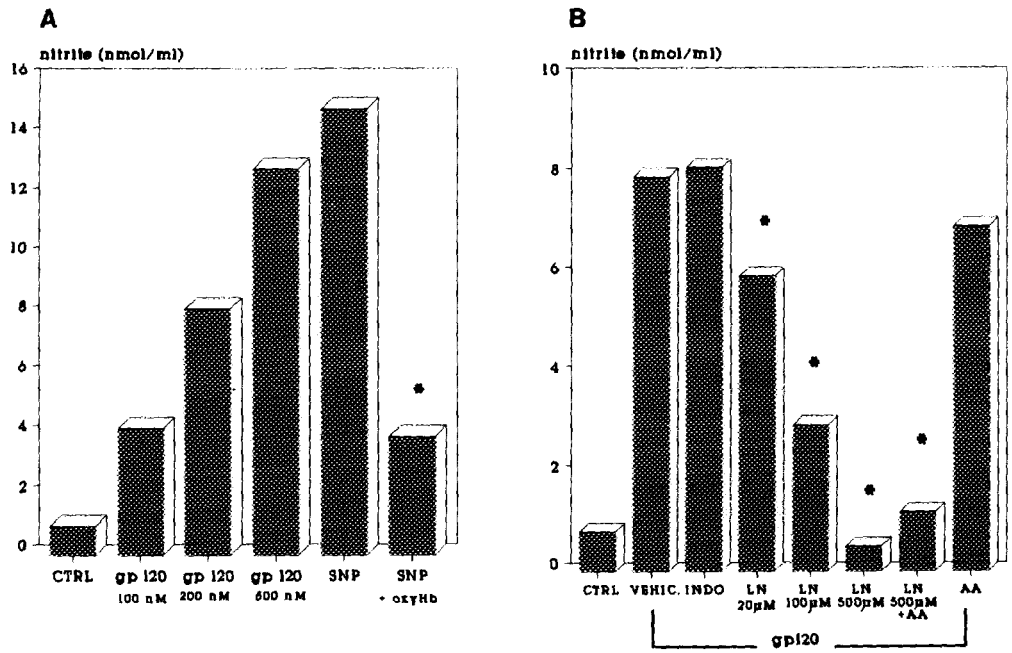

Figure 1. A) Gp 120 glycoprotein (100-500 nM) and SNP enhances the generation of nitrite in the supernatant of human cultured astrocytoma cells. OxyHb (10 μM) inhibited the effect of SNP. Each column represents the mean of 4 experiments. * $P<0.01$ SNP vs SNP in the presence of oxyHb. B) The effect of gp 120 (200 nM) on nitrite levels detectable in supernatant of astrocytoma cells is reduced by the NO synthase inhibitor L-NAME (20-300 μM). Indomethacin (INDO; 10 μM) and arachidonic acid (AA; 30 μM) failed to affect nitrite formation. Columns represent means of 4 experiments. * $P<0.01$ gp 120 alone vs gp 120 in the presence of L-NAME.

DISCUSSION

The present experiments demonstrate that gp 120 enhances the release of PGE_2 by human cultured T 67 astrocytoma cell line and that this effect is mediated by the formation of NO. In fact, L-NAME, an inhibitor of NO synthase, signifcantly inhibited both nitrite formation and PGE_2 levels in supernatants of gp120-treated astrocytoma cells. The inhibition of PGE_2 release by L-NAME was restored by addition of AA, an effect inhibited by indomethacin, thus demonstrating that inhibition of NO-generating machinery interferes with cyclo-oxygenase enzymes in astrocytoma cells. This is in agreement with previous data reporting that both endogenous or exogenous NO increase cyclo-oxygenase activity in activated mouse macrophages (11). In addition, evidence exists showing that NO stimulates prostanoid formation in peripheral tissues as well as in rat hipotalami (12, 13). The interaction of NO with the iron-heme center of cyclo-oxygenase, could well explain the enhancement of PGE_2 release in human astrocytoma cells by NO. Indeed, methylen bleu failed to reduce PGE_2 levels in supernatants of

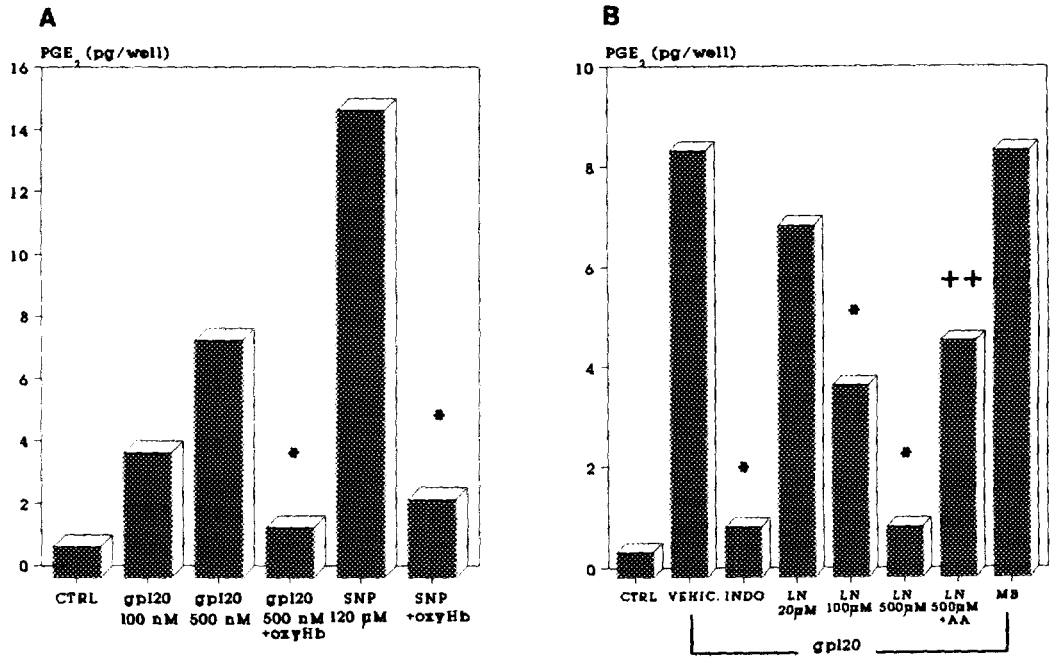

Figure 2. A) Gp 120 glycoprotein (100,500 nM) and SNP enhance the release of PGE_2 in the supernatant of human cultured astrocytoma cells. OxyHb (10 µM) inhibited this effect. Each column represents the mean of 4 experiments. * $P<0.01$ gp 120 and SNP alone vs pretreatment with oxyHb. B) The effect of gp 120 (500 nM) on PGE_2 levels detectable in supernatant of astrocytoma cells is reduced by the NO synthase inhibitor L-NAME (20-300 µM) and by indomethacin (INDO; 10 µM). Arachidonic acid (AA; 30 µM) reverted the effect of L-NAME. Methylene blue failed to affect gp-120-dependent PGE_2 release. Columns represent means of 4 experiments. * $P<0.01$ gp 120 alone vs gp 120 in the presence of L-NAME and ++ $P<0.01$ L-NAME vs L-NAME in the presence of AA.

cultured astrocytoma cells, showing that the stimulation of guanylate cyclase by NO is not involved in gp 120-dependent elevation of PGE_2 release by astrocytoma cells.

The evidence that gp120 simultaneously activates NO synthase and cycloxygenase in astroglial cells is relevant in explaining HIV- related brain damage. Indeed, both NO and arachidonic acid metabolites possess neurotoxic activity (7, 8, 14). In addition, astroglial cells represent a source of such neurotoxins in the brain (15,10), thus playing a role in gp 120-mediated neurotoxicity. The mechanism underlying gp120 activity and the subsequent NO/prostanoid release remains to be elucidated, but the possible involvment of cytokine network could play a role. In fact, cytokines (IL1 and TNF) are known to stimulate the inducible isoform of NO synthase in atstroglial cells and are suggested to mediate the increase of NO following incubation of astrocytes with gp 120 (16, 5).This is also supported by the evidence that the protein synthesis inhibitor cyclohexemide attenuates both the inducible release of NO by astroglial cells (17) and the prostanoid- and cytokine-dependent neurodegenerative effect due to interactions between macrophage/astroglial cells and neurons in the presence of gp 120 (4).

In conclusion, the HIV coating gp 120 glycoprotein stimulates the release of PGE_2 by cultured astrocytoma cells with a mechanism that involves the formation of NO and, subsequently, the activation of cyclo-oxygenase enzyme(s). Therefore, astroglial cells could represent a site of cooperation of NO synthase(s) with cyclo-oxygenase(s) which occurs during HIV infections and may represent a target for a novel pharmacological approach to AIDS-related cerebral disorders.

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