Vol. 194, No. 1, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1993 Pages 439-445

HIV gp120 GLYCOPROTEIN STIMULATES THE INDUCIBLE ISOFORM OF NO SYNTHASE IN HUMAN CULTURED ASTROCYTOMA CELLS

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Received June 4, 1993

SUMMARY: The effect of the HIV coating glycoprotein gp 120 on the generation of NO by human cultured T67 astrocytoma cells was investigated. Preincubation of astrocytoma cells with gp 120 (10 pM, 100 and 500 nM) produced a significant, dose-dependent increase of nitrite levels in supernatant of pretreated cells which was higher when compared to untreated cells. This effect was prevented by coincubation of cells with monoclonal antibodies directed against gp 120, or by pretreatment of cells with the selective NO synthase inhibitor L-NAME (100 μM). The rise of nitrite following pretreatment of astrocytoma cells with gp 120 was accompanied by an increase in NO synthase activity which was mainly Ca^{++}-independent. Also this effect was inhibited by antibodies against gp 120, showing the specificity of the activation of the L-Arg-NO pathway subsequent to incubation of astrocytoma cells with the HIV coating protein. In conclusion, the present results are consistent with an activation of the inducible, Ca^{++}-independent isoform of NO synthase in cultured astrocytoma cells following coincubation with gp 120. This may contribute to explain some of the neuropathological changes accompanying HIV-related cognitive disorders.

The involvement of Central Nervous System (CNS) in Acquired Immunodeficiency Syndrome (AIDS) has recently been reported in up to 80% of cases at *postmortem* (1). In particular, cortical neuronal loss is frequently associated to HIV-related cognitive disorders (see 2), though the mechanism remains still unclear.
Increased intraneuronal Ca^{++} has been described to occur in cultured neurons following incubation with gp120, a component of HIV envelope (see 2) thus

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Abbreviations: NO: nitric oxide; L-NAME: N^{ω}-nitro-L-arginine methyl ester; L-Arg: L-arginine, anti-gp120 Ab: antibodies against gp 120.

0006-291X/93 $4.00

leading to the suggestion that an "excitotoxic" mechanism may be involved. On the other hand, gp 120 has been demonstrated to stimulate glial cells and this also occurred via activation of the cytokine network (3).

It has recently been shown that glial cells release NO (4, 5, 6, 7), a nitrogen free radical generated by the bioconversion of L-Arg into citrulline and spontaneously degradating into nitrite, which is supposed to be neurotoxic under several experimental conditions (8,9). In addition, human cultured astrocytoma cells release NO following incubation with cytokines through activation of an inducible, Ca^{++}-independent isoform of NO synthase, the NO generating enzyme (10).

Therefore, the aim of the present work was to study the effect of gp120 on the L-arginine-NO pathway in cultured astrocytoma cells. This was assessed by measuring nitrite (the breakdown product of NO) in cell supernatant or by detecting the activity of NO synthase in gp 120-pretreated astrocytoma cells.

MATERIALS AND METHODS

Materials. The composition of the modified Krebs' bicarbonate buffer was previously described (see in 7). Sodium hydrosulphyte, hemoglobin (from bovine blood), sodium nitrite, sulphanilamide, naphtylethylendiamine dihydrochloride, N^{ω}-nitro-L-arginine methyl ester (L-NAME) were obtained from Sigma (Milan). Gp 120 (Recombinant HIV-1 gp 120 from Baclovirus expression system; 90 % pure following immunoaffinity chromatography; binding to CD4 detected using ABT's CD4/gp 120 Capture ELISA assay) and monoclonal antibodies directed against gp 120 (anti-gp120Ab; Isotype IgG1 in ascitic fluid, specific immunoneutralization titre 1:40) were a generous gift of Dr. Holmes (NIBSAC, South Mimms, U.K.). Oxyhemoglobin was prepared by reduction of bovine hemoglobin with sodium hydrosulphite as previously described (see in 7).

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, as previously described (11). T67 cells were grown in monolayers within 80-cm^2 Flasks (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.). The neutralization of gp 120 was previously obtained by coincubation with anti gp 120 Ab, diluted 1:20 for 1 h (37° C).Gp 120, BSA and neutralized gp 120 were added to cell cultures 12, 24 and 48h beforehand. The day of the experiments, cells were washed with Hanks' Balanced Salt Solution (HBSS) and gently trypsinized, washed twice in Krebs' buffer without Ca^{++} and resuspended to reach a final concentration of 1x 10^6 cells ml^{-1}. Cell viability in the presence or absence of gp120, BSA and neutralized gp120 was more than 95% as assessed by trypan blue uptake. Incubation of cells with L-NAME for 20 min did not alter viability.

Nitrite analysis. Nitrite (NO_2^-) in supernatants of 5 x 10^5 astrocytoma cells stirred for 5 min at 1,000 rpm and centrifuged at 13,000 x 5 min, either untreated or pretreated with gp 120 or neutralized gp 120 was measured by the Griess reaction. Aliquots of the cell supernatants were mixed with an equal volume of Griess reagent (1% sulphanilamide/0.1% naphthylethylendiamine dihydrochloride/2.5% 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_2^- ml^{-1}.

Determination of NO synthase activity in control or pretreated astrocytoma cells. Astrocytoma cells (5 x 10^5) were homogenized in buffer containing 0.32 M sucrose, 20 mM Hepes buffer, 1 mM EDTA and 1mM dithiothritol and centrifuged at 39,000 x g for 30 min.. The NO synthase activity was then assayed using 340 µl of the supernatant, which was incubated with 60 µl of an incubation medium of the following composition: 2mM NADPH, 0.45 mM $CaCl_2$, 10 µM of calmodulin ml^{-1}, 200 mM arginine, [^{3}H] L-arginine (5 µCi ml-1) in a total volume of 400 µl. After 45 min incubation at 37° C the mixture was loaded on 1 ml Dowex AG50WX-8 (Na^+ form) column and eluted by 5 ml of bidistilled H_2O.

[^{3}H] citrulline obtained by this procedure was measured by a β counter and the ratio between labelled citrulline (dpm) and µg protein assayed in the homogenate was taken as NO synthase activity. Data were expressed as % changes of NO synthase activity in gp 120- or neutralized gp 120- pretreated astrocytoma cells when compared to untreated cells. In order to verify whether Ca^{++} could affect gp 120-dependent changes of NO synthase activity, the astrocytoma cell homogenates were also resuspended in Ca^{++} free buffer (EGTA 1mM). The absence of endotoxin contamination of bidistilled water used for preparing all the assay solutions was assessed as described previously (12).

Statistics. Results are expressed as mean ± s.e.m. 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

Astrocytoma cells (1 x 10^6 cells ml^{-1}, n=10), resuspended in 500 µl of Tyrode buffer, were stirred for 5 min (1000 rpm). The measurement of nitrite level in the supernatant of stirred cells showed detectable concentrations of this breakdown product in a range of 0.8-2 nmol/ ml. OxyHb (10 µM; n=10), a trapping agent for NO coincubated for 5 min with astrocytoma cells, or pretreating cells (30 min) with the NO synthase inhibitor L-NAME (100 µM; n=10), inhibited nitrite generation in supernatant of stirred astrocytoma cells (by 70±3 and 65±2.5%, respectively). Coincubation of cells with gp 120 (10 pM, 100 and 500 nM, n=4 for each concentration) for 48h, produced a significant increase of nitrite levels in astrocytoma cell supernatant (Fig. 1). This effect was reduced by L-NAME (100 µM, n=4) and by coincubation of cells with neutralized gp 120 (n=4), thus demonstrating that the source of nitrite was represented by NO and that gp 120 selectively enhanced its release by astrocytoma cells (Fig. 1).

The increase of nitrite production by gp 120-pretreated astrocytoma cells was accompanied by a time-dependent increase of NO synthase activity in cell homogenates. In fact, gp 120 (100 nM, n=4), coincubated with cells for 12, 24 and 48h beforhand, significantly ($P<0.05$) enhanced the rate of [^{3}H] citrulline generated from [^{3}H] arginine in cell homogenates (Fig. 2A). Furthermore, the removal of Ca^{++} from the incubation medium did not greatly affect [^{3}H] citrulline formation, showing

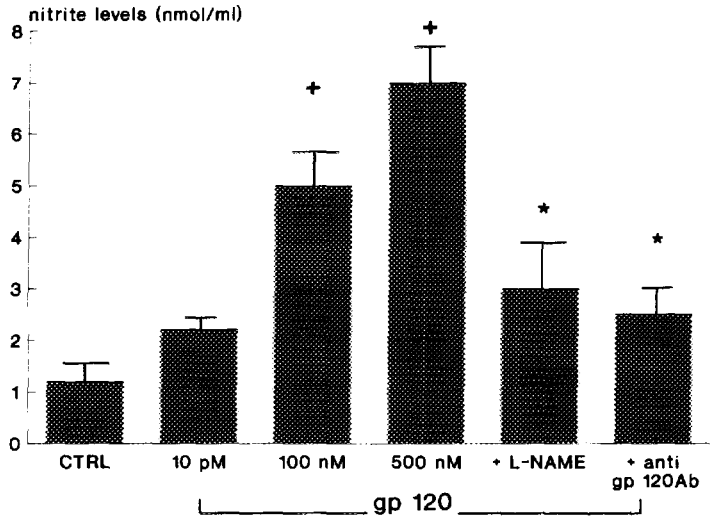

Figure 1. Gp 120 glycoprotein (10 pM, 100 and 500 nM), incubated for 48 h with astrocytoma cells, enhances the production of nitrite (nmol/ml) in supernatants of stirred (1000 r.p.m.) cells. L-NAME (100 μM) or antibodies directed against gp 120 (anti gp 120 Ab; 1:20 dilution) incubated with gp 120 before addition to cells, antagonized the effect of gp 120 (500 nM).
Bars represent the mean ± SE of 4 experiments.
+ $P< 0.05$ between CTRL and gp 120-pretreated astrocytoma cells.
* $P<0.05$ gp 120-pretreated astrocytoma cells vs L-NAME or anti gp 120Ab.

that gp 120 enhanced the inducible, Ca^{++}-independent isoform of NO synthase (n=4, Fig. 2B). Similarly to that described for nitrite production by astrocytoma cells, the effect of gp 120 on NO synthase activity was reversed by coincubation of cells with neutralized gp 120.

The effect of gp 120 was not due to changes in osmolarity of colture medium, nor to aspecific effect depending on its high molecular weight, since bovine serum albumine (BSA; equimolar concentrations) incubated with astrocytoma cells, did not alter nitrite production or NO synthase activity (n=4, not shown). In addition, gp 120 was tested, before being incubated with astrocytoma cells, with anti gp120 Ab using a blot analysis showing up to 60% immunoneutralization under concentrations used in our experiments (dilution 1:20).

DISCUSSION

The present data demonstrate, for the first time, that the HIV coating glycoprotein gp120 enhances the biosynthesis and release of NO for human cultured astrocytoma cells. In fact, coincubation of gp 120 with astrocytoma cells significantly increased the amount of nitrite (the breakdown product of NO) in supernatant of pretreated cells and stimulated the inducible, Ca^{++}-independent isoform of the NO generating enzyme. This may be relevant for explaining some aspects of the HIV-related neuropathology. In fact, evidence exists showing that gp 120 possesses neurotoxic activity both *in vitro*

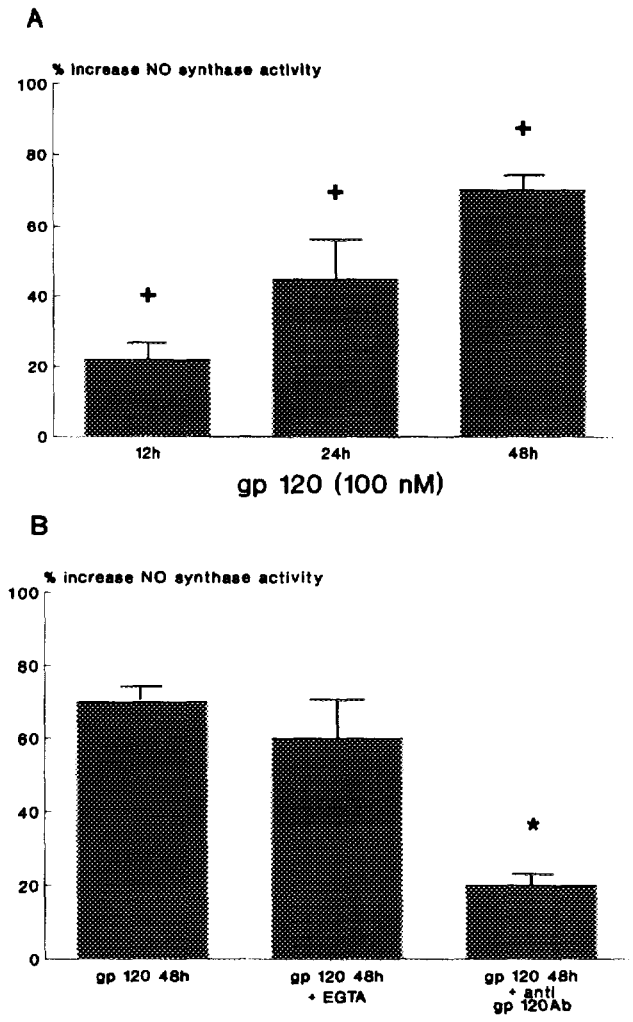

Figure 2. A) Gp 120 glycoprotein (100 nM), coincubated for 12, 24 and 48 h with astrocytoma cells, enhances NO synthase activity in cell homogenates. B) Antibodies directed against gp 120 (anti gp 120 Ab; 1:20 dilution) incubated with gp 120 before addition to cells, antagonized this effect. EGTA (1 mM) failed to affect NO synthase activity.
Bars represent the mean ± SE of 4 experiments.
+ $P < 0.05$ between CTRL and gp 120-pretreated astrocytoma cells.
* $P < 0.05$ gp 120-pretreated astrocytoma cells vs anti gp 120Ab.

and *in vivo* (see 2). In addition, the involvement of glial cells in gp 120-dependent neuropathological effect has recently been supported by evidence that gp 120 may compete with endogenous peptides for a receptor, located on astrocyte cell membrane, that is relevant for neuronal function (13, 14). On the other hand, gp 120 has been reported to produce a significant decrease of Glial Fibrillary Acid Protein (GFAP) staining for astrocytes in human brain aggregates (15). Therefore, the possible release of a "neurotoxic" factor from astrocytes by gp 120 or a modified gp 120 (possibly via

activation of infected macrophages or microglia) has been suggested as a mechanism of action for gp 120 in producing brain damage (see 2).

The present results support the idea that this so far unknown factor may well be represented by NO. In fact, it has recently been demonstrated that NO release, *in vivo*, is involved in NMDA-mediated experimental epilepsy and in neuronal cell death produced by administration of tacrine in LiCl-pretreated rats (8, 9). We have also reported that astrocytoma cells release NO under basal conditions and that an inducible isoform of NO synthase is activated by cytokines (10). In addition, NO seems to be involved in the stimulation of the immunocompetent activity of astrocytoma cells, following stimulation with γ-interferon (16). Since gp 120 enhances the release of cytokines (IL-1ß and TNFα) from glial cells (see 3) it is likely that the activation of L-Arg-NO pathway in astrocytoma cells we have found in our experiments, may occur as a consequence of an increased release of cytokines by astrocytoma cells. Indeed, due to their position between neurons and blood vessels of cerebral microcirculation, astrocytes may well represent a relevant step in the development of HIV-related neuropathology.

Thus, the metabolism of HIV envelope and the release of gp 120 by macrophages or microglia could enhance the release of cytokines from astrocytoma cells and the generation of NO which, in turn, may affect neuronal function and produce brain damage also in the absence of a direct extension of the infection of HIV into the brain, as frequently demonstrated in patients with HIV-related cognitive disorders (see 2).

In conclusion, gp 120 activates the generation of NO from astrocytoma cells through an enhancement of the inducible isoform of NO synthase. This could represent a target for the effect of gp 120 glycoprotein in the brain and may contribute to explain some of the neurological disorders accompanying AIDS.

ACKNOWLEDGMENTS. This work was supported by the VIth 1993 AIDS Project (grant n. 8204-88), Ist. Sup. Sanita', Rome, Italy. The MRC AIDS Directed Programme Reagent Project and Dr. H. C. Holmes (NIBSAC, South Mimms, U.K.) are gratefully acknowledged for generous supply of gp 120. Our thanks to Dr. A.M. Paoletti, Dr. P. Rodino', Dr. R. Massoud, Dr. G. Perno and Mr. Giovanni Politi (Rome, Italy) for their valid collaboration.

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