BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **238,** 916–919 (1997)
ARTICLE NO. RC977155

Spontaneous Induction of Nitric Oxide- and Prostaglandin E_2-Release by Hypoxic Astroglial Cells Is Modulated by Interleukin 1β

Vincenzo Mollace,[1] Carolina Muscoli,* Domenicantonio Rotiroti, and Giuseppe Nisticó
*Department of Biology, University of Rome "Tor Vergata", Rome, Italy; and *Faculty of Pharmacy, University of Reggio Calabria, Catanzaro, Italy*

Received July 15, 1997

The effect of 0, 30, 60, 120, 240, 360 min hypoxia on the release of NO and PGE_2 was investigated in human cultured astroglial cells. Exposure of astroglial cells to hypoxic injury produced a dose-dependent increase of the nitrite (the breakdown product of NO) level in the cell supernatant. In addition, a significant activation of the inducible isoform of NO synthase was seen, demonstrating that the enhancement on NO release produced by hypoxic injury was related to an increased biosynthesis of NO-generating enzyme(s). This effect was strongly antagonised by pretreating cells with dexamethasone (20 μM). The increase in NO release by hypoxic astroglial cells was accompanied by sustained release of PGE_2, which was antagonised by the cycloxygenase inhibitor indomethacin (10 μM), and partially attenuated by L-NAME (100 μM), a nitric oxide synthase inhibitor, showing that the release of PGE_2 was driven by NO. Finally, inducible NOS activity elicited by hypoxic injury, was antagonised by incubating astroglial cells with antibodies directed against type2 receptor for IL1β. In conclusion, hypoxia stimulates cytokine network in astroglial cells leading to enhanced release of NO and prostanoids and this may represent a key mechanism in cerebral blood flow disturbances.

Cerebral blood flow disturbances are accompanied by biochemical derangement within neurones and astroglial cells including severe loss in energy phosphates, profound disruption of ion homeostasis and delayed neuronal damage (see1). Several pathophysiological mechanisms have been proposed in order to explain this complex form of toxicity, including an overproduction of excitatory amino-acids, oxygen-free radicals and acidosis (2), though the exact mechanism of post-ischemic brain damage remains to be elucidated.

Evidence exists that many glial functions are either directly or indirectly energy-dependent and became impaired during substrate depletion as can be found during brain ischemia (3-4). However, the role of astroglial cells in hypoxic/ischemic injury in the brain is still unclear. Activation of astrocytes in vivo after transient ischemia has been demonstrated, leading to appearance of isoforms of several enzymes such as NADPH-diaphorases, which are normally not expressed in astroglial cells (5). In particular, it is known that NADPH diaphorase reflects the activity of nitric oxide synthase (NO synthase), the enzyme which makes NO, a nitrogen free radical species, through biotransformation of L-arginine into citrulline (see 6). Since NO has been shown to affect both vessel tone and neuronal performances either directly or via enhancement of other vasodilating agents such as prostanoids (see 6), it is likely that changes in NO production by astroglial cells may represent a key event in the mechanisms underlying cerebral blood flow disturbances. In addition, the release of NO by astroglial cells has been shown to occur as a consequence of activation of inducible enzymes, mainly via an involvement of cytokine network (7), leading to neuromodulatory activities under physiological conditions as well as in some neurological disorders.

The present experiments have been performed in order 1) to evaluate the release of NO and PGE_2 by human cultured astroglial cells undergoing experimentally induced hypoxic injury and 2) to verify the possible role of the endogenous release of cytokines, such as IL1β, in the activation of astroglial cells which follows hypoxic/ischemic insult.

MATERIALS AND METHODS

Materials. Dexamethasone, superoxide dismutase (from bovine erythrocytes), sodium nitrite, sulphanilamide, naphtylethylendia-

[1] To whom correspondence should be addressed.

Abbreviations used: NO, nitric oxide; L-NAME, N^{ω}-nitro-arginine-methyl ester; PGE_2, prostaglandin E_2; IL1β, interleukin 1β.

0006-291X/97 $25.00

mine dihydrochloride, indomethacin, N^{ω}-nitro-L-arginine methyl ester were obtained from Sigma(Milan). Antibodies directed against type2 receptor for IL1β were a gift from Sclavo (Siena, Italy).

Preparation of astroglial cells and experimental procedures. Human astrocytoma cells (GO-G-UVW-European College for Cell Culture) were characterized by means of monoclonal and polyclonal antibodies directed against Glial Fibrillary Acidic Protein (GFAP), S 100 protein, Fibronectin, Factor VIII and Vimentin. Astroglial cells were grown in monolayers within 80 cm_2 flasks (NUNC, Denmark) containing Ham's-F 10 (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 confluent, cells were exposed for 0, 30, 60, 120, 240 and 360 min to 100% N_2 in an air-tight humidified chamber leading to hypoxic insult (8). When required, Dexamethasone (20 μM), L-NAME (100 μM), indomethacin (10 μM), and antibodies directed against type2 receptor for IL1β were added to the colture medium before induction of hypoxia. In some experiments, after exposure to 100 N_2, cells were washed and resuspended for 30 min in oxygenated Krebs solution in the presence or in the absence of superoxide dismutase (60 U/ml). In all experiments, supernatants were aspirated and then assayed for PGE_2 and nitrite. Cell viability in the presence or absence of hypoxic injury, and after addition of all compounds used in our experiments was more than 95% as assessed by trypan blue exclusion test.

Measurement of PGE_2 in supematant of astrocytoma cells. PGE_2 was assayed by using a specific enzymeimmunoassay (EIA) system (Amersham, U.K.). Briefly, supernatant of astrocytoma cells or standard PGE_2 were incubated for 3h 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 further I 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 measurement. Nitrite (NO_2^-) in astrocytoma cell supernatant, was measured by the Griess reaction. Aliquots of the cell supenatants 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.

Determination of NO synthase activity in astroglial cells. Astrocytoma cells (5×10^5) were homogenized in buffer containing 0.32 M sucrose, 20mM Hepes buffer, 1mM EDTA and 1mM didthiothritol and centrifuged at 39,000 $\times$ 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 $CcCl_2$, 10μM of calmodulin/ml, 200 μM arginine, [$_3$H] L-arginine (5 mCi/ml) in a total volume of 400μl. After 45 min incubation at 37 the mixture was loaded on 1 ml Dowex AG50WX-8 (NA+form) column and eluted by 5 ml of bidistilled H2O.

[3H] citrulline obtained by this procedure was measured by a b counter and the ratio between labelled citrulline (dpm) and mg protein assayed in the homogenate was taken as NO synthase activity. Data were expressed in % changes NO synthase activity when compared to untreated cells. In order to quantify the inducible isoform of NO activity, the astrocytoma cell homogenates were also resuspended in Ca++-free buffer (EGTA 1 mM).

Results are expressed as mean $\pm$ 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

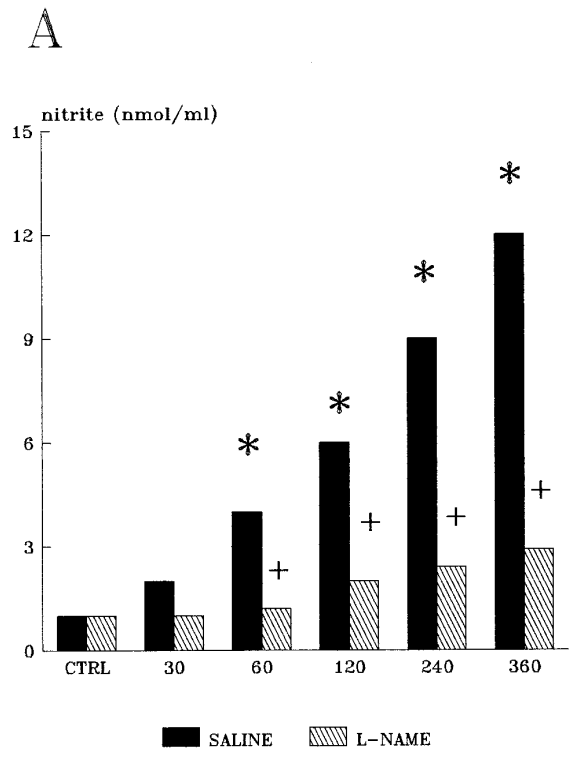
Incubation of human cultured astrocytoma cells into an air tight, oxygen poor chamber for 0, 30, 60, 120, 240 and 360 min produced a significant ($P<0.05$) and time-dependent increase of nitrite in cell supernatant when compared to control, oxygenated astroglial cells (n= 8; Fig. 1). This effect was totally abolished by coincubation of cells with the NO-synthase inhibitor L-NAME (100 μM; n=8), thus suggesting that the increase in nitrite level was due to enhanced formation of NO. In addition, a significant ($P<0.05$), time-dependent increase of inducible, Ca^{++}-calmodulin-independent NO synthase was found in homogenates of cells undergoing hypoxic treatment, mainly after 240 and 360 min, an effect which was blocked by dexamethasone (20 μM; n=8; Fig. 2).

The effect of hypoxic treatment was accompanied by a relevant rise in PGE_2 concentration in supernatant of astroglial cells (Fig. 1; n=8). This effect was blocked when pretreating cells with indomethacin (10 μM; n=8 not shown), an inhibitor of cycloxygenase, being this effect reversed when indomethacin pretreatment was followed by addition of arachidonic acid (40 mM; n=8, not shown), the precursor of prostanoids, showing that hypoxic insult produced an activation o arachidonic acid cascade. Interestingly, the enhanced release of PGE_2 by astroglial cells was not only affected by indomethacin, but also by pretreating cells with L-NAME (100 μM), thus demonstrating that the activation of cycloxygenase by hypoxic injury was, at least in part, driven by NO.

In order to explore the role of endogenous IL1-β on changes seen following hypoxic injury in astroglial NO synthase and cycloxygenase activity, antibodies directed against type 2 receptor for IL-1β were incubated with astroglial cells and nitrite and PGE_2 levels were measured and compared with untreated cells. In cells pretreated with anti-IL1-β type2 antibodies, a significant decrease of nitrite and PGE_2 levels occurred, but only after 240 and 360 min hypoxia, thus suggesting that endogenous release of IL-1β affected only activation of NO synthase and cycloxygenase occurring at later times, probably via enhancement of inducible enzymes (Fig. 3).

DISCUSSION

Evidence has recently been accumulated indicating that the biosynthesis of NO by both endothelial cells and nervous tissues (neurones and astroglial cells) may play a role in the regulation of cerebral blood flow under basal conditions as well as during cerebral ischemia (9-10). In particular, it has been shown that the formation



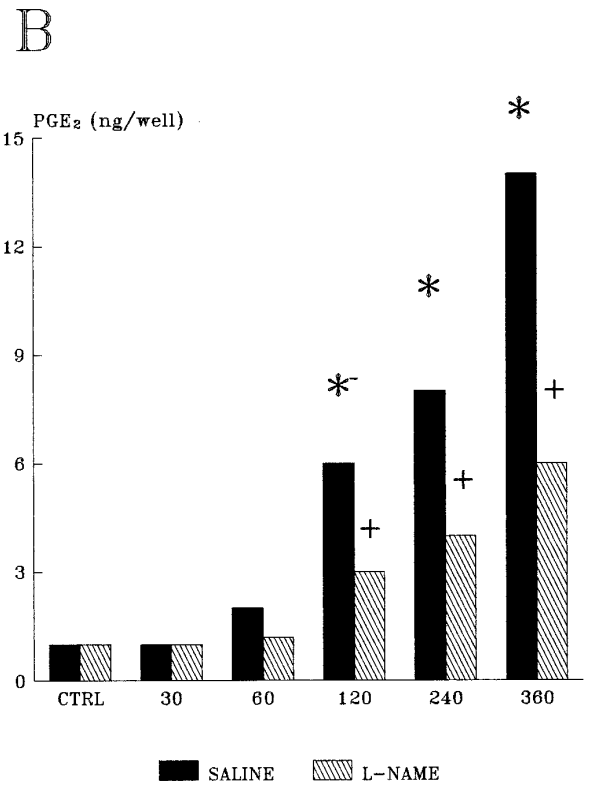


FIG. 1. Induction of nitrite- (A) and PGE_2-release (B) in supernatant of human cultured astroglial cells undergoing 30, 60, 120, 240, and 360 min hypoxic injury. L-NAME (100 μM), reversed this effect. Bars represent the mean of at least 8 experiments. *$P<0.05$ hypoxic vs control cells. †$P<0.05$ between L-NAME-untreated vs pretreated astroglial cells.

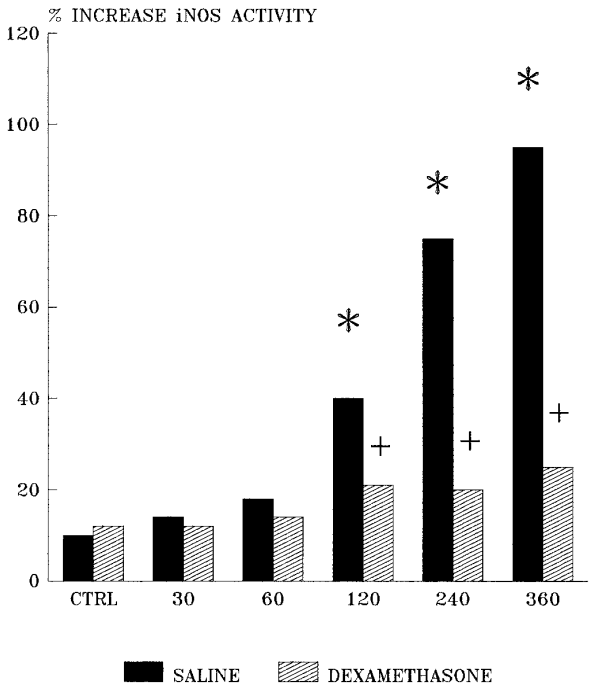


FIG. 2. Enhancement of inducible nitric oxide synthase activity (iNOS) in homogenates of human cultured astroglial cells undergoing 30, 60, 120, 240, and 360 min hypoxic injury. Dexamethasone (20 μM) reversed this effect. Bars represent the mean of at least 8 experiments. *$P<0.05$ hypoxic vs control cells. †$P<0.05$ between dexamethasone-untreated vs pretreated astroglial cells.

of NO from L-arginine becomes important in order to maintain an adequate blood flow (via collateral vessels) during reperfusion which follows global ischemia in mongolian gerbils (11). On the other hand, the inhibition of NO synthesis attenuates NMDA neurotoxicity in neuronal cultures (12) and reduces brain damage produced by occlusion of the middle cerebral artery, strongly suggesting that the release of NO may represent a key mechanisms in post-ischemic brain damage (13).

Cerebral ischemia produces relevant effect on NO biosynthesis in the brain, whose pathophysiological role seems to depend upon cell type in which this free radical is generated and released. Indeed, an early activation of NO biosynthesis in the ischemic area can be found following mean cerebral artery occlusion in rats, an effect clearly mediated by the release of glutamate via NMDA receptors, and this seems to mainly involve constitutive neuronal NO synthase (see 14). On the other hand, after later times, infiltrating neutrophils and astroglial cells has been described to become activated thus expressing high mRNA levels of the inducible, macrophage type NO synthase (see 14). Therefore, almost two of the described isoforms of NO synthase are significantly enhanced by brain ischemia, according to the cell type in which those enzymes are expressed.

The present experiments demonstrate that astroglial

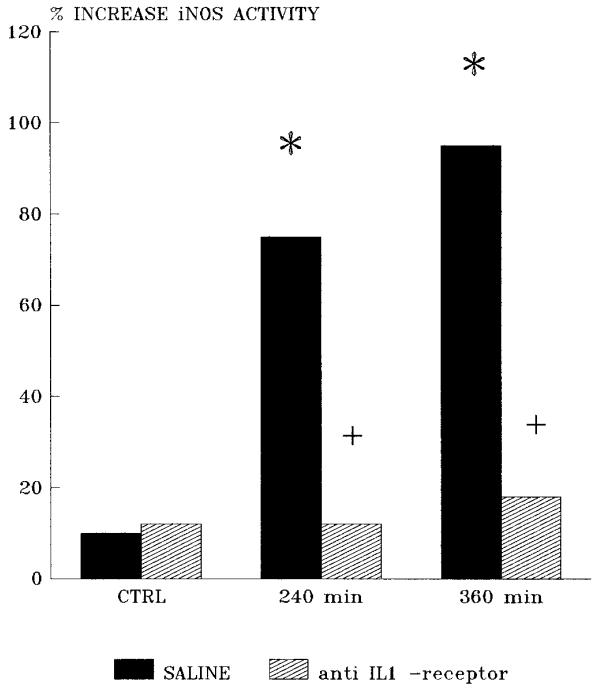


FIG. 3. The increase of iNOS activity in homogenates of astroglial cells undergoing 240 and 360 min hypoxia is reversed by incubating cells with antibodies directed against IL-1β type 2 receptor (see text). Bars represent the mean of at least 8 experiments. *$P<0.05$ hypoxic vs control cells. †$P<0.05$ between anti IL-1β type 2 Ab-untreated vs pretreated astroglial cells.

cells in colture time-dependently react to hypoxia by releasing large amounts of NO, an effect which is associated to a significant activation of cycloxygenase(s), as expressed by the increase on PGE_2 levels in cell supernatant. This effect appears very early and is strictly correlated to a significant increase of both isoforms, being the inducible NO synthase detectable after 4h exposure of astroglial cells to hypoxic insult. In addition, the latter effect is clearly related to an activation of the endogenous release of IL1β by astroglial cells, as recently supposed by others, indicating that the cytokine network may be involved in functional changes occurring in astroglial cells undergoing hypoxia/ischemia insult. This is confirmed by recent observation that many cytokines, and IL1β in particular, are overexpressed in brain tissues after induction of focal as well as global ischemia (see 15), thus participating in the modulation of biochemical changes seen throughout the ischemic area.

In conclusion, our results show that astroglial cells are prone to react to hypoxic insult by releasing NO and prostanoids since early phases of their exposure to oxygen poor environmen. This effect is in part mediated by spontaneous activation of constitutive NO synthase and by activation of cytokine network in the later times of hypoxic insult, leading to activation of inducible NO synthase. Taken togheter, the present data suggest that the coupled NO and prostanoid release by hypoxic astrocytes may strongly interfere with hemodynamic and neuromodulatory processes underlying post-ischemic brain damage and may represent a target for new therapeutical strategies in cerebral blood flow disturbances.

ACKNOWLEDGMENTS

We are indebted to Mr. Giovanni Politi (Rome) for excellent technical assistance and to Mrs. Elisabetta Baboro (Rome) for typing the manuscript. This work was supported by 40 and 60% grants of MURST (Rome, Italy) by National Council for Research (CNR, Rome, Italy) and by Istituto Mondino-Tor Vergata, (Rome, Italy).

REFERENCES

1. Siesjo, B. K. (1981) *J. Cereb. Blood Flow* **1,** 155–185.
2. Schmidt-Kastner, R., and Freund, T. F. (1991) *Neuroscience* **40,** 599–636.
3. Kauppinen, R. A., Enkvist, K., Holopinen, I., and Akerman, K. E. O. (1988) *Neuroscience* **26,** 283–289.
4. Yu, A. C. H., Scusboe, A., and Hertz, L. (1982) *J. Neurochem.* **39,** 954–960.
5. Endoh, M., Maiese, K., Pulsinelli, W. A., and Wagner, J. A. (1993) *Neurosci. Lett.* **154,** 125–128.
6. Moncada, S., Palmer, R. M. J., and Higgs, E. A. (1991) *Pharmacol. Rev.* **43,** 109–142.
7. Mollace, V., and Nisticó, G. (1995) *Adv. Neurimmunol.* **5,** 421–430.
8. Tombaugh, G. C., and Sapolsky, R. M. (1992) *Brain Res.* **588,** 154–158.
9. Faraci, F. M. (1992) *Am. J. Physiol.* **261,** H1038–H1042.
10. Toda, N., and Okamura, T. (1990) *Biochem. Biophys. Res. Commun.* **170,** 308–313.
11. Sancesario, G., Iannone, M., D'Angelo, V., Nisticó, G., and Bernardi, G. (1992) *Funct. Neurol.* **7,** 123–127.
12. Dawson, V. L., *et al.* (1991) *Proc. Natl. Acad. Sci. USA* **88,** 6368–6371.
13. Nowicki, J. P., Duval, P., Poigner, H., and Scatton, B. (1991) *Eur. J. Pharmacol.* **204,** 339–340.
14. Iadecola, C. (1997) *Trends Neurol. Sci.*
15. Minami, M., Kuraishi, Y., and Satoh, M. (1992) *J. Neurochem.* **58,** 390–392.