

Hypoxia/Reoxygenation-mediated Induction of Astrocyte Interleukin 6: A Paracrine Mechanism Potentially Enhancing Neuron Survival

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Summary

To elucidate mechanisms underlying neuroprotective properties of astrocytes in brain ischemia, production of neurotrophic mediators was studied in astrocytes exposed to hypoxia/reoxygenation (H/R). Rat astrocytes subjected to H/R released increased amounts of interleukin (IL) 6 in a time-dependent manner, whereas levels of tumor necrosis factor and IL-1 remained undetectable. IL-6 transcripts were induced in hypoxia and the early phase of reoxygenation, whereas synthesis and release of IL-6 antigen/activity occurred during reoxygenation. Elevated levels of IL-6 mRNA were due, at least in part, to increased transcription, as shown by nuclear runoff analysis. The mechanism stimulating synthesis and release of IL-6 antigen by astrocytes was probably production of reactive oxygen intermediates (ROIs), which occurred within 15–20 minutes after placing hypoxia cultures back into normoxia, as the inhibitor diphenyl iodonium inhibited the burst of ROIs and subsequent IL-6 generation (blockade of nitric oxide formation had no effect on ROI generation or IL-6 production). Enhanced IL-6 generation was also observed in human astrocytoma cultures exposed to H/R. Survival of differentiated PC12 cells exposed to H/R was potentiated by conditioned medium from H/R astrocytes, an effect blocked by neutralizing anti-IL-6 antibody. In a gerbil model of brain ischemia, IL-6 activity was lower in the hippocampus, an area sensitive to ischemia, compared with IL-6 activity in the cortex, an area more resistant to ischemia. IL-6 antigen, demonstrated immunohistochemically, was increased in astrocytes from ischemic regions of gerbil brain. These data suggest that H/R enhances transcription of IL-6, resulting in increased translation and release of IL-6 antigen after the burst of ROI generated early during reoxygenation. Release of IL-6 from astrocytes could exert a paracrine neurotrophic effect in brain ischemia.

Astrocytes are the most abundant cell type in the central nervous system (CNS),¹ and they have an important role in the maintenance of neuronal functions, including regulation of ionic/metabolic milieu (1), transport of neurotransmitters (2), as well as neurotrophic effects (3, 4). This varied spectrum of astrocyte functions confers survival advantage

of neurons cocultured with astrocytes (3) and enables astrocytes to activate immune mechanisms (5–7). The potential of astrocytes to contribute to the host response in ischemia is suggested by their proliferation at sites subjected to ischemia/reperfusion and by their capacity to phagocytose cellular debris (8, 9).

Cultured mononuclear phagocytes have been shown to produce mediators on exposure to hypoxia/reoxygenation (H/R) that are likely to contribute to the pathogenesis of the tissue response in ischemia/reperfusion (10). In this context, Colletti et al. (11) showed that hepatic lobar ischemia induced release of TNF, which increased pulmonary vascular permeability. IL-6, apparently derived from ischemia lym-

¹ Abbreviations used in this paper: CNS, central nervous system; DPI, diphenyl iodonium; H/R, hypoxia/reoxygenation; LDH, lactate dehydrogenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; L-NMMA, N⁶-monomethyl L-arginine; NAC, N-acetyl cysteine; NGF, nerve growth factor; ROI, reactive oxygen intermediate; RT-PCR, reverse transcriptase PCR; SOD, superoxide dismutase.

phocytes, has been reported to promote expression of intercellular adhesion molecule 1 in cardiac myocytes (12). In the brain, astrocytes, as well as microglia and infiltrating macrophages, have the capacity to synthesize cytokines, which alter the properties of myriad target cells (13). This led us to hypothesize that cytokine(s) released by astrocytes exposed to H/R might exert a neuroprotective effect in the setting of ischemia.

The results of our study indicate that exposure of cultured rat astrocytes to hypoxia induces IL-6 transcription, which continues in the early phase of reoxygenation, and that subsequent reoxygenation results in a burst of oxygen free radical production stimulating translation and release of bioactive IL-6 from the cell. Enhanced production of IL-6 was observed in a cultured human astrocyte line and in a gerbil model of bilateral brain ischemia. IL-6 derived from H/R astrocytes promoted the survival of differentiated PC12 pheochromocytes exposed to H/R. Taken together, these studies indicate that the astrocyte response to H/R sets in motion events that could contribute to minimization and repair of ischemic lesions.

Materials and Methods

Cell Culture. Astrocytes and microglial cells were obtained from neonatal rats by a modification of a previously described method (14, 15). In brief, cerebral hemispheres were harvested from neonatal Sprague-Dawley rats within 48 h of birth, carefully separated from meninges, and digested at 37°C with Dispase II (3 mg/ml; Boehringer-Mannheim, Mannheim, Germany) in MEM with Joklick's modification (GIBCO BRL, Gaithersburg, MD). Cells were collected by centrifugation (300 g for 10 min), resuspended in MEM supplemented with FCS (10%; Filtron, Technology Corp., Northborough, MA), and plated in 175-cm² culture flasks (approximately two brains/flask). After 10–14 d, cells were incubated for 48 h with cytosine arabinofuranoside (10 µg/ml; Wako Chemicals, Osaka, Japan) to prevent growth of fibroblasts and oligodendroglial cells. After agitating culture flasks on a shaking platform (Bioshaker BR-30L; Tautek, Tokyo, Japan) for 3 h at 37°C, adherent cells were collected and characterized as astrocytes (see below). Cells floating in the medium were then distributed in 24-well plates, incubated for 30 min at 37°C, and washed vigorously with HBSS. The cells adherent after this wash were harvested and characterized as microglia cells. Their identification was confirmed immunohistochemically by use of monoclonal antibodies for glial fibrillary acidic protein and MAC-1 (generously provided by Dr. A. Wanaka, Second Department of Anatomy, Osaka University Medical School), respectively. Cultures with an homogeneous cell population (>98% staining for one of these two markers) were used for experiments. U373 cells, a human astrocyte cell line, were generously provided by Dr. T. Hirano (Osaka University Medical School). Cells used in the cytokine activity assays—A375S2 cells (IL-1), L-M cells (TNF), and MH-60 cells (IL-6)—were generously provided by Otsuka Pharmaceutical Company (Tokyo, Japan), Dainippon Pharmaceutical Company (Osaka, Japan), and Dr. T. Hirano, respectively.

Conditions for Exposure of Cell Cultures to H/R. Cells were plated in either 24 or 6-well plates or 75-cm² or 150-cm² dishes (Corning, Vernon, NY) and exposed to H/R as described (16, 24). At the indicated time points, pH and oxygen partial pressure in the culture medium were monitored by use of a blood gas analyzer (ABL-2; Radiometer, Copenhagen, Denmark). Viability of astrocytes

during the experiments was assessed by several criteria, including general morphologic characteristics, phase contrast microscopy, trypan blue exclusion, and release of lactate dehydrogenase (LDH) into culture supernatants (the latter determined with a kit obtained from Sigma Chemical Co., St. Louis, MO). In addition, maintenance of general cellular biosynthetic and energetic metabolism was evaluated by incorporation of [³H]leucine into TCA-precipitable material, as described (17), and measurement of cellular ATP levels by chromatography of cell lysates on reversed-phase HPLC (18). Cellular protein content was assessed by the method of Lowry et al. (19). In some experiments, the entire protocol was performed in the hypoxia chamber, whereas in other instances, cultures were exposed to ambient air for varying times after hypoxia (i.e., H/R).

Cytokine Activity Assays. Activities of IL-1, TNF, and IL-6 in medium conditioned by astrocytes during exposure to hypoxia or H/R were studied by use of the IL-1-sensitive A375S2 cell growth inhibition assay (20), the TNF-sensitive L-M cell viability assay (21), and the IL-6-dependent MH-60 cell proliferation assay (22), respectively. In each assay, cell proliferation/viability was assessed by use of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide), as described (23). The MTT assay was performed by plating either A375S2 cells, L-M cells, or MH-60 cells in 96-well plates (~10⁵ cells/well), incubating them for the indicated period, adding astrocyte- (or microglia-) conditioned medium for 24 h, and then MTT. 6 h later, cells were lysed in HCl (0.04 N)/isobutanol (96%, vol/vol), and reduction of MTT was measured by absorbance at 570 nm by use of an ELISA reader (UV-160; Shimadzu, Kyoto, Japan). Bioactivity of each cytokine was determined by comparison with a standard curve generated with A375S2 cells, MH-60 cells, or L-M cells and known concentrations of recombinant human IL-1 (generous gift from Otsuka Pharmaceutical Co.), IL-6 (generously provided by Dr. T. Hirano) or TNF (Dainippon Pharmaceutical Company). In certain assays, neutralizing anti-murine IL-1α antibody (10 µg/ml; Genzyme Corp., Cambridge, MA) or anti-murine TNFα antibody (10 µg/ml; Genzyme Corp.) was added to astrocytes 4 h before reoxygenation. In other experiments, either diphenyl iodonium (DPI), N-acetyl cysteine (NAC), N⁶-monomethyl L-arginine (L-NMMA), or allopurinol (all from Sigma Chemical Co.) was added to cultures 10 min before reoxygenation or at the indicated time points. In some experiments, hypoxic astrocyte-culture medium was replaced with arginine-free MEM (GIBCO BRL) 4 h before reoxygenation.

Immunoprecipitation of Metabolically Labeled IL-6 Antigen. Rat astrocytes (~10⁷ cells) were incubated in methionine/cysteine-poor MEM (GIBCO BRL) under normoxic or hypoxic conditions for 24 h, and the [³⁵S]methionine (0.2 mCi/ml; 1,134 Ci/mmol; New England Nuclear, Boston, MA) and [³⁵S]cysteine (0.1 mCi/ml; 1,038 Ci/mmol; New England Nuclear) were added; labeling continued for another 8 h under each condition. For reoxygenated samples, metabolic labeling was started 29 h after the onset of hypoxia and was continued in the presence of [³⁵S]methionine/cysteine for another 3 h under hypoxia and up to 5 h after reoxygenation (total labeling time was 8 h in each case). Where indicated, cycloheximide (10 µg/ml; Sigma Chemical Co.) or DPI (50 µM; Sigma Chemical Co.), a neutrophil-type NADPH oxidase inhibitor (25), was added at the beginning of reoxygenation and remained present until the end of the experiment. The positive control for induction of IL-6 antigen was obtained from the supernatant of astrocytes treated with LPS (10 ng/ml; Sigma Chemical Co.) for 4 h. Supernatants of astrocytes radiolabeled under each condition were subjected to immunoprecipitation with rabbit anti-murine IL-6 IgG (20 µg/ml; R&D Systems, Inc., Minneapolis, MN) by addition of antibody to conditioned medium (15 ml derived from ~10⁷

cells) and incubation for 12 h at 4°C. Then, a suspension of *Staphylococcus* protein A (0.4 ml/tube; 10%; IgGSorb, Enzyme Center, Malden, MA) was added. The mixture was incubated for 30 min at 4°C, and the precipitate was collected by centrifugation (5,000 rpm for 10 min). The pellet was washed three times in Tris (20 mM; pH 7.4)-buffered saline (0.1 M) containing Triton X-100 (0.05%) and EDTA (1 mM). Samples were then boiled in reducing sample buffer (26) and applied to SDS-PAGE (12.5%). Gels were fixed and soaked in EN³HANCE (New England Nuclear), dried, and then autoradiography was performed. Immunoprecipitation of IL-6 from metabolically labeled cell lysates was performed according to the same method as described above, except that cell pellets were washed three times with Tris (20 mM; pH 7.4)-buffered saline (0.1 M), frozen at -80°C, thawed, and lysed in the same buffer, also containing EDTA (5 mM), PMSF (1 mM), and trasylol (100 U/ml). In some experiments, supernatant and cell lysates from reoxygenated astrocytes were preincubated with murine recombinant IL-6 (1 µg) for 2 h before the addition of anti-IL-6 antibody. Reverse transcriptase (RT) PCR was performed to assess IL-6 transcripts and Southern blot hybridization.

Rat astrocyte total RNA (derived from $\sim 2 \times 10^7$ cells) was extracted by use of the acid guanidium-thiocyanate phenol chloroform method (27) at the indicated time points, and cDNA was prepared by incubating 5 µg RNA for 1 h with RT (BRL, Kyoto, Japan) and oligo dT primer. PCR was performed by use of 24 cycles (94°C for 30 s, 64°C for 40 s, and 72°C for 90 s) and the following primers derived from the rat IL-6 sequence (28): 5' GAC TGA TGT TGT TGA CAG CCA CTG C 3' (sense) and 5' TAG CCA CTC CTT CTG TGA CTC TAA CT 3' (antisense). The reaction mixture consisted of primers (0.2 µM), RT product (1 µg), dNTP (25 µM), and α -³²P]dCTP (0.1 µM). Products were subjected to agarose gel (1%) electrophoresis and autoradiography using x-ray film (Fuji Film, Tokyo, Japan). The amount of IL-6 transcript was semiquantitatively assessed by comparison with the PCR product of actin transcript amplified simultaneously as an internal control (10). PCR products with IL-6 primers amplified by the same procedure described above without α -³²P]dCTP were identified by digestion with HinfI (28) and Southern blot hybridization using radiolabeled murine IL-6 cDNA probe (generously provided by Dr. T. Hirano) according to the method described previously (29).

Nuclear Run-off Analysis for IL-6. To evaluate IL-6 transcription, nuclear run-off assay analysis was performed by use of previously described methods (30). In brief, nuclear suspension (0.2 ml), obtained from $\sim 2 \times 10^7$ astrocytes at the indicated time points, was incubated with CTP, ATP, and GTP (0.5 mM each) in the presence α -³²P]UTP (250 µCi, 3,000 Ci/mmol; New England Nuclear). The positive controls for induction of IL-6 transcription were nuclei isolated from astrocytes treated with LPS (10 ng/ml; Sigma Chemical Co.) for 4 h. Samples were subjected to phenol/chloroform extraction, and RNA was precipitated and resuspended in hybridization buffer. Hybridization to denatured murine IL-6 (20 µg) and human β -actin (5 µg; control) dot blotted onto nylon membranes was performed at 42°C for 2 d. Filters were then washed, dried, and exposed to x-ray film.

Assays for Reactive Oxygen Intermediates (ROIs) and NADPH Oxidase Activity. ROIs were measured by lucigenin-based chemiluminescence by adding bis *N*-methyl acridinium nitrate (lucigenin; 0.2 mg/ml; Sigma Chemical Co.)/CaCl₂ (10 mM) to hypoxic astrocytes ($\sim 10^7$ cells) suspended in PBS at the time of reoxygenation. Chemiluminescence was detected by use of a luminescence reader (BRL-301; Aloka, Tokyo, Japan) after equilibration of the cell suspension with 5% CO₂/room air (31). NADPH activity was measured as described (32) by incubating astrocytes ($\sim 10^7$

cells) for 3 h at 37°C in 2 ml PBS, CaCl₂ (5 mM), and cytochrome C (5 µg/ml). Where indicated, DPI (50 µM), an inhibitor of NADPH oxidase, was added to the incubation mixture. After the incubation period, cell suspensions were centrifuged (1,000 g for 5 min), and OD_{540nm} in the supernatant was measured with a spectrophotometer (UV-160; Shimadzu, Kyoto, Japan). Superoxide production was determined based on the difference of absorbance at 540 nm in the absence and presence of superoxide dismutase (SOD; 1 µg/ml; Sigma Chemical Co.). NADPH oxidase activity was calculated based on the difference of superoxide production in the absence and presence of DPI.

Effect of ROIs on the Production of IL-6. Rat astrocyte cultures plated on 24-well plates were incubated with hydrogen peroxide (0–12 mM, Wako Chemicals) under either hypoxic or normoxic conditions for 1 min, washed three times with MEM containing FCS (0.1%) pre-equilibrated with each atmospheric condition, and further incubated for the indicated period under that condition. IL-6 activity elaborated into the conditioned medium was measured by use of the MH-60 proliferation assay.

Effect of Astrocyte-conditioned Medium on the Survival of PC12 Cells. Rat astrocytes were plated on 150-cm² dishes, exposed to hypoxia (32 h), and reoxygenated in serum-free MEM. Astrocyte-conditioned medium (~ 40 ml) was collected 16 h after reoxygenation and concentrated ~ 20 -fold by ultrafiltration (Amicon, Beverly, MA). PC12 cells (generously provided by Dr. A. Wanaka) were plated in 24-well plates ($\sim 5 \times 10^4$ cells/well) and differentiated as previously described (33). Differentiated PC12 cells were exposed to hypoxia (24 h) in MEM containing FCS (0.1%) and Hepes (7 mM; pH 7.4). At the onset of reoxygenation, medium was replaced with either MEM containing FCS (0.1%), murine recombinant IL-6 (10 ng/ml; Genzyme) or concentrated astrocyte-conditioned medium exposed to H/R and supplemented with FCS (0.1%). In some experiments, concentrated astrocyte-derived medium was preincubated with either neutralizing antimurine IL-6 antibody (10 µg/ml; R&D Systems) or neutralizing antimurine nerve growth factor (NGF) antibody (10 µg/ml; generous gift from Dr. A. Wanaka) for 60 min at 37°C. The viability of differentiated PC12 cells was assessed by morphologic criteria and trypan blue dye exclusion 24 h after reoxygenation.

Assays of IL-6 Activity from Brain Extract and Immunohistochemistry. Adult Mongolian gerbils anesthetized by ether inhalation were subjected to transient forebrain ischemia by bilateral clipping of common carotid arteries for 5 min by the method described previously (34). In sham-operated animals, carotid arteries were exposed without any further manipulations. At the indicated times, gerbils were killed by decapitation and the brain was separated on a cooled plate (4°C) under sterile conditions. The cerebral cortex and the hippocampus were quickly frozen in liquid nitrogen and kept at -70°C. Brain tissues were then homogenized with a polytron in 2 ml (cortex) or 0.5 ml (hippocampus) of PBS (pH 7.4). After centrifugation at 10,000 g for 10 min at 4°C, tissue homogenates were filtered twice through 0.22-µm cellulose filters (Sumilon, Osaka, Japan) and subjected to the IL-6 activity assay, as described above. Protein content was determined by use of a microprotein assay kit (BioRad Laboratories, Richmond, CA). At each time point, blood samples were collected from the tail vein of animals, plasma was separated by centrifugation (1,000 g for 5 min), and cytokine activity was assessed as above.

For immunohistology, gerbil brain was fixed in paraformaldehyde (4%) and embedded in paraffin. Sections were reacted with goat anti-mouse IL-6 (20 µg/ml; R&D Systems) or nonimmune goat IgG (20 µg/ml; Sigma Chemical Co.). Sites of binding of primary antibody were visualized with rabbit anti-goat peroxi-

dase-conjugated affinity-purified IgG (1 $\mu\text{g/ml}$). Where indicated, after sections were reacted with anti-mouse IL-6 and the peroxidase conjugate, sections were decolorized, antibodies were eluted with ethanol (100%), and peroxidase was blocked by hydrogen peroxide (0.5% for 30 min). Then, sections were reacted with rabbit anti-glial fibrillary acidic protein IgG (75 $\mu\text{g/ml}$; Sigma Chemical Co.), and sites of antibody binding were visualized with goat anti-rabbit IgG conjugated to alkaline phosphatase (0.5 $\mu\text{g/ml}$; Sigma Chemical Co.).

Statistical analysis was performed by Neuman-Kuehl's methods for multiple comparison after analysis of variance.

Results

Astrocyte Viability During Exposure to H/R. Oxygen tension in the medium fell to ~ 8 torr within 3–5 h after transfer of astrocyte cultures to the hypoxia chamber. Cell viability was well maintained throughout H/R, based on multiple criteria including release of LDH into the culture supernatant, trypan blue exclusion, and morphologic features. Total cellular protein synthesis in cultures exposed to hypoxia for 48 h, assessed by incorporation of [^3H]leucine, decreased to $68.4 \pm 1.8\%$ after 48 h, compared with normoxic control cultures, and was paralleled by a decrease in cellular ATP content ($70.2 \pm 2.8\%$ of normoxic culture). During the period of reoxygenation, protein synthesis appeared to rebound, achieving levels ~ 1.7 times that seen in normoxia within 3 h.

General protein synthesis and ATP returned to pretreatment (i.e., normoxic) levels within 24 h of replacement into the ambient atmosphere. Cellular protein content was not significantly diminished under these conditions compared with cultures maintained in normoxia.

Induction of Cytokines from Cultured Astrocytes by H/R. Conditioned medium from astrocytes exposed to H/R promoted the proliferation of MH-60 cells, suggesting that increased amounts of IL-6 activity were present (Fig. 1, A and B). Consistent with this concept, neutralizing anti-murine IL-6 antibody blocked the enhanced proliferation of MH-60 cells resulting from H/R astrocyte-conditioned medium (Fig. 1 B). Release of IL-6 by astrocytes required a period of hypoxia (≥ 16 h) followed by reexposure to normoxia (i.e., reoxygenation); IL-6 activity was only found in culture supernatants during the subsequent reoxygenation period, being detectable within 8 h and increasing up to 32 h (Fig. 1 A). No IL-6 activity was detectable in the conditioned medium or cell lysates of astrocytes exposed only to hypoxia or normoxia. The degree of hypoxia was also critical, as only severe oxygen depletion ($p\text{O}_2 \sim 8\text{--}24$ torr) induced astrocyte IL-6 production (Fig. 1 B). The specificity of H/R for induction of astrocyte IL-6 was suggested by the observation that cultured microglia subjected to the same conditions did not produce IL-6, although exposure of microglia to LPS did result in release of IL-6 activity (Fig. 1 C). In contrast to H/R-

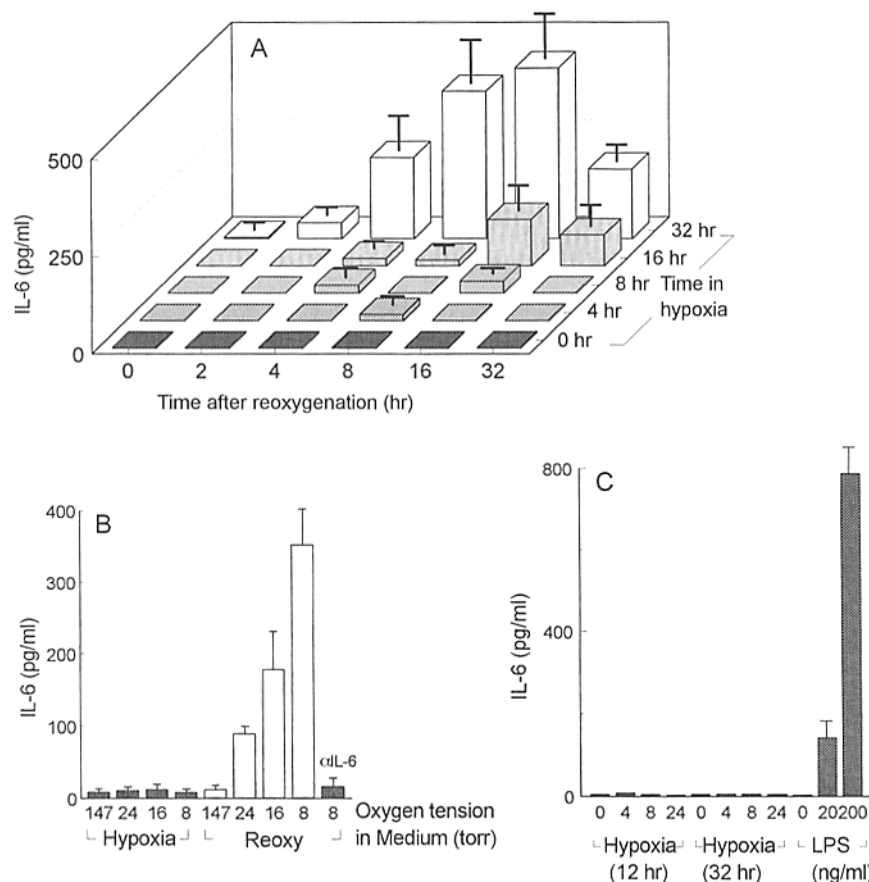


Figure 1. Elaboration of IL-6 activity by cultured astrocytes and microglia exposed to H/R. (A) Rat astrocytes or microglia ($\sim 10^5$ cells/well) were exposed to hypoxia for the indicated periods (0–32 h) and then reoxygenated as described in Materials and Methods. Then, culture supernatants were harvested and IL-6 activity in the medium (500 μl /well) was measured with the MH-60 cell proliferation assay. (B) Astrocytes were exposed to the indicated oxygen tensions for 32 h and then reoxygenated (i.e., returned to normoxia). Conditioned medium was harvested at the end of hypoxia (Hypoxia) and 16 h after reoxygenation (Reoxy), and IL-6 activity was assessed with the MH-60 proliferation assay. Samples from reoxygenated astrocytes were preincubated with neutralizing antimurine IL-6 antibody (10 $\mu\text{g/ml}$) for 30 min before MH-60 assay (indicated by α -IL-6). (C) Microglial cultures were exposed to hypoxia (12 or 32 h), and samples were harvested at the end of hypoxia (0) and 4, 8, and 24 h after reoxygenation. Microglia were also activated by exposure to LPS (20 or 200 ng/ml). In each case, $n = 8$, and values are expressed as the mean \pm SD.

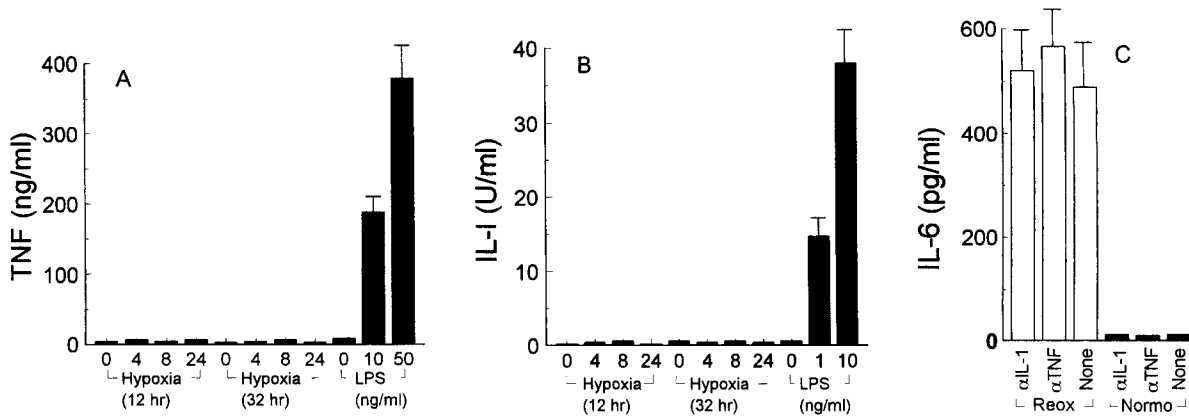


Figure 2. Effect of H/R on astrocyte production of TNF and IL-1, and the effect of anti-TNF and anti-IL-1 antibody on astrocyte release of IL-6 in response to H/R. (A and B) Cultured rat astrocytes ($\sim 10^5$ cells/well) were exposed to hypoxia for either 12 or 32 h followed by reoxygenation. At each time point (0, 4, 8, and 24 h after reoxygenation), TNF (A) and IL-1 (B) activity in the conditioned medium was measured by use of the L-M cell cytotoxicity assay and the A375S2 cell growth inhibition assay, respectively. Activity of cytokines present at the end of the period of hypoxia was expressed as time point 0. Positive controls included astrocytes exposed to the indicated concentration of LPS for 32 h. (C) Either blocking antimurine TNF IgG (10 μ g/ml) or antimurine IL-1 IgG (10 μ g/ml) was added to culture medium of normoxic astrocytes (Normo) or 4 h before reoxygenation (Reox); these cultures had already been subjected to hypoxia for 32 h, and after 12 h of incubation, release of IL-6 activity was measured by use of the MH-60 assay. In each case, $n = 8$, and values are expressed as the mean \pm SD.

induced astrocyte production of IL-6, no TNF or IL-1 activity was detected even after prolonged exposure to hypoxia or H/R (Fig. 2, A and B), but astrocytes exposed to LPS (positive controls) produced both IL-1 and TNF (Fig. 2, A and B). Neither neutralizing antibody to murine TNF nor IL-1 altered production of IL-6 by astrocytes exposed to H/R (Fig. 2 C). These data indicate that H/R-induced astrocyte production of IL-6 is not likely to result from prior endogenous production of TNF or IL-1.

PCR analysis using IL-6 primers indicated that IL-6 mRNA was induced in astrocytes during hypoxia (Fig. 3 A), even though there was no evidence of increased IL-6 activity until the subsequent reoxygenation period. Levels of IL-6 transcripts increased at the end of hypoxia compared with normoxia (Fig. 3 A), whereas amplification of β -actin mRNA showed no significant change in transcript levels (Fig. 3 C). During reoxygenation, there was a further increase in the level of IL-6 transcripts for up to 1 h, thereafter falling off to the normoxic baseline by 8 h (Fig. 3 A). Identity of amplicons with IL-6 primers as derived from IL-6 mRNA was confirmed by probing blots with radiolabeled IL-6 cDNA and cleavage of PCR products by *Hinf*I (Fig. 3 B) (28). Elevation of steady-state IL-6 message levels during hypoxia and early in reoxygenation was associated with increased transcription as demonstrated by the nuclear run-off assay (Fig. 4).

Taken together, these data suggest that release of IL-6 occurred from H/R-astrocytes during reoxygenation, whereas the levels of IL-6 mRNA began to increase substantially during hypoxia. To gain further insights into mechanisms underlying the synthesis of IL-6 antigen, immunoprecipitation of metabolically labeled astrocyte cultures exposed to hypoxia or H/R was performed. Immunoprecipitation of supernatants from astrocytes exposed to hypoxia alone showed no evidence of an immunoreactive IL-6 band, as was also ob-

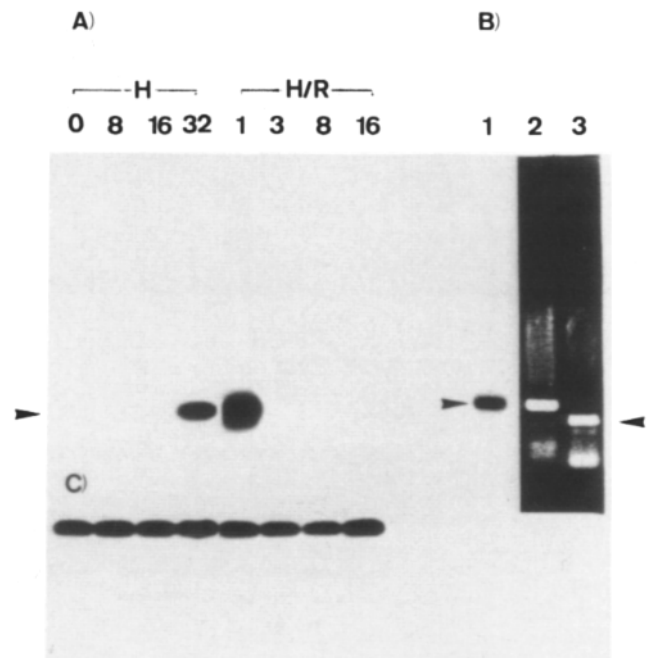


Figure 3. PCR analysis of IL-6 transcripts, identification of amplified IL-6 transcripts, and internal control. RNA extracted from cultured astrocytes ($\sim 2 \times 10^7$ cells) exposed to H/R (0, 8, 16, or 32 h under hypoxia and 1, 3, 8, or 16 h after reoxygenation) was processed as described and amplified by use of either rat IL-6 primers (A) or rat β -actin primers (control; C). In B, the identity of PCR-amplified IL-6 transcripts after 32 h of hypoxia (lane 2) was confirmed by either Southern blotting (lane 1) using murine 32 P-labeled IL-6 cDNA or by cleavage of the IL-6 amplicon with the restriction enzyme *Hinf*I (lane 3).

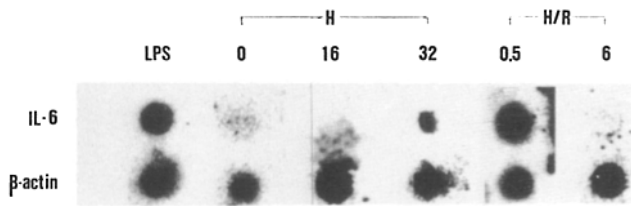


Figure 4. Nuclear run-off analysis of IL-6 transcripts in astrocytes exposed to hypoxia and H/R. Astrocytes (2×10^7 cells) were exposed to either LPS (10 ng/ml for 4 h) or H/R (0, 16, or 32 h of hypoxia [H] and 0.5 or 6 h of subsequent reoxygenation), and nuclei were extracted with Tris-buffered saline containing NP-40 (1%). Nuclear run-off analysis was performed as described, then hybridized with murine IL-6 and β -actin (control) cDNA probes, and subjected to autoradiography.

served with cultures exposed to normoxia (Fig. 5 A, *Hypo* and *Normo*). Consistent with these data, immunoprecipitates of cell lysates from hypoxia astrocytes gave no immunoreactive band with anti-IL-6 antibody (see below), although multiple background bands were observed (Fig. 5 B, *Hypo* and *Normo*). In contrast, immunoprecipitation of supernatants from astrocytes exposed to H/R yielded an ~ 26 -kD band whose appearance was blocked by addition of excess unlabeled IL-6 during incubation of astrocyte-derived samples with the primary antibody (Fig. 5 A, *Reox* and *R-Cold*, respectively). Similarly, precipitation of metabolically labeled astrocyte lysates gave an ~ 26 -kD band (as in the conditioned medium) whose appearance was also blocked by excess unlabeled IL-6 (Fig. 5 B, *Reox* and *R-Cold*, respectively). Inclusion of cycloheximide in the medium prevented astrocyte

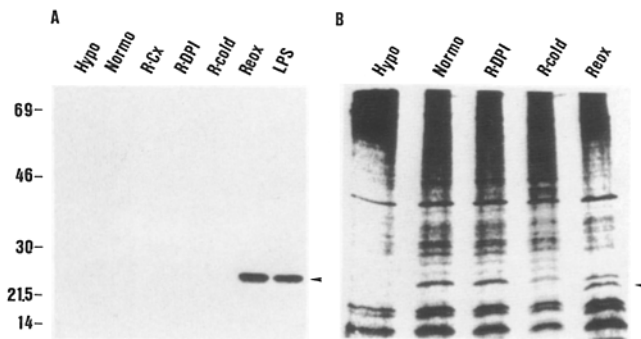


Figure 5. Immunoprecipitation of IL-6 from (A) metabolically labeled astrocyte culture supernatant and (B) cell lysate. Astrocytes ($\sim 10^7$) were maintained in cysteine/methionine-poor medium and were labeled after either hypoxia (*Hypo*) alone, hypoxia followed by reoxygenation (*Reox*), or exposure to normoxia (*Normo*) alone by adding to [35 S]methionine and [35 S]cysteine for the final 8 h of the experiment (details of the protocol are described in Materials and Methods). As a positive control, supernatant was obtained from astrocytes incubated with LPS (10 μ g/ml) for 4 h. At the time of reoxygenation, either cycloheximide (10 μ g/ml; *R-Cx*) or DPI (50 μ M; *R-DPI*) was added to the medium as indicated. In some experiments, unlabeled murine recombinant IL-6 (1 μ g) was preincubated 2 h before addition of anti-IL-6 IgG to reoxygenated cultures (*R-cold*). Immunoprecipitates were solubilized in SDS-PAGE sample buffer and subjected to SDS-PAGE (12.5%). Migration of molecular weight standards run simultaneously is shown in kilodaltons. Details of procedures are described in Materials and Methods.

production/release of material immunoprecipitated by anti-IL-6 antibody (Fig. 5 A, *R-Cx*), indicating a mechanism involving de novo protein synthesis.

Generation of ROIs by Astrocytes Exposed to H/R and their Role in IL-6 Production. Since expression of IL-6 antigen occurred only after reoxygenation of hypoxic astrocytes, we considered the hypothesis that ROIs are important in triggering synthesis of IL-6 antigen. First, the time course for generation of ROIs by astrocytes exposed to H/R was studied by lucigenin-dependent chemiluminescence: a peak of ROIs was evident within 15 min of replacement of cultures from hypoxia back to normoxia, lasting for ~ 60 min (Fig. 6 A). Addition of NAC to the medium suppressed ROI generation

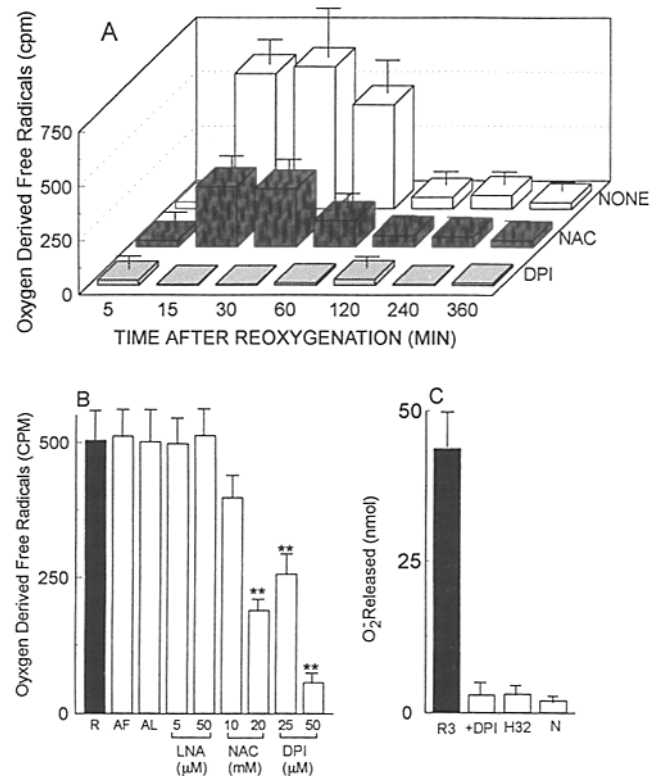


Figure 6. Generation of ROIs by cultured astrocytes exposed to H/R: effect of arginine-free medium (AF), L-NMMA (LNA), DPI, NAC, allopurinol (AL), and SOD. (A) Astrocytes ($\sim 10^7$) were exposed to hypoxia for 32 h, and cells were resuspended in PBS containing CaCl_2 (5 mM) and lucigenin (0.2 mg/ml). Either DPI (50 μ M) or NAC (20 mM) was added at the time of reoxygenation, and chemiluminescence was monitored at the noted time intervals. (B) The experiment was performed as in A except that cells were exposed to hypoxia for 32 h and reoxygenation for 60 min after the addition of the indicated concentration of allopurinol (AL; 10 μ M), L-NMMA (LNA), NAC, or DPI. AF indicates experiments in which cultures were preincubated with arginine-free medium 4 h before and during reoxygenation. $**p < 0.01$ by Neuman-Keuhl's analysis. (C) Superoxide generation by astrocytes ($\sim 10^7$ cells) during the first 3 h of reoxygenation after 32 h of hypoxia was measured with the cytochrome C reduction assay by determining the difference in OD at 540 nm in the absence minus the presence of SOD (1 μ g/ml; *R3*). Suppression of superoxide generation on addition of DPI (50 μ M) to astrocytes subjected to H/R as above is shown by the bar denoted by +DPI. Superoxide generation by astrocytes exposed to hypoxia (32 h) or normoxia (N) alone is also shown. For these experiments, $n = 6$, and the mean \pm SD is shown. Details of procedure are described in Materials and Methods.

(20 mM), and DPI blocked ROIs virtually completely (Fig. 6, *A* and *B*). In contrast, allopurinol (Fig. 6 *B*, *AL*) was not an effective inhibitor of ROI formation by H/R astrocytes, consistent with lack of involvement of xanthine oxidase. Since DPI also inhibits nitric oxide synthetase [(35); the latter enzyme can be induced in cultured astrocytes (36)], it was necessary to assess the contribution of nitric oxide to induction of IL-6. Preincubation astrocyte cultures with arginine-free medium or L-NMMA had no effect on the subsequent generation of ROIs (Fig. 6 *B*, *AF* and *LNA*). These data suggest that NADPH oxidase-like activity in astrocytes likely accounted for generation of ROIs. The nature of the ROIs generated by H/R astrocytes was further characterized in the presence of SOD by use of the cytochrome C reduction assay (Fig. 6 *C*). Superoxide generation, the component of cytochrome C reduction suppressed in the presence of SOD (Fig. 6 *C*, *R3*) was blocked by inclusion of DPI (Fig. 6 *C*, *R3*, +DPI), whereas astrocytes exposed to hypoxia or normoxia alone (Fig. 6 *C*, *H32*, *N*) demonstrated no ROI formation. These data suggest that superoxide formation in astrocytes subjected to H/R was due to an NADPH oxidase-like activity.

To determine if there was a link between generation of ROIs by astrocytes exposed to H/R and production of IL-6 antigen, the effect of DPI on IL-6 expression was examined. When H/R-astrocytes were subjected to immunoprecipitation in the presence of DPI, suppression of the IL-6-immunoreactive band was observed in both supernatants and cell ly-

sates (Fig. 5, *A* and *B*, *R-DPI*). A similar inhibition of IL-6 release by astrocytes exposed to H/R was observed when IL-6 activity was studied in conditioned medium in the presence of DPI (Fig. 7 *A*) (DPI had no effect on determination of IL-6 activity in the bioassay when tested in the presence of exogenous IL-6). In addition, inclusion of L-NMMA had no effect on subsequent generation of IL-6 (Fig. 7 *A*, *LNA*). Thus, generation of ROIs, which occurs relatively early during the period of reoxygenation, has an important stimulatory effect on the synthesis of IL-6 antigen. Consistent with this concept, to inhibit IL-6 release, DPI had to be added either before or very close to the beginning of reoxygenation, as 30 min later it was ineffective in preventing IL-6 release/production (Fig. 7 *B*).

If our hypothesis concerning ROIs as the stimulus for translation and release of IL-6 is correct, we reasoned that hypoxic astrocytes, with increased levels of IL-6 transcripts, should be capable of producing increased amounts of IL-6 if exposed to an exogenous source of ROIs, even in continued hypoxia. To test this, astrocytes preincubated under hypoxia were exposed to hydrogen peroxide for 1 min (cells were still in the hypoxic environment during this period) followed by continued incubation under hypoxia. Oxygen tension in the medium of hypoxic astrocytes declined to <10 torr within 5 min after the displacement of hydrogen peroxide. Release of IL-6 activity was dependent on the concentration of hydrogen peroxide (Fig. 8 *A*), and occurred in a time-dependent manner, first evident by 2 h and increasing up to

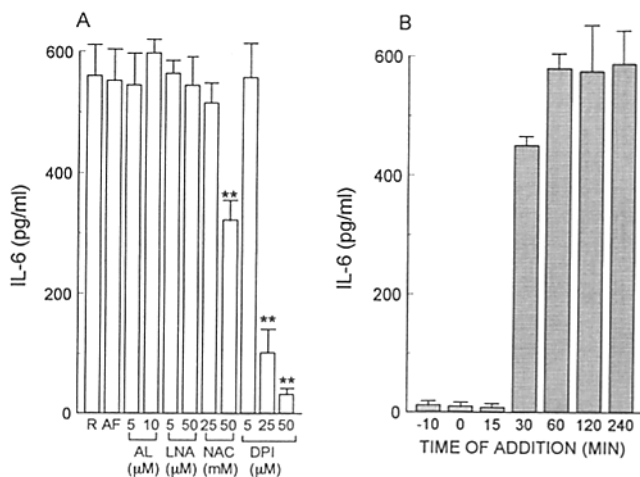


Figure 7. Effect of arginine-free medium, L-NMMA, DPI, NAC, and allopurinol on induction of IL-6 activity by astrocytes exposed to H/R. (A) Astrocytes ($\sim 10^5$ cells/well) were exposed to hypoxia (32 h) followed by 16 h of reoxygenation. Culture medium was then replaced with either arginine-free medium (*AF*) 2 h before and during reoxygenation, or, in other experiments, L-NMMA (*LNA*), DPI, NAC, or allopurinol (*AL*), at the indicated concentrations, was added to the culture 10 min before and during reoxygenation. IL-6 activity elaborated into the medium was assessed after 16 h by use of the MH-60 cell proliferation assay. $***p < 0.01$ by Neuman-Keuhl's analysis. (B) Astrocytes (10^5 cells/well) were exposed to hypoxia for 32 h. DPI (50 μ M) was then added 10 min before (–10) reoxygenation and at the indicated time points after reoxygenation (the start of reoxygenation is designated as time 0). Data shown indicate the mean \pm SD, and $n = 8$.

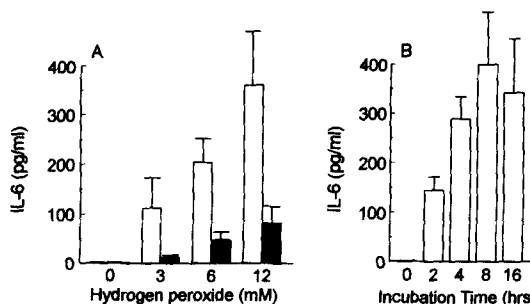


Figure 8. Induction of astrocyte IL-6 by hydrogen peroxide. (A) Astrocytes in 24-well plates ($\sim 10^5$ cells/well) were incubated under hypoxic (open bars) or normoxic (solid bars) conditions for 24 h. Hydrogen peroxide at the indicated concentration (0–12 mM) was added for 1 min, and then cultures were washed three times and incubated for an additional 12 h in the same environment as used previously (hypoxia or normoxia). Conditioned medium was harvested after 12 h for determination of IL-6 activity. (B) Astrocytes were exposed to hypoxia as above for 24 h, hydrogen peroxide (12 mM) was added for 1 min, followed by extensive washing, and the incubation was continued for the indicated times under hypoxia. Astrocyte-conditioned medium was harvested and IL-6 activity assessed. In each case, "0" denotes the activity of IL-6 present in the culture supernatant before exposure to hydrogen peroxide. The means \pm SD ($n = 8$) are shown.

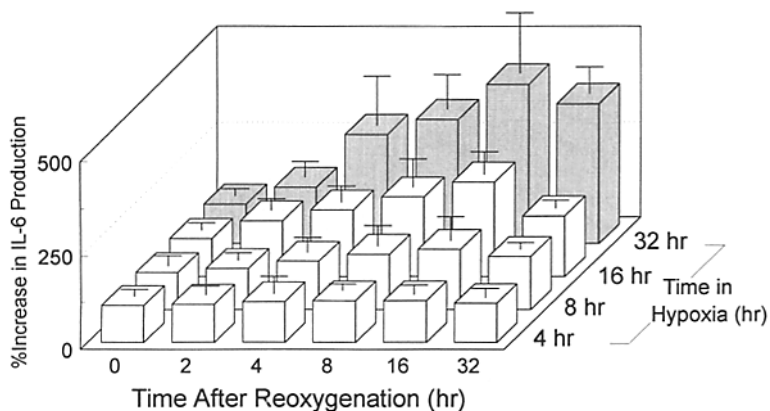


Figure 9. Effect of H/R on IL-6 production by the human astrocyte cell line U373. U373 cells ($\sim 3 \times 10^5$ cells/well) were exposed to hypoxia (4–32 h) followed by reoxygenation. At the indicated times, conditioned medium was harvested, and IL-6 activity was measured by use of the MH-60 cell proliferation assay. Values are expressed as the percentage increase over basal IL-6 production by U373 cells in normoxic condition. Data represent the means \pm SD ($n = 6$).

8 h (Fig. 8 B). Elaboration of IL-6 activity by hypoxic astrocytes exposed to hydrogen peroxide (Fig. 8 A, *open bars*) was much greater than in normoxic cultures subjected to the same treatment (Fig. 8 A, *solid bars*). The time course of IL-6 release was somewhat more rapid when exogenous hydrogen peroxide was added to hypoxic astrocyte cultures (Fig. 8 B) compared with reoxygenation, probably due to addition of such a strong oxidant in a concentrated fashion (compared with the more sustained production of lower levels of ROIs in reoxygenated astrocytes).

H/R-mediated Induction of IL-6 Activity in a Human Astrocyte Cell Line. H/R-mediated enhancement of astrocyte IL-6 production was also observed in the U373 human astrocyte cell line. U373 cells have been shown to generate IL-6 con-

stitutively under normoxic conditions (122.3 ± 20.1 pg·ml⁻¹·well⁻¹ in 12 h) (37). Exposure of U373 cells to H/R resulted in IL-6 release above the normoxic baseline by $\sim 410 \pm 20.3\%$ after 16 h of reoxygenation. This increase was most apparent in reoxygenated cultures previously exposed to hypoxia for 32 h (Fig. 9). Hypoxia alone had no effect on U373 cell IL-6 production (data not shown).

Effect of Astrocyte-conditioned Medium After R on Survival of Differentiated PC12 Cells. To assess a potentially neurotrophic role of astrocyte-derived IL-6, the effect of conditioned medium of astrocytes to H/R on the viability of PC12 cells exposed to H/R was assessed. Differentiated PC12 cells (Fig. 10, 1) were vulnerable to injury by H/R under serum-poor conditions; almost all cells became nonviable after 24 h hypoxia

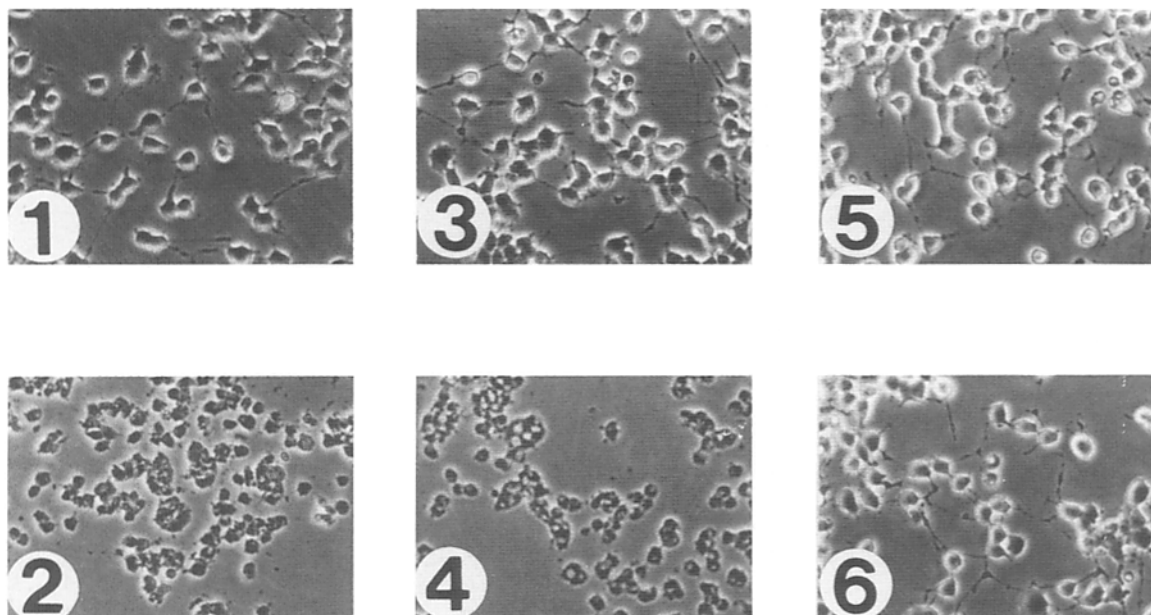


Figure 10. Effect of astrocyte-conditioned medium on the viability of PC12 cells exposed to H/R. PC12 cells in 24-well plates ($\sim 5 \times 10^4$ cells/well), differentiated as described (1), were exposed to hypoxia (24 h) followed by reoxygenation (12 h). At the start of reoxygenation, culture medium was changed to either MEM containing IL-6-free fetal bovine serum (0.1%; 2), purified recombinant IL-6 (10 ng/ml; 6), or conditioned medium, concentrated 20-fold, derived from astrocytes exposed to H/R (32 h/16 h; 3). In 4 and 5, concentrated astrocyte-conditioned medium was preincubated with either antimurine IL-6 IgG (10 μ g/ml; 4) or antimurine NGF IgG (10 μ g/ml; 5) for 60 min at 37°C before addition to PC12 cultures. Phase contrast micrographs under each condition are shown ($\times 400$).

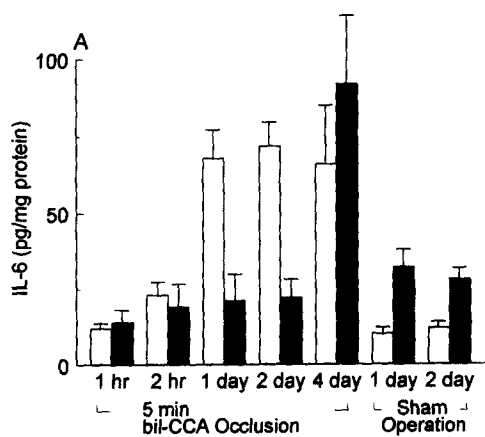
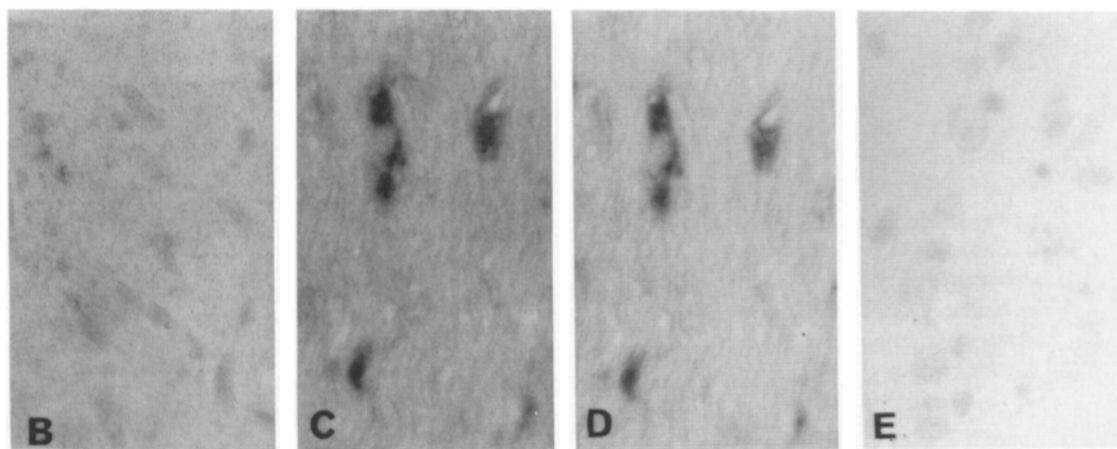


Figure 11. Expression of IL-6 activity and immunohistochemical evidence of IL-6 antigen in gerbil brain after bilateral occlusion of the carotid artery. (A) Tissues were homogenized and assayed for IL-6 activity. Protein content of samples was assessed by BioRad microprotein assay. Cerebral cortex (open bars) and hippocampus (shaded bars) were separated from adult Mongolian gerbils subjected to transient forebrain ischemia for 5 min or sham-operated controls. Values shown represent means \pm SE ($n = 4$). (B–E) Tissues were immunostained for IL-6 antigen as described in Materials and Methods. B and C show normoxic ($\times 340$) and ischemic ($\times 560$) gerbil cerebral cortex, respectively, stained with anti-IL-6 IgG. D shows the same section as in C, now double stained to reveal the astrocyte marker glial fibrillary acidic protein ($\times 560$). E shows ischemic gerbil cortex stained with nonimmune IgG ($\times 340$).



followed by 12 h of reoxygenation (cell viability was $<20\%$ by trypan blue exclusion; Fig. 10, 2). Murine recombinant IL-6 (10 ng/ml) or concentrated astrocyte-conditioned medium added at the time of reoxygenation had a protective effect, increasing the viability of PC12 cells subjected to H/R to $>80\%$ (Fig. 10, 3 and 6). The protective effect of astrocyte-conditioned medium was diminished by preincubation with neutralizing antimurine IL-6 antibody (10 $\mu\text{g/ml}$; Fig. 10, 4), but not by neutralizing antimurine NGF antibody (10 $\mu\text{g/ml}$; Fig. 10, 5). These data indicate that astrocytes may exert a neurotrophic effect during H/R through production of IL-6.

Expression of IL-6 in the Brain After Ischemia/Reperfusion. To complement our studies in cell culture, expression of IL-6 activity was studied in brain tissue of gerbils after transient bilateral occlusion of the common carotid artery. In the cerebral cortex, where viability of neurons is well maintained during such an ischemic challenge (34), IL-6 activity in extracts became higher 1 d after ischemia/reperfusion compared with sham-operated controls (Fig. 11 A, open bars). In contrast, in extracts of the hippocampal area, where loss of neurons becomes apparent 3–4 d after ischemia/reperfusion (34), a peak of IL-6 activity was observed only 4 d after the ischemic insult (Fig. 11 A, shaded bars). Consistent with these data, immunostaining of ischemic gerbil cerebral cortex with anti-IL-6 antibody demonstrated that, compared with nor-

moxia, increased expression of IL-6 was observed in ischemic astrocytes (Fig. 11, B and C, respectively). The identity of the cells expressing immunoreactive IL-6 (Fig. 11 C) was confirmed by double staining with antibody to glial fibrillary acidic protein (Fig. 11 D). Nonimmune IgG did not stain ischemic gerbil brain (Fig. 11 E).

Discussion

Astrocytes, the major glial cell in the CNS, have recently been recognized as participants in the response of the brain to environmental perturbations. While astrocytes retain their viability even in the face of extreme conditions, as can occur during ischemia/reperfusion, they become activated under such conditions, allowing them to assume new roles, as in tissue remodelling in the CNS (8). These considerations suggest parallels between astrocyte functions in the CNS and mononuclear phagocyte properties in peripheral vasculature. Consistent with this view, astrocytes have been shown to produce cytokines, including IL-1, IL-6, interferons, and TGF- β , allowing them to orchestrate a cytokine network in the brain, as reviewed by Benveniste (13). Another important property of astrocytes is their “neuroprotective” function, as exemplified by regulation of the ionic milieu, removal of excitatory neurotransmitters, and induction/maintenance of the blood-brain barrier (1, 2, 38). In addition to these “housekeeping” func-

tions, astrocytes directly influence the differentiation and survival of neurons by producing extrinsic growth/neurotrophic factors, such as NGF, basic fibroblast growth factor, and ciliary neurotrophic factor (39–41).

Our previous studies demonstrated that mononuclear phagocytes and endothelial cells elaborate cytokines after exposure to H/R (10, 42). This led us to assess whether astrocytes might respond similarly by production of a cytokine(s), but one with protective, neurotrophic properties, such as IL-6. The data presented here indicate that astrocytes produce IL-6 when exposed to hypoxia followed by a period of reoxygenation. The range of potential functions of astrocyte-derived IL-6 in ischemia is suggested by previous studies in tissue culture showing that IL-6 stimulates astrocyte proliferation and production of NGF (8, 39), has a neuroprotective effect on dopaminergic and cholinergic neurons (43, 44), and an NGF-like action on PC12 cells (45). Although elucidation of the role of astrocyte-derived IL-6 in the CNS will require further investigation, the enhanced production of IL-6 in the brain in response to ischemia, along with previous studies indicating that IL-6 administered into the brain exerts a protective effect on cholinergic neurons subjected to injury by *N*-methyl-D-aspartate (NMDA) agonists (46), suggests that IL-6 could have important beneficial effects.

Production of IL-6 by astrocytes in response to H/R occurs in a highly specific manner. Hypoxia upregulates transcription of IL-6 without inducing translation or release of the IL-6 gene product. This is similar to the increase in platelet-derived growth factor B-chain mRNA observed in hypoxic endothelium, which also occurs without production of mitogenic activity or demonstrable protein (30). During reoxygenation, activation of an NADPH oxidase-like activity results in astrocyte generation of superoxide, which serves as a stimulus for further transcription, as well as translation and

release of IL-6. Thus, moderated formation of ROIs is a trigger for protein biosynthesis and is not accompanied by astrocyte cytotoxicity, in keeping with the capacity of lower levels of oxygen free radicals to serve as intracellular second messengers in pathways activated by cytokines and other agents (10, 47). The validity of this hypothesis is suggested by experiments in which hypoxic astrocytes, with their increased levels of IL-6 mRNA, can be triggered to synthesize/release IL-6 activity after exposure to hydrogen peroxide (while still maintained in hypoxia), whereas normoxic astrocytes, with very low levels of IL-6 mRNA, did not produce comparable amounts of IL-6 in response to the same stimulus. The reasons for lack of translation of the IL-6 mRNA during hypoxia and the apparent delay of several hours between generation of ROIs and detection of IL-6 are not clear. This could be analogous to observations in spermatogenesis, in which untranslated mRNA is apparently translated at a later time after induction of a specific set of proteins (48). Our results emphasize the importance of elucidating mechanisms through which hypoxia and reoxygenation induce expression of IL-6 mRNA and translation and release of active IL-6.

Taken together, these data suggest that H/R activates astrocytes, resulting in their expression of IL-6 neurotrophic activity, as exemplified by enhanced PC12 viability (pilot studies with primary rat cortical neurons exposed to supernatants from H/R astrocytes have yielded similar results). Generation of ROIs, though associated with neuronal toxicity (49, 50) in CNS ischemia, can, when moderated, also trigger neuroprotective/neurotrophic properties of astrocytes through expression of IL-6. Future studies will be required to dissect the potential contribution of IL-6 to reparative mechanisms from that of other mediators implicated in ischemia, such as basic fibroblast growth factor and ciliary neurotrophic factor (51).

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