Hypoxia/Reoxygenation Induces Nitric Oxide and TNF-α Release from Cultured Microglia But Not Astrocytes of the Rat

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Abstract

Hypoxia/reoxygenation (H/R) elicits neuronal cell injury and glial cell activation within the central nervous system (CNS). Neuroinflammation is a process that primarily results from the acute or chronic activation of glial cells. This overactive state of glial cells results in the increased release of nitric oxide (NO) and/or tumor necrosis factor alpha (TNF-α), a process which can lead to neuronal damage or death. In this study, we found that hypoxia for eight or twelve hours (h) followed by 24 h reoxygenation (H8/R24 or H12/R24) induced NO production and TNF-α release from cultures of enriched microglial or mixed glial cells. However, microglial cells could not survive longer periods of hypoxia (≥ 12 h) in microglia-enriched culture. While astrocytes retained a 95 % viability following longer periods of H/R in astrocyte-enriched cultures, they did not produce any significant quantities of NO and TNF-α. Reoxygenation for prolonged periods (three and five days) following H24 resulted in progressively greater increases in NO production (about two-fold greater level in hypoxia as compared to normoxic conditions) accompanied by relatively less increases in TNF-α release in mixed glial cell cultures. Our data indicate that inflammatory mediators such as NO and TNF-α are released from glia-enriched mix culture in response to H/R. While microglial cells are more vulnerable than astrocytes during H/R, they survive longer in the presence of astrocyte and are the major cell type producing NO and TNF-α. Furthermore, the TNF-α release precedes NO production in response to a prolonged duration of reoxygenation following hypoxia for 24 h.

Key Words: hypoxia-reoxygenation, astrocyte, microglial cell, NO, TNF-α

Introduction

Hypoxia/reoxygenation (H/R) is involved in a wide range of pathophysiological responses. In the central nervous system (CNS), H/R not only causes neuronal cell injury, but also induces glial cell activation (40). The mammalian CNS is composed of neurons and supporting cells, collectively called glial cells, which comprise greater than 85% of the total population of brain cells. It is known that the early phase of hypoxic/ischemic insult and associated neuronal cell injury is due to glutamate excitotoxicity, the damage of tissue by the presence of free radicals and cellular energy supply failure (4, 6, 18, 45). Consistent with the notion that neurons are more vulnerable than glial cells to H/R (31), our previous study has demonstrated that nearly 50% of cultured neurons died following H/R, while 80% of cultured glial cells were still able to survive (45). The glial cells of CNS can be classified into four types including astrocytes, microglial cells, oligodendrocytes and ependymal cells. Astrocytes that perform numerous critical functions under normal physiological conditions, one of which functions is the protection of the CNS from insults. Microglial cells,

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with functions equivalent to macrophages, are able to migrate and engulf cellular debris, foreign particles, or microbes in the brain, and they also play a participatory role in the CNS immune response. Both astrocytes and microglial cells are among the first cells to respond to injury within the CNS (30). Evidence appears to indicate various levels of activation of astrocytes and microglial cells following H/R (10, 19, 22, 39, 41, 44). H/R-induced cell damage suffered by astrocytes and microglial cells has not been frequently discussed (28).

Neuroinflammation is a process that results primarily from the acute or chronic activation of glial cells, resulting in increased levels of oxidative stress and inflammatory molecules which can lead to neuronal damage or even cell death (5). A number of molecules associated with inflammation, and their associated pathways, have been identified within the CNS and may be activated in various pathophysiological conditions (1, 23).

One of the molecules principally involved in the brain's response to H/R is NO, an important intra/intercellular messenger involved in neuromodulation, reproductive function and immune response (25). NO is synthesized from L-arginine by NO synthase (NOS). The NO production that arises at the onset of ischemia is driven by the up-regulation of neuronal NOS (nNOS) and endothelial NOS (eNOS) (16). Subsequent to such an event, the activated glial cells are able to express an inducible isoform of NOS (iNOS) in producing high levels of NO, which is responsible for the killing of neurons (32, 35).

Tumor necrosis factor-α (TNF-α), a pleiotropic cytokine, is produced by glial cells during CNS injury (2, 42). This is another important mediator involved in the brain's response to H/R. The effects of acute TNF-α exposure in the ischemic brain include blood-brain barrier disruption, leukocyte recruitment, and activation of glial cells (9, 15). Several types of cells (e.g. neurons and astrocytes) may be the cellular source of TNF-α, and the actions of TNF-α may be related to the specific type of activated cells following brain ischemia (3, 12, 21, 29).

Glial cells may play an important role in the process of cerebral inflammation in response to hypoxic/ischemic stress, although the particular type of cell injured and the cellular sources of NO and TNF-α are not clear. In this study, we intended to examine [1] glial cell survival and [2] NO and TNF-α production in different glial cell types (including astrocytes and microglial cells) at various time periods following H/R.

Materials and Methods

Reagents

All cell culture reagents were obtained from Gibco-BRL (Gaithersburg, MD, USA). Monoclonal antibody against glial fibrillary acid protein (GFAP) was purchased from Chemicon International (Temecula, CA, USA), monoclonal antibody against rat macrophage/monocyte ED1 was purchased from Serotec (Oxford, UK).

Various Primary Cultures of Glial Cells (Including Mixed Glial, Astrocyte-Enriched and Microglial Cells-Enriched Cultures)

Primary cultures of mixed glial cells were prepared from one-day-old neonatal rat tissue (43, 44, 45). The cells were incubated at 37°C in a humidified 5% CO_2/95% air atmosphere. Medium was initially replenished five days subsequent to plating and changed every four days thereafter. The cell cultures were used 15 to 20 days subsequent to plating. Immunocytochemical staining (methodology as below) with antibodies against GFAP (an astrocyte marker) or against ED1 (a microglial marker) indicated that our mixed glial-cell cultures consisted of approximately 75 to 80% astrocytes and 15 to 20% microglial cells (43). The other unstained cell types (such as oligodendrocytes, fibroblasts or smooth-muscle cells) comprised less than 5% of total cells.

Microglial cell-enriched cultures were prepared from primary mixed glial cultures. Briefly, on day 12 of the mixed glial-cell cultures, any floating cells and any weakly attached microglial cells were shaken off by gentle shaking (180 rpm) of the culture flasks for 5 min. The resultant cell suspension was transferred to 12-well tissue-culture plates and allowed to adhere at 37°C for 2 h under standard incubation conditions. Unattached cells were removed after 2 h of incubation, and microglial cells were isolated as adherent cells. About 95 to 98% of attached cells proved to be positive for ED1 immunocytostaining. For experimental purposes, the cell suspension collected from the culture flask was transferred into 12-well tissue-culture plates at a concentration of 2×10^5 cell/ml/well (in 1 ml per well).

Astrocyte-enriched cultures were also prepared from primary mixed glial cultures (44). Briefly, on day 12 the microglial cells were shaken off the mixed glial-cell cultures, and astrocytes were obtained by treating the attached cells with 0.25% trypsin/0.05% EDTA. The cells were centrifuged at 1,200 rpm for 10 min, following which the pellets were suspended with DMEM/F12 media containing 10% heat-inactivated fetal bovine serum (Gibco, Los Angeles, CA, USA). Subsequent to two subcultures at ten-day intervals, the cells were plated into 12-well tissue-culture plates at 2×10^5 cell/1 ml/well. Approximately 90 to 95% of these cells were identified as being positive for anti-GFAP immunocytochemical staining.

Process of H/R

The culture medium for glial cultures was replaced with serum-free DMEM/F12 (without phenol red) immediately prior to the cells' use for experimental purposes. Then, cultures were placed into a modular incubator chamber (Billups-Rothenberg, Del Mar, CA, USA) which was maintained at 100% humidity, and cultures perfused, for 30 min, with a primary-grade mixed anoxic gas (5% CO_2 and 95% N_2) at a rate of 2 l/min (38, 44). Following the exposure for the designated time period, the hypoxic chamber was sealed tightly and placed in an incubator (at 37°C in humidified 5% CO_2/95% air) for various periods of time for the "hypoxia" group. At the completion of hypoxic exposure, cultures were removed from the chamber and maintained in the regular incubator under normal incubation conditions for the requisite "reoxygenation" period prior to culture assessment. "Normoxia" cultures were incubated under normoxic conditions for the corresponding periods. Each H/R group was classified with an abbreviated class name according to the particular duration of hypoxia/reoxygenation that applied to the H/R group, *i.e.*, H24/R24 represents a condition of hypoxia for 24 h followed by reoxygenation for a further 24 h. For longer periods of reoxygenation the abbreviation d (for day) was used instead. Thus H24/R1d indicated hypoxia for 24 h followed by reoxygenation for one day. Treatment groups included H8/R24, H12/R24, H24/R24 (= H24/R1d), H24/R3d, and H24/R5d.

Assessment of Cell Viability

Measurement of lactate dehydrogenase (LDH) activity. Cell death was quantified by measurement of LDH activity within the culture medium. LDH is a stable cytoplasmic enzyme released into the culture medium as an indicator of cell damage or lysis. The method of LDH level assessment was conducted according to a modification of a previously published procedure (20, 43). Absorbance of the mixture at 340 nm incident radiation was recorded automatically by microplate reader (ELX 808UV, Bio-TEK Instruments) immediately subsequent to removal of cultures from the incubator. One unit of LDH activity was defined as the quantity of enzyme that would be needed to catalyze the consumption by cells of 1 M of NADH per min.

3-(4,5-Dimethylthianol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) reduction assay. Cell viability at the end of each experiment was analyzed by a MTT reduction assay (26). This assay is based upon the capacity of mitochondrial enzymes to transform MTT to MTT formazan. Briefly, MTT stock solution (5 mg/ml in PBS) was added to each well of the 12-well-plate containing glial cells to a final concentration of 0.5 mg/ml. Following incubation at 37°C for a period of 2 h, the medium was aspirated and an equal volume of dimethyl sulfoxide (DMSO) was added to each well in order to dissolve the reduced MTT-formazan crystals. Quantitation of MTT reduction was accomplished by measuring absorbance at 570 nm against the 630 nm reference using the 96-well microplate reader. Results were presented as the mean optical density values from at least four separate cultures and were converted to the proportion (percentage) of the corresponding value for "Normoxia" wells.

Measurement of Nitrite Level

Since NO itself is quite unstable, NO production by cultured cells was determined by the measurement of total nitrite, a stable oxidation product of NO. The level of total nitrite was determined in serum-free media (without phenol red), subsequent to the reoxygenation experiments by means of a spectrophotometric assay based upon the Griess reaction (13). Griess reagent consists of 0.1% N-(1-naphthyl) ethylene diamide dihydrochloride, 1% sulfanilic acid, and 2.5% phosphoric acid. Duplicate aliquots (100 µl) of culture medium were removed and mixed with an equal volume of Griess reagent. Subsequent to a reaction time of 30 min, the absorbance at 540 nm was measured. Sodium nitrite in assay medium, over a concentration range of between 2.5 and 40 µM was used to prepare a standard absorbance curve for quantification of total nitrite in cultured media.

Measurement of TNF-α Level

Levels of TNF-α in the culture media were measured by use of enzyme-linked immunoadsorbent assay (ELISA) kits (BioSource International, Camarillo, CA, USA). This assay was based upon the quantitative sandwich-enzyme-immunoassay-principle using antibodies directed against rat TNF-α. The sensitivity of this assay was < 1 pg/ml.

Statistical Analysis

All data are expressed as the mean ± S.E.M. Statistical significance was assessed with an analysis of variance (ANOVA) test followed by Bonferroni's *t*-test using the SigmaStat program (Jandel Scientific, Inc., San Rafael, CA, USA). A value of $P < 0.05$ was considered to be statistically significant.

Results

Cell Viability, Nitrite and TNF-α Levels for Various Primary Cultures of Glial Cells Exposed to Hypoxia Followed by Reoxygenation for 24 h

The LDH release was expressed as a proportion

(percentage) of the corresponding value for control "sister" cultures (*i.e.*, normoxia conditions). The level of LDH release for the normoxia group was 38 ± 4, 30 ± 2 and 25 ± 8 units/ml in mixed glial cultures, astrocyte-enriched cultures and microglial cell-enriched cultures, respectively. The LDH activities within the culture media of mixed glial cultures increased significantly following experimental conditions of H12/R24 and H24/R24 (139 ± 8% and 182 ± 9% of normoxic levels, respectively); however, we did not observe any significant change in LDH activity for astrocyte-enriched and microglial cell-enriched cultures (Fig. 1A).

The total nitrite within culture media increased significantly under H12/R24 and H24/R24 experimental conditions for mixed glial cultures (normoxia: 1.95 ± 0.27 μM; H12/R24: 3.74 ± 0.45 μM; H24/R24: 4.49 ± 0.44 μM). There were significant increase in nitrite production under H8/R24, H12/R24 and H24/R24 conditions for microglial cell-enriched cultures (normoxia: 1.54 ± 0.20 μM; H8/R24: 3.12 ± 0.46 μM; H12/R24: 3.26 ± 0.81 μM; H24/R24: 5.84 ± 0.29 μM); however, we did not observe any response under the same experimental conditions for astrocyte-enriched cultures (Fig. 1B). A significant increase in medium TNF-α level was noted for mixed glial cultures under H12/R24 and H24/R24 conditions (normoxia: 19 ± 8 pg/ml; H12/R24: 295 ± 142 pg/ml; H24/R24: 206 ± 69 pg/ml) and for microglial cell-enriched cultures under H8/R24 (normoxia: 20 ± 20 pg/ml; H8/R24: 373 ± 256 pg/ml), although the levels of TNF-α did not change significantly for astrocyte-enriched (Fig. 1C).

The morphology of cells from astrocyte-enriched or mixed glial cultures did not change significantly subsequent to H12/R24 experimental conditions (data not presented). In contrast, cells from microglial cell-enriched cultures did reveal some cell damage following 12 h of hypoxia prior to reoxygenation, most of the microglial cells becoming rather rounded and of a smaller cell size than was the case for these cells' normoxic morphology (Fig. 2B). Following the reoxygenation treatment of cell cultures for 24 h following 12 h of hypoxia, a number of cells died with apparent cellular disruption, we noted quite a substantial amount of cellular debris being present (Fig. 2C). Furthermore, the MTT assay was assessment the microglial cell viability. After exposure to hypoxia, the microglial cells of cultures were almost disrupted and floating in media during reoxygenation for 24 h. However, the MTT reduction assay is based on the mitochondrial enzyme activity of attached cells on cultures. So we only measured the values of MTT reduction from microglial cell-enriched cultures at H8/R0, H12/R0 and H24/R0. The levels of MTT reduction at H8/R0 (71.2 ± 2.7%), H12/R0 (58.5 ± 4.0%) and

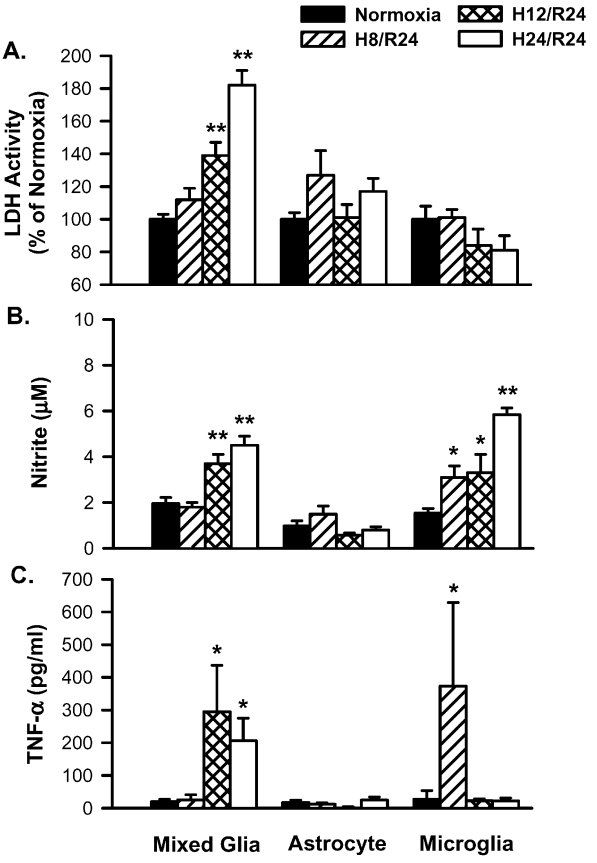

Fig. 1. Levels of (A) LDH, (B) NO and (C) TNF-α release in culture media of control (normoxia) cultures, cultures exposed to H8/R24, H12/R24 or H24/R24 for mixed glial cultures, astrocyte-enriched cultures, and microglial cell-enriched cultures, respectively. Data represent the mean ± S.E.M. of at least four separate experiments measured in duplicate. Asterisk(s) indicate(s) significant difference in parameter value at indicated time point when compared to corresponding value for normoxia group: $*P < 0.05$; $**P < 0.01$.

H24/R0 (34.5 ± 5.0% of normoxia) were significant decrease (Fig. 3)

Cell Viability, Nitrite and TNF-α Levels for Mixed Glial-Cell and Astrocyte-Enriched Cultures Exposed to Hypoxia for 24 h Followed by Longer Periods of Reoxygenation

After 24 h hypoxic exposure, the microglial cells of microglial cells-enriched cultures were almost disrupted and floating in media at reoxygenation for 24 h. Since we found that microglial cells were quite susceptible to hypoxic injury, we further examined the response of mixed glial and astrocyte-enriched cultures to 24 h hypoxia following by various (longer-duration) periods of reoxygenation. The cell viability (as reflected by MTT level reduction) of glial cells

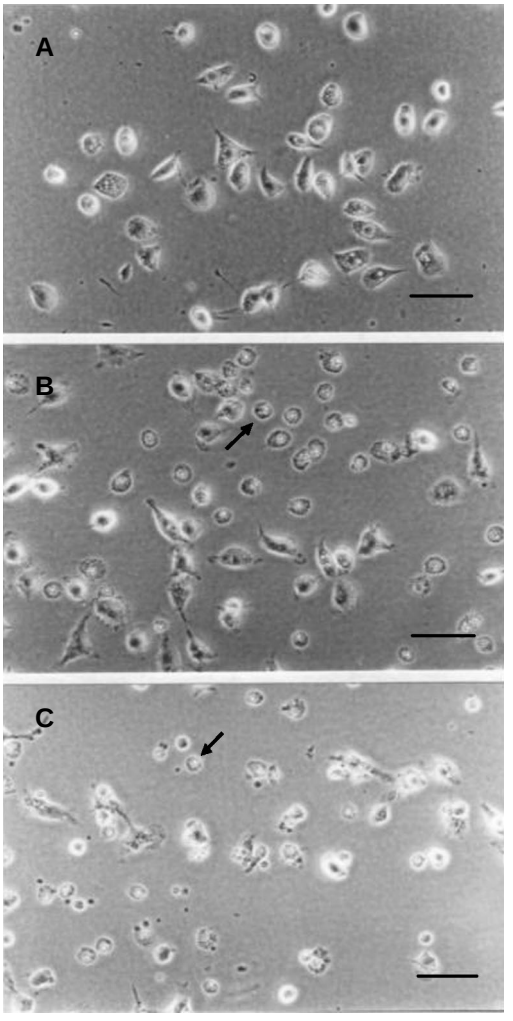

Fig. 2. Phase-contrast photomicrographs of microglial cell-enriched cultures in (A) normoxia, (B) H12/R0 and (C) H12/R24 conditions. Arrows indicate the damaged microglial cells. Scale bars = 50 μm.

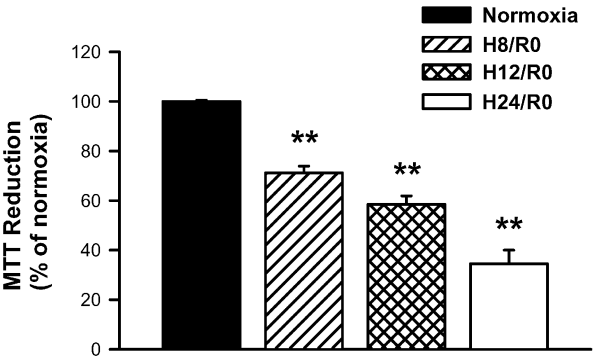

Fig. 3. The MTT reduction from microglial cell-enriched cultures that exposed to H8/R0, H12/R0 and H24/R0 conditions. Data represent the mean ± S.E.M. of at least three separate experiments measured in duplicate. Asterisk(s) indicate(s) significant difference in parameter value at indicated time point when compared to corresponding value for normoxia group: $^{**}P < 0.01$.

and astrocytes appeared to remain at almost 100% of normoxic group (including N24/R1d, N24/R3d and N24/R5d). The intra-media LDH activity (H24/R1d: 163 ± 6%, H24/R3d: 144 ± 12% and H24/R5d: 144 ± 17% of normoxia) increased significantly and the corresponding decline in MTT level (H24/R1d: 85 ± 4%, H24/R3d: 88 ± 7% and H24/R5d: 82 ± 3% of normoxia) at H24/R1d, H24/R3d and H24/R5d for mixed glial cultures. Astrocyte-enriched cultures, however, did not reveal any significant change either in LDH activity or MTT reduction (Fig. 4, A and B).

Total nitrite within mixed glial cultures was elevated significantly subsequent to H24/R1d (3.94 ± 0.45 μM) as compared to N24/R1d (1.85 ± 0.27 μM) and further increased following prolonged periods of reoxygenation (*i.e.* H24/R3d and H24/R5d). There was an approximately two-fold greater nitrite level for hypoxic (H24/R5d) as compared to normoxic experimental conditions (N24/R5d) (Fig. 4C).

The significant increase in TNF-α level following H24/R1d experimental conditions (H24/R1d: 169 ± 60 pg/ml *vs*. N24/R1d: 17 ± 8 pg/ml) appeared to decline somewhat following reoxygenation of the mixed glial cultures for prolonged periods of time (*i.e*. three and five days), especially for H24/R5d (H24/R3d: 160 ± 47 pg/ml *vs*. N24/R3d: 24 ± 10 pg/ml; H24/R5d: 82 ± 20 pg/ml *vs*. N24/R5d: 26 ± 13 pg/ml) conditions (Fig. 4D). When compared with the corresponding results for the "normoxia" group, the "H/R" groups exhibited an increased TNF-α level (3- to 9-fold). In contrast to such a result, however, both the total nitrite and TNF-α production in astrocyte-enriched cultures did not reveal any significant change subsequent to prolonged periods of reoxygenation following hypoxia for 24 h (Fig. 4, C and D).

Discussion

Our data reveal that the LDH release following H12/R24 and H24/R24 increased with respect to cell injury for mixed glial cultures although no such change was apparent for astrocyte-enriched cultures. Astrocytes were able to maintain 95% viability even subsequent to hypoxia for 24 h followed by longer-duration periods (including three- and five-day periods) of reoxygenation. However, our photomicrographs did reveal a significant degree of cell damage in cultured microglial cells following H12/R0. The different types of glial cells have different sensitivity in response to H/R. Our results suggest that microglial cells are sensitive to cell damage induced by H/R.

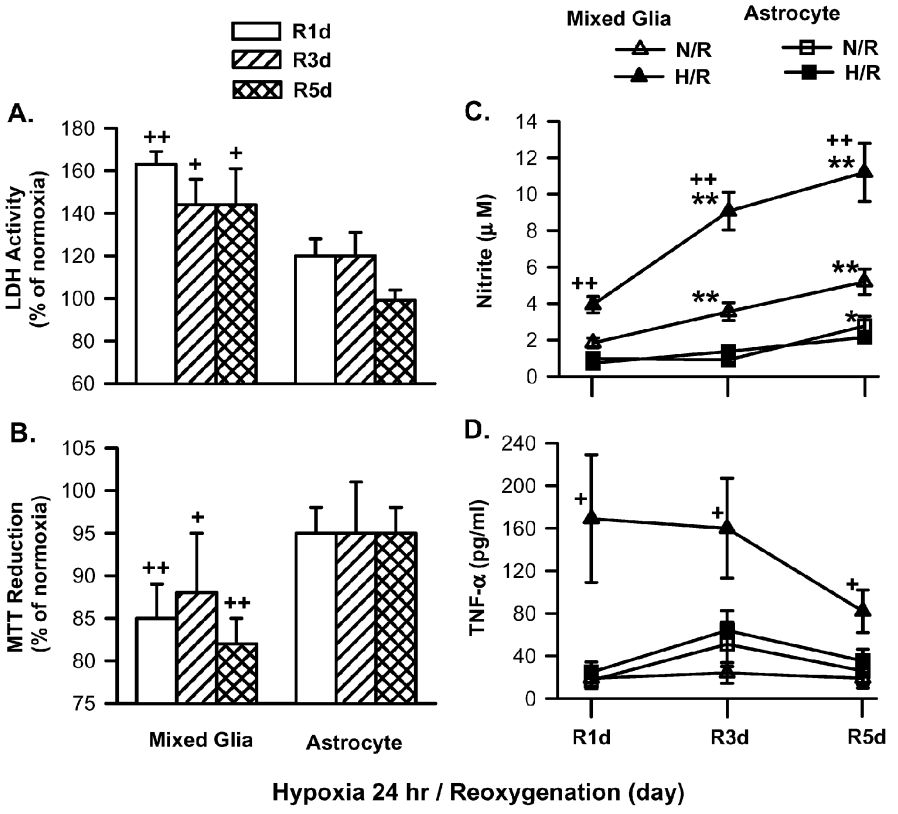

Fig. 4. Levels of (A) LDH activity, (B) MTT reduction, (C) NO production and (D) TNF-α release from mixed glial and astrocyte-enriched cultures that had been exposed to conditions of normoxia (N24/R1d, N24/R3d and N24/R5d), H24/R1d, H24/R3d, or H24/R5d. Data represent the mean ± S.E.M. of at least four separate experiments measured in duplicate. The positive sign(s) indicate significant difference from corresponding normoxia-group value at indicate(s) time points: $+P < 0.05$; $++P < 0.01$. Asterisk(s) indicate(s) significant difference in level of LDH, MTT, NO or TNF-α between cells subjected to H24/R3d or H24/R5d and H24/R1d: $*P < 0.05$; $**P < 0.01$.

The astrocytes may be more resistant to the damage than microglial cells under the same condition. Furthermore, astrocytes may secrete the essential growth factors for microglia that may even protect microglia such as colony-stimulating factor-1 (CSF-1), granulocyte macrophage colony-stimulating factor (GMCSF) and transforming growth factor-beta (TGF-β) (8, 11, 37). This could also be the reason why microglia in mixed glial cultures survived longer with sustained release of NO and TNF in H/R.

Although most cells in microglial cell-enriched cultures died immediately following a 12 h period of hypoxia (Fig. 2 and Fig. 3), the LDH release from microglial cells did not increase significantly for H12/R24 and H24/R24 as compared to simple normoxic conditions (Fig.1A). Such a discrepancy in morphological or MTT assay results and LDH activity only revealed for microglial cell-enriched cultures but not for astrocyte-enriched and mixed glial cultures. This indicates that assessment of LDH activity in culture supernatant may not be the most suitable indicator of microglial cell damage following H/R. The precise mechanism underlying cell injury following hypoxia for microglial cells clearly warrants further investigation.

Glial cells play an important role in the initiation of cerebral inflammation. Inflammation occurs in many types of neurological disorders, including cerebral ischaemia/hypoxia injury (27, 36). Associated with neuronal injury caused by ischaemia/hypoxia insults is the activation of glial cells (particularly astrocytes and microglial cells), an outcome which may further damage neurons at the site of the inflammation (14). The over-activation of glial cells (both astrocytes and microglial cells) typically results in increased levels of NO and TNF-α in cell cultures and *in vivo* (7, 17), which may be the actual chemical mediators to cause further neuronal injury (24, 33, 34). Using mixed glial cultures (consisted of approximately 75 to 80% astrocytes and 15 to 20% microglial cells), astrocyte-enriched cultures (90 to 95% astrocytes) and microglial cell-enriched cultures (95 to 98% microglial cells), we found that both NO production and TNF-α release

following H/R were significantly enhanced for mixed glial cultures and microglial cells-enriched cultures as compared to normoxic condition, but not in astrocyte-enriched cultures. Similar results were obtained with H12/R24, H24/R24 and H24/R1d, H24/R3d, H24/R5d. Furthermore, our previous paper (44) had provided evidence that microglial cells were more activated than astrocytes on mixed glial-cell cultures in response to H/R. We thus suggest that the inflammatory mediators, NO and TNF-α, may be induced by H/R from microglial cells, but not from astrocytes.

In mixed glial cultures, longer periods of reoxygenation (H24/R1d, H24/R3d, and H24/R5d) induced progressively greater increases in NO production and progressively smaller increases in TNF-α release when compared to normoxic condition (Fig. 4, C and D). Our results suggest that the proinflammatory cytokine TNF-α may play an important role at an early phase insult in reoxygenation following hypoxia, possibly initiate and propagate further inflammatory responses following the initial insult. Furthermore, the role of NO may be at the late phase insult in reoxygenation. It is not clear whether the TNF-α released by microglial cells during H/R would be responsible for the subsequent induction of NO production from glial cells. The relationship between TNF-α release and NO production by glial cells warrants further investigation and elucidation. The findings of this study demonstrate that NO and TNF-α production by activated microglial cells in response to H/R and TNF-α release preceding NO production in prolonged duration of reoxygenation following hypoxia for 24 h.

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