Journal of Neuroscience Research 79:208–215 (2005)

Nitric Oxide From Inflammatory-Activated Glia Synergizes With Hypoxia to Induce Neuronal Death

Palwinder Mander,[1] Vilma Borutaite,[1] Salvador Moncada,[2] and Guy C. Brown[1]*
[1]Department of Biochemistry, University of Cambridge, Cambridge, United Kingdom
[2]Wolfson Institute for Biomedical Research, University College London, London, United Kingdom

Inflammatory-activated glia are seen in numerous central nervous system (CNS) pathologies and can kill nearby neurons through the release of cytotoxic mediators. Glia, when activated, can express the inducible isoform of nitric oxide synthase (iNOS) producing high levels of nitric oxide (NO), which can kill neurons in certain conditions. We show, however, that inflammatory activation of glia in a mature culture of cerebellar granule neurons and glia causes little or no neuronal death under normal (21%) oxygen conditions. Similarly, hypoxia (2% oxygen) or low levels of an NO donor (100 μM DETA/NO) caused little or no neuronal death in nonactivated cultures. If inflammatory activation of glia or addition of NO donor was combined with hypoxia, however, extensive neuronal death occurred. Death in both cases was prevented by the *N*-methyl-D-aspartate (NMDA) receptor blocker MK-801, implying that death was mediated by the glutamate receptor. Low levels of NO were found to increase the apparent K_M of cellular oxygen consumption for oxygen, probably due to NO-induced inhibition of mitochondrial respiration, in competition with oxygen, at cytochrome oxidase. Necrotic death, induced by hypoxia plus DETA/NO, was increased further by deoxyglucose, an inhibitor of glycolysis, suggesting that necrosis was mediated by energy depletion. Hypoxia was found to be a potent stimulator of microglia proliferation, but this proliferation was not significant in inflammatory-activated cultures. These results suggest that low levels of NO can induce neuronal death under hypoxic conditions, mediated by glutamate after NO inhibition of respiration in competition with oxygen. Brain inflammation can thus sensitize to hypoxia-induced death, which may be important in pathologies such as stroke, neurodegeneration, and brain aging.

Key words: cytochrome oxidase; glutamate; microglia; inflammation

Activated glia (astrocytes and microglia) are seen in a range of central nervous system (CNS) pathologies, including brain infections, neurodegenerative diseases, inflammation, ischemia, and normal aging (Eddleston and Mucke, 1993; Kreutzberg, 1996). Activated glia can release cytotoxic mediators that kill invading pathogens, but these mediators may also kill nearby neurons (Marty et al., 1991; Boje and Arora, 1992). Glial activation involves changes in cell phenotype and the expression of new proteins, such as the inducible isoform of nitric oxide synthase (iNOS). The mechanism by which activated glia induce neuronal death has been shown to involve nitric oxide (NO) (Chao et al., 1996; Bolaños et al., 1997; Loihl and Murphy, 1998; Bal-Price and Brown, 2001) and glutamate (Bal-Price and Brown, 2001; Barger and Basile, 2001), as well as reactive oxygen species (ROS) and proinflammatory cytokines (Beckman et al., 1994; Chao et al., 1995a,b). Glial activation and iNOS expression alone do not always lead to neuronal death (Demerle-Pallardy et al., 1993; Hewett et al., 1994), however, suggesting that particular conditions are required for NO-induced neuronal death.

The brain is extremely sensitive to changes in oxygen and glucose supply. Low oxygen or glucose levels can lead to decreased ATP synthesis, resulting in depletion of cellular ATP and subsequent neuronal death (Pulsinelli and Duffy, 1983; Yager et al., 1992). Such death is mediated largely by *N*-methyl-D-aspartate (NMDA)-type glutamate receptors, which become activated by ischemia-induced glutamate release or ischemia-induced neuronal depolarization (Simon et al., 1984; Pohorecki et al., 1990). Hypoxia/anoxia initiates neuronal death by removal of the substrate oxygen from cytochrome oxidase. Cytochrome oxidase, however, normally has a K_M for oxygen below 1 μM, making it insensitive to all but the most extreme levels of hypoxia. NO is a potent reversible inhibitor of cytochrome oxidase in competition with oxygen, resulting in a marked increase in the apparent K_M for oxygen (Brown and Cooper, 1994; Cleeter et al., 1994; Brown and Borutaite, 2002; Moncada and Erusalimsky, 2002).

Contract grant sponsors: BBSRC; European Commission and MRC.

*Correspondence to: Guy C. Brown, Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge, CB2 1QW UK. E-mail: gcb@mole.bio.cam.ac.uk

Received 1 July 2004; Revised 6 August 2004; Accepted 6 August 2004

Published online 22 November 2004 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jnr.20285

High NO levels result in neuronal death by effectively the same mechanism as anoxia, i.e., blockage of cytochrome oxidase (Bal-Price and Brown, 2001). Because of the competition between NO and oxygen at cytochrome oxidase, the possibility arises that low levels of NO synergize with low levels of hypoxia to induce death via cytochrome oxidase inhibition.

Such synergy might be important in a variety of pathologies where iNOS is expressed. For example, after ischemia, glia become activated and express iNOS (Endoh et al., 1994; Iadecola et al., 1995; Kawase et al., 1996). NO from iNOS is thought to mediate postischemic neuronal damage, as inhibitors of iNOS reduce the infarction volume (Iadecola et al., 1995; Zhang et al., 1996) and iNOS knockout mice are strongly protected against such damage (Zhao et al., 2003). Activated glia expressing iNOS are found in Alzheimer's disease (AD) patients (Hu et al., 1998), and neuronal death in AD is now thought to be at least partly ischemic (Jendroska et al., 1995; Kim et al., 1998). Vascular dementias, which may have some overlap with AD, result from atherosclerosis, which is inflammatory in origin and causes brain ischemia (Desmond, 1996). Normal aging is accompanied by low-level glial activation and iNOS expression (Vernet et al., 1998; Kyrkanides et al., 2001), which might sensitize to hypoxia. Systemic infection, inflammation, or prior strokes are known to be strong risk factors for subsequent strokes (Syrjanen, 1993; Macko et al., 1996), suggesting the possibility that inflammation sensitizes to ischemia. Brain infection or trauma can also cause iNOS expression, which may be accompanied by ischemia (Clark et al., 1996; Sharshar et al., 2003). For these reasons, it is important to determine whether NO and hypoxia are synergistic in inducing neuronal death.

MATERIALS AND METHODS

Materials

The following materials were purchased from the indicated sources: DETA/NO, and 1400W dihydrochloride from Alexis and MK-801 maleate from Calbiochem. All other reagents were ordered from Sigma.

Neuronal–Glial Culture

Cerebellar granule cell (CGC) cultures were prepared from 7-day-old Wistar rats as described previously (Bal-Price and Brown, 2001). Briefly, the pups were anesthetized, using 5% halothane in oxygen, followed by decapitation. Brains were removed under sterile conditions and the cerebellum dissected. Meninges were removed and the cerebella dissociated in Versene solution (1:5,000; Gibco BRL) and plated at 0.25×10^6 cells/cm^2 in 24-well plates (in 500 μl Dulbecco's minimum Eagle medium [DMEM; Gibco BRL]) coated with 0.001% poly-L-lysine. Cultures were maintained in DMEM supplemented with 5% horse serum, 5% fetal calf serum, 38 mM glucose, 5 mM HEPES, 2 mM glutamine, 25 mM KCl, and 10 μg/ml gentamicin. Cells were kept at 37°C in a humidified atmosphere of 5% CO_2/95% air and used for experiments at 16–18 days in vitro (DIV). CGC cultures contained 22 ± 4% astrocytes and 2 ± 1% microglia as assessed by immunocytochemistry using antibodies against glial fibrillary acidic protein (GFAP; a marker for astrocytes) and OX-42 (a marker for microglia). All experiments were undertaken in accordance with the UK Animals (Scientific Procedures) Act 1986.

Treatments of Culture

Lipopolysaccharide (LPS), a cell wall component of Gram-negative bacteria and interferon-γ (IFN-γ), a proinflammatory cytokine, are potent activators of glia when administered together. Neuronal–glial cultures were treated with 100 ng/ml LPS (from *Salmonella typhimurium*) and 10 ng/ml IFN-γ for approximately 20 hr before starting hypoxic or normoxic incubations. iNOS expression in the culture was assessed by NADPH diaphorase staining (data not shown) and measurements of nitrite in the medium. Nitrite levels in the medium were measured using the Griess reaction. Briefly, after treatments, aliquots of medium were centrifuged ($0.1 \times g$) for 5 min, 6 mM HCl was then added to the supernatant, and then 1 mM sulfanilamide and 1 mM N-1 (1-naphthyl)ethylenediamine (NEDA) were also added. Absorbance at a wavelength of 548 nm was measured by a plate reader (BMG; Fluostar Optima) before and after the addition of NEDA. Nitrite concentrations in samples were calculated from a standard curve of sodium nitrite in DMEM. Where present, inhibitors were added at the same time as LPS/IFN-γ.

We used 2-deoxyglucose (DOG) to inhibit glycolysis in some experiments. DOG is taken up by glucose carriers and phosphorylated by hexokinase to 2-deoxyglucose-6-phosphate, which is not used by phosphoglucose isomerase and increases in concentration to inhibit hexokinase, thus blocking glycolysis (Woodward and Hudson, 1954). The glucose concentration in the culture medium at the start of experiments (16–18 DIV) was 29 ± 3 mM, whereas the DOG concentration (when present) was 10 mM. Others have shown that similar ratios of DOG to glucose are sufficient to inhibit glycolysis in neurons and other cells (Woodward and Hudson, 1954; Yang et al., 1999; Munoz-Pinedo et al., 2003).

Assessment of Cell Viability

CGC viability was assessed by propidium iodide (PI; 2 μg/ml) and Hoechst 33342 (6 μg/ml) staining, using a fluorescence microscope (Axiovert S-100) and filters for excitation at 365 nm and emission at 420 nm. The cell-impermeable nuclear dye PI stains the nuclei of cells that have lost plasma membrane integrity and are considered necrotic. Using the cell-permeable DNA dye Hoechst 33342, the nuclear morphology of the CGCs was studied. Cells with noncondensed Hoechst staining but lacking PI staining were considered to be viable, whereas cells showing irregular Hoechst staining, nuclear shrinkage, chromatin condensation, and/or nuclear fragmentation but were PI negative were classified as showing chromatin condensation (CC). Individual cells exhibiting both CC and PI staining were included in the PI data. Cells were counted in three microscopic fields in each well (three wells per treatment) and expressed as a percentage of the total number of neurons. Each treatment was repeated at least three times.

Hypoxia Treatments

Hypoxic conditions were achieved by incubating cultures in humidified sealed containers perfused with a gas mixture consisting of 2% oxygen, 5% carbon dioxide, and 93% nitrogen at 37°C. Cultures were incubated under these conditions for 12 hr. For ambient oxygen conditions (normoxia), cultures were placed in similar containers but perfused with a gas mixture consisting of 21% oxygen, 5% carbon dioxide, and 74% nitrogen. Both gas mixes were obtained from BOC gases (Surrey, UK).

Oxygen Consumption Measurements

Oxygen consumption of cells was measured using Clark-type oxygen electrode (Rank Brothers, Bottisham). Cultures were plated in 25 cm^2 flasks ($\sim 8.3 \times 10^6$ cells) and used at 16 DIV. Cells were scraped gently and resuspended in 1 ml of HEPES-buffered saline supplemented with 38 mM glucose, 2 mM glutamine, 25 mM KCl, and 1 mM L-arginine. Cultures were either untreated or activated with 100 ng/ml LPS and 10 ng/ml IFN-γ for 20 hr. The apparent K_M ($K_{1/2}$) was calculated by measuring the O_2 concentration at which the rate of O_2 consumption was half the maximal (initial) rate.

Assessment of Microglia Proliferation

Microglia cells were identified using isolectin IB_4 (from *Griffonia simplicifolia*), which has strong affinity for microglia but not astrocytes. An Alexa Fluor 488 conjugate of isolectin IB_4 (10 ng/ml) was added to cultures (after hypoxic or normoxic incubations) and incubated for 15 min at 37°C. Stained cells (microglia) were visualized and counted by viewing under a fluorescence microscope (excitation, 488 nm; emission, 530 nm).

Statistical Analysis

Data are expressed as mean ± SEM. Significance was determined using analysis of variance (ANOVA).

RESULTS

Hypoxia Is Synergistic With NO in Inducing Neuronal Death

Cocultures of cerebellar granule neurons and glia were exposed for 12 hr to hypoxia (2% O_2), an NO donor (100 μM DETA/NO), or both. Immediately after this period, cultures were stained with PI and Hoechst 33342 to assess the number of necrotic (PI) neurons or neurons with condensed/fragmented chromatin (CC), a measure of "apoptosis." Hypoxia alone caused very little neuronal death, (PI, 2%; CC, 1%) (Fig. 1a,b). DETA/NO (100 μM) did not induce neuronal death in normoxic (21% O_2) conditions (PI, 2%; CC, 1%), but under hypoxic conditions high levels of neuronal death were seen (PI, 26%; CC, 71%). These changes were prevented by the NMDA receptor blocker MK-801 (added 30 min before hypoxia/DETA/NO), which reduced death (to PI, 3%; CC, 3%), implicating glutamate or the NMDA receptor as a mediator of death. In some experiments, glycolysis was inhibited by the addition of 10 mM DOG, which induced little death alone under normoxic conditions (PI, 2%; CC, 6%) but synergized with either hypoxia (PI, 26%; CC, 45%) or NO (PI, 11%; CC, 14%) or hypoxia and NO together (PI, 81%; CC, 19%) to induce neuronal necrosis. Although addition of DOG to the NO plus hypoxia-treated cultures reduced the number of chromatin-condensed, PI-negative (i.e., apoptotic) neurons (Fig 1b), it did so not by reducing the total number of chromatin-condensed neurons, but rather by converting chromatin-condensed, PI-negative (PI−) neurons to chromatin-condensed PI-positive (PI+) neurons (see Fig 1c; hypoxia plus DETA/NO: PI−/CC− 3%, PI+/CC− 4%, PI−/CC+ 71%, PI+/CC+ 22%; hypoxia plus DETA/NO and deoxyglucose: PI−/CC− 0%, PI+/CC− 9%, PI−/CC+ 19%, PI+/CC+ 72%). No astrocytes or microglia showed any signs of chromatin condensation or necrosis in any of the conditions used (Fig 1c), indicating that cell death in NO and hypoxic conditions was specific to neurons.

NO From Inflammatory Activated Glia Sensitizes Neurons to Hypoxia-Induced Death

LPS and IFN-γ induce iNOS expression in both astrocytes and microglia. Glial activation and iNOS expression after LPS/IFN-γ treatment was confirmed by measuring nitrite levels in the medium. Nitrite levels in nonactivated cultures were not different between hypoxic (2.7 ± 3.0 μM) and normoxic incubations (3.0 ± 2.3 μM). After LPS/IFN-γ treatment, nitrite levels were significantly higher, but not different between hypoxic (11.2 ± 4.3 μM) and normoxic treatments (12.1 ± 2.8 μM). Addition of an iNOS inhibitor decreased the nitrite levels in the activated cultures to control levels (3.1 ± 0.3 μM in normoxia, 1.6 ± 1.1 μM in hypoxic conditions). Additionally, glia were stained intensely for

Fig. 1. (Continued.) Neuronal death induced by 100 μM DETA/NO under normoxic (gray) or hypoxic (black) conditions. Cultures were stained with the cell-impermeable dye propidium iodide (PI) to count necrotic cells (**a**) and the cell-permeable dye Hoechst 33342 to count neuronal nuclei showing chromatin condensation or fragmentation as a marker of apoptosis (**b**). Neuronal death was prevented by 10 μM MK-801, an NMDA receptor antagonist. Deoxyglucose (DOG, 10 mM; an inhibitor of glycolysis) increased the number of neurons dying by necrosis. **c:** Neuronal chromatin was PI negative and not condensed after hypoxia alone (i), but there was nuclear shrinkage and chromatin condensation after hypoxia with 100 μM DETA/NO (ii) or hypoxia with 10 mM DOG (iii). The combination of DOG and DETA/NO together under hypoxic conditions resulted in extensive necrosis indicated by PI staining of neurons, in addition to nuclear shrinkage and chromatin condensation (iv). Note the large, elongated nuclei, visible particularly in (ii) and (iv), belonging to astrocytes, which are unaffected by the treatments used. Magnification 40×. Statistical differences were determined using ANOVA at $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$. All significant differences are in comparison to either normoxic control or hypoxic control. The symbol # replaces * for significance when comparing protection against 100 μM DETA/NO. Error bars indicate SEM; $n = 3$ or more.

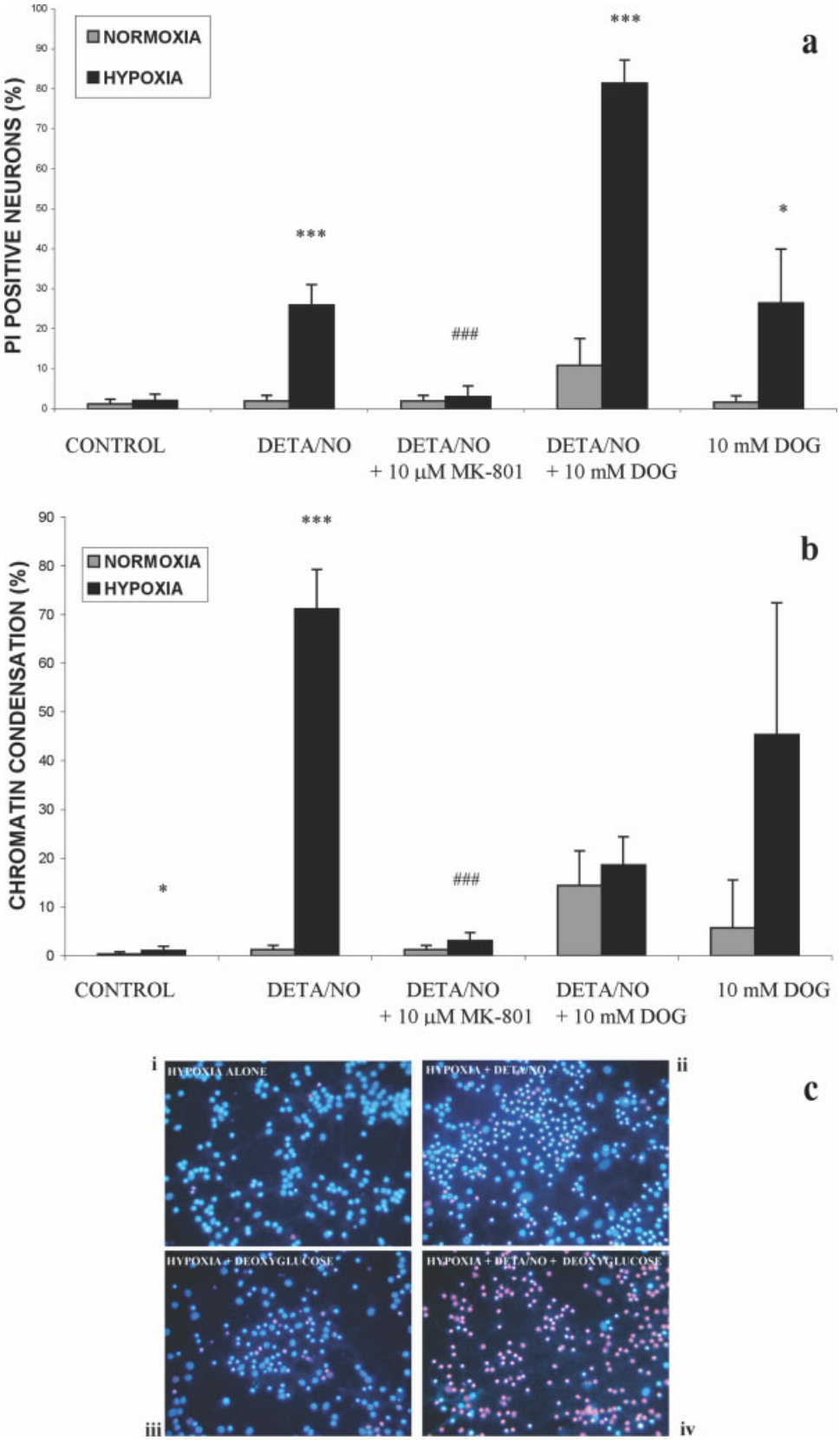
a
NORMOXIA
HYPOXIA
PI POSITIVE NEURONS (%)
***
***
*
###
CONTROL
DETA/NO
DETA/NO + 10 μM MK-801
DETA/NO + 10 mM DOG
10 mM DOG
b
NORMOXIA
HYPOXIA
CHROMATIN CONDENSATION (%)
***
*
###
CONTROL
DETA/NO
DETA/NO + 10 μM MK-801
DETA/NO + 10 mM DOG
10 mM DOG
c
i
HYPOXIA ALONE
ii
HYPOXIA + DETA/NO
HYPOXIA + DEOXYGLUCOSE
HYPOXIA + DETA/NO + DEOXYGLUCOSE
iii
iv

Fig. 1.

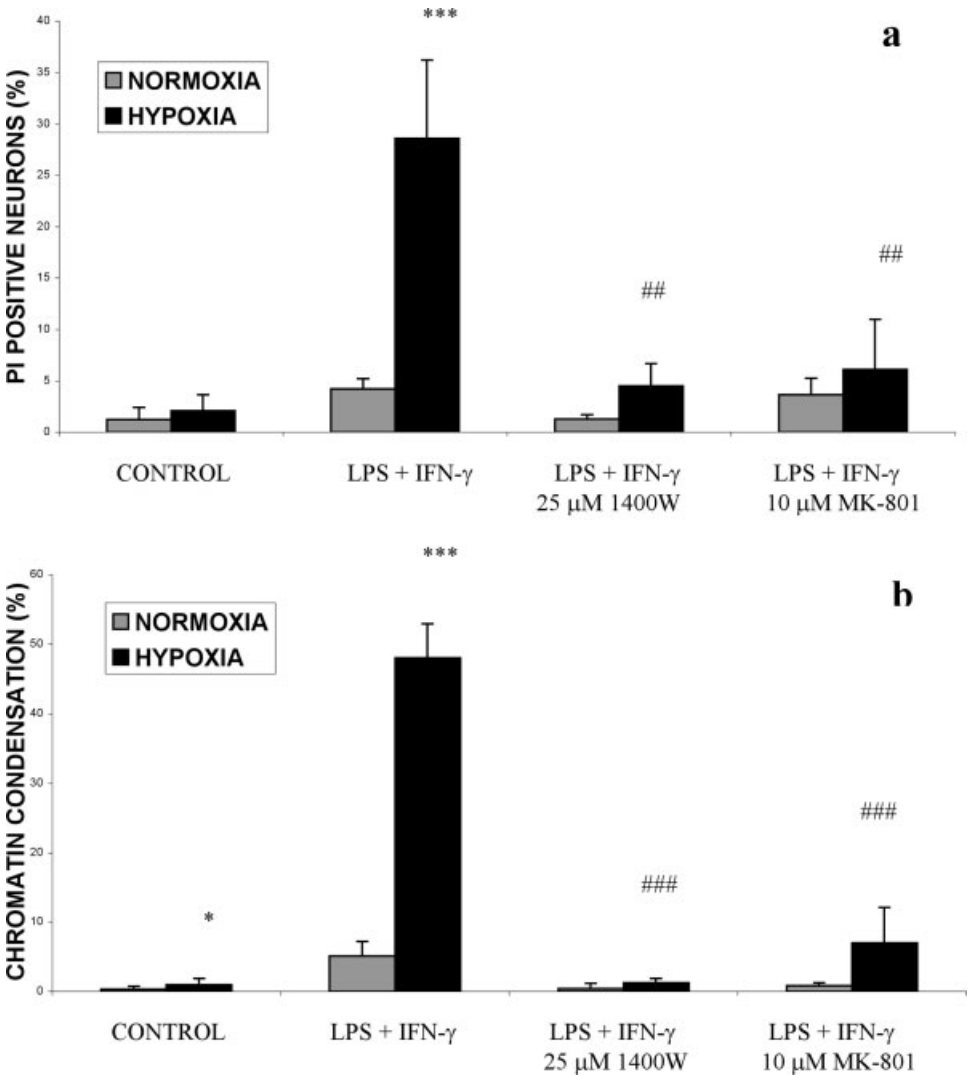

Fig. 2. Cultures activated with LPS and IFN-γ induced neuronal death under hypoxic (black), but not normoxic (gray) conditions. Death assessed with propidium iodide (**a**) for necrotic neurons and Hoechst 33342 (**b**) for chromatin condensation or fragmentation. Neuronal death was prevented by an inhibitor of iNOS (25 μM 1400W) and a blocker of the NMDA receptor (10 μM MK-801). Statistical differences were established using ANOVA at $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$. All significant differences are in comparison to their corresponding normoxic or hypoxic control. The symbol # replaces * for significance when assessing protection against LPS and IFN-γ activated cultures. Error bars indicate SEM; $n = 3$ or more.

NADPH diaphorase activity after LPS/IFN-γ activation but not in nonactivated cultures (data not shown), confirming that glia had become activated and that they were the source of increased nitrite found in the medium. The NADPH diaphorase activity of neurons was low, and unchanged by LPS/IFN-γ activation (data not shown).

Glial activation by LPS/IFN-γ caused little neuronal death under normoxic conditions (PI, 4%; CC, 5%; Fig. 2a,b). Under hypoxic conditions, however, glial activation caused high levels of neuronal death (PI, 29%; CC, 48%). This death was prevented by the NMDA receptor antagonist MK-801 (PI, 6%; CC, 7%) or by the iNOS inhibitor 1400W (PI, 4%; CC, 2%).

NO From iNOS Raises the Apparent K_M for O_2 of Cellular Respiration

NO inhibits respiration in competition with O_2, raising the apparent K_M of respiration for oxygen into the physiologic range, potentially sensitizing cells to hypoxia. Using an oxygen electrode, we found that low levels of NO from 100 μM DETA/NO did indeed increase the apparent K_M ($K_{1/2}$) for oxygen of the neuronal–glial cultures, from 1.8 ± 1.0 μM O_2 in untreated control cultures to 50.5 ± 3.5 μM O_2 in treated cultures. Similarly, in LPS/IFN-γ activated cultures, the $K_{1/2}$ of respiration for O_2 was raised to 12.0 ± 8.0 μM O_2, inhibiting NO synthesis with 25 μM 1400W prevented this increase ($K_{1/2}$ 1.5 ± 0.8 μM O_2).

Hypoxia Induces Rapid Proliferation of Microglia

Microglia in mixed neuronal-glial cultures were counted before and after normoxic or hypoxic incubations, using the microglia-specific stain, isolectin IB_4 (Fig. 3). Almost twice as many microglia were seen in cultures after 12 hr of hypoxia, but not normoxia. The number of microglia in LPS/IFN-γ activated cultures was assessed before and after hypoxic and normoxic incubations. There was no significant increase in microglia in activated cultures after hyp-

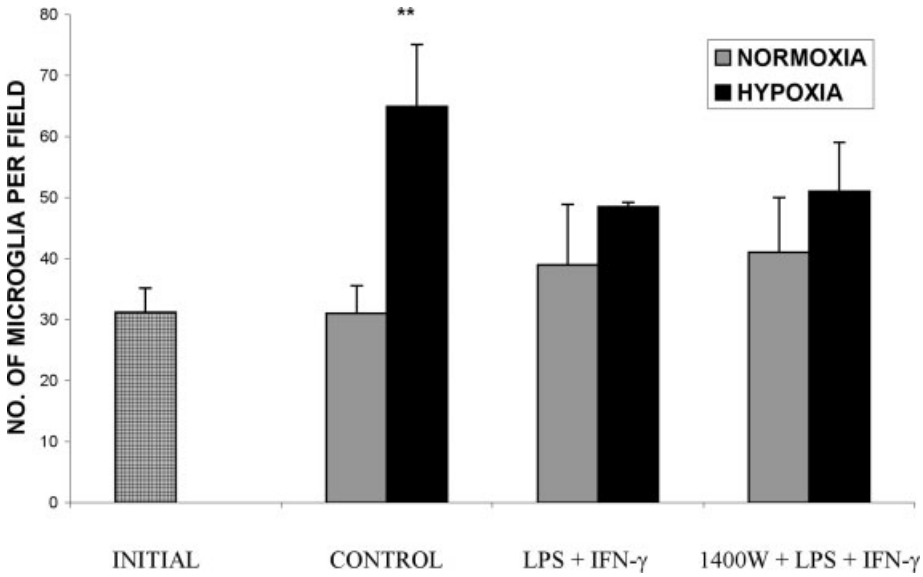

Fig. 3. Hypoxia induces rapid proliferation of microglia. Microglia were counted before (INITIAL) or after 12 hr of hypoxic or normoxic incubations. A significant increase in the number of microglia was seen after hypoxia in comparison to the normoxic control; however this proliferation was not present in activated cultures (LPS/IFN-γ treated). The lack of microglia proliferation in activated cultures under hypoxic conditions was not due to nitric oxide, as the addition of 1400W (an iNOS inhibitor) did not restore microglia proliferation. Statistical differences were established using ANOVA at $^{**}P < 0.01$. All significant differences are in comparison to the corresponding normoxic treatment. Error bars indicate SEM; $n = 3$ or more.

oxia or normoxia either in the presence or absence of 1400W (Fig. 3), ruling out the possibility that neuronal death in activated cultures under hypoxic conditions was due to an increase in the number of microglia.

DISCUSSION

Hypoxic conditions can arise in the brain during ischemia, strokes, trauma, or atherosclerosis (Bouma and Muizelaar, 1992; Sweeney et al., 1995; Desmond, 1996). The brain is exceptionally sensitive to reduced oxygen or glucose supply, resulting in neuronal death (Juurlink and Sweeney, 1997). Necrotic neurons are often at the core of the ischemic infarction, with apoptotic neurons in the penumbra (Choi, 1996). Our in vitro data presented here shows that neuronal–glial cultures are able to survive hypoxia (2% oxygen) for 12 hr, with little neuronal death (2% necrosis and 1% apoptosis). An oxygen level of 2% is equivalent to about 20 μM O_2, which is well above the K_M of respiration for oxygen in the absence of NO (although oxygen consumption by the cells may reduce the local level of oxygen (Mamchaoui and Saumon, 2000). In the presence of NO, the apparent K_M of respiration for oxygen was increased (to 50 μM in the presence of 100 μM DETA/NO), so that cellular respiration was inhibited severely at 20 μM O_2 but not at 200 μM O_2. The amount of NO released from this concentration of DETA/NO did not cause any neuronal death under normal oxygen concentrations (21%) but induced 26% neuronal necrosis and 71% neuronal apoptosis under hypoxic conditions.

The presence of DOG, an inhibitor of glycolysis, further increased hypoxia plus DETA/NO-induced neuronal necrosis (necrosis increased from 26 to 81%), indicating that glycolysis protects against NO/hypoxia-induced neuronal necrosis, presumably by supplying an alternative source of cellular ATP when mitochondrial ATP production is inhibited by NO/hypoxia. In the absence of DOG, however, the hypoxia/NO-treated neurons died largely by apoptosis (PI, 26%; CC, 71%), whereas in the presence of DOG they died largely by necrosis (PI, 81%; CC, 19%). This apparent switch from apoptosis to necrosis seems to have been due to increased necrosis rather than decreased induction of apoptosis, as the hypoxia/NO/DOG-treated neurons were both PI positive and had condensed chromatin (Fig. 1c), i.e., they had induced apoptosis but were also necrotic. This is consistent with results by others (Lee et al., 2002) who found that NO and hypoxia were synergistic in inducing apoptosis in fibroblasts, but DOG greatly increased the amount of necrosis. The astrocytes in our cocultures were not killed by NO, hypoxia, or NO/hypoxia presumably because, unlike neurons, they have a high glycolytic capacity that is upregulated by NO (Almeida et al., 2001, 2004).

Glia (astrocytes and microglia) become activated postischemia, and may be protective, partly through the removal of debris. During activation the inducible isoform of nitric oxide synthase (iNOS) is expressed. The NO generated is harmful to neurons, and inhibitors of iNOS are able to significantly reduce the infarct volume (Iadecola et al., 1995; Zhang et al., 1996). Activation of the mixed neuronal-glial culture with LPS/IFN-γ caused little neuronal death at normal oxygen concentrations (4% necrosis and 5% apoptosis). Under hypoxic conditions,

inflammatory-activated glia induced death of the cocultured neurons (29% necrosis and 48% apoptosis). Neuronal death was prevented by 1400W (an iNOS inhibitor), showing that it was due to NO from iNOS. The increase in K_M of respiration for oxygen in activated cultures (12 μM) was also prevented by treatment with 1400W (K_M = 1.5 μM). These results are similar to those seen with DETA/NO under hypoxic conditions, suggesting that it is the NO rather than other molecules or cytokines produced by activated glia that sensitizes to hypoxia.

It has been suggested that physiologic concentrations of NO are not able to significantly inhibit respiration in neurons (Bellamy et al., 2002). Even if physiologic levels of NO (produced by nNOS stimulation) are not able to inhibit respiration, NO produced by iNOS (during inflammation) may reach the higher, pathologic concentrations shown to inhibit respiration (120 nM NO, as measured by Bellamy et al., 2002) and lower concentrations of NO would be sufficient to inhibit respiration under hypoxic conditions. Studies using an in vivo model of ischemia showed that cytochrome oxidase is not inhibited by NO under physiologic conditions or during reoxygenation (after a brief period of anoxia) (De Visscher et al., 2002). iNOS is not likely to be expressed in these conditions, however, and it would be interesting to repeat these studies during brain inflammation.

Hypoxia was found to be a rapid stimulator of microglia proliferation, with a twofold increase in just 12 hr. The mechanism by which hypoxia triggers proliferation of microglia is unclear, but is thought to involve TNF-α (Bruce et al., 1996; Walton et al., 1999) or colony-stimulating factors (Walton et al., 1999). LPS is known to irreversibly inhibit microglia proliferation (Gebicke-Haerter et al., 1989; Ganter et al., 1992). In addition, we observed no increase in the number of microglia in LPS/IFN-γ-activated cultures after incubations in hypoxia, indicating that hypoxia-induced neuronal death in activated cultures was not due to an increase in microglia.

Glutamate has been implicated in ischemia-induced neuronal death and its actions at the NMDA receptor are concurrent with excitotoxic death of neurons (Simon et al., 1984; Pohorecki et al., 1990). Our data presented here also show that glutamate mediates neuronal death induced by hypoxia in the presence of NO (from DETA/NO or from activated glia). We found that the NMDA receptor blocker MK-801 prevented neuronal death in both cases. Higher NO concentrations have been shown to cause glutamate release from neurons, as a result of inhibition of mitochondrial respiration (McNaught and Brown, 1997; Bal-Price and Brown, 2001) and through Ca^{2+}-dependent pathways from neurons (Leist et al., 1997) and astrocytes (Bal-Price et al., 2002). These results suggest that glial-derived NO in hypoxia causes activation of the NMDA receptor either via respiratory inhibition induced-glutamate release, or respiratory inhibition-induced depolarization of the plasma membrane.

Our findings presented here show that because NO production inhibits cellular respiration, inflammation sensitizes to hypoxia, inducing neuronal death. Reduced cerebral blood flow can lead to low oxygen (and glucose) levels, which in the presence of NO may cause inhibition of mitochondrial respiration and subsequent excitotoxic neuronal death. These findings may have relevance to brain pathologies such as stroke, vascular dementias, and trauma where both hypoxia/ischemia and iNOS expression occur, and suggest a mechanism by which the aged, traumatized, or degenerating brain may become more sensitive to hypoxic/ischemic damage.

REFERENCES

Almeida A, Almeida J, Bolaños JP, Moncada S. 2001. Different responses of astrocytes and neurons to nitric oxide: the role of glycolytically generated ATP in astrocyte protection. Proc Natl Acad Sci USA 98:15294–15299.

Almeida A, Moncada S, Bolaños JP. 2004. Nitric oxide switches on glycolysis through the AMP protein kinase and 6-phosphofructo-2-kinase pathway. Nat Cell Biol 6:45–51.

Bal-Price A, Brown GC. 2001. Inflammatory neurodegeneration mediated by nitric oxide from activated glia-inhibiting neuronal respiration, causing glutamate release and excitotoxicity. J Neurosci 21:6480–6491.

Bal-Price A, Moneer Z, Brown GC. 2002. Nitric oxide induces rapid, calcium-dependent release of vesicular glutamate and ATP from cultured rat astrocytes. Glia 40:312–323.

Barger SW, Basile AS. 2001. Activation of microglia by secreted amyloid precursor protein evokes release of glutamate by cystine exchange and attenuates synaptic function. J Neurochem 76:846–854.

Beckman JS, Chen J, Crow JP, Ye YZ. 1994. Reactions of nitric oxide, superoxide and peroxynitrite with superoxide dismutase in neurodegeneration. Prog Brain Res 103:371–380.

Bellamy TC, Griffiths C, Garthwaite J. 2002. Differential sensitivity of guanylyl cyclase and mitochondrial respiration to nitric oxide measured using clamped concentrations. J Biol Chem 277:31801–31807.

Boje KM, Arora PK. 1992. Microglial-produced nitric oxide and reactive nitrogen oxides mediate neuronal cell death. Brain Res 587:250–256.

Bolaños JP, Almeida A, Fernandez E, Medina JM, Land JM, Clark JB, Heales SJ. 1997. Potential mechanisms for nitric oxide-mediated impairment of brain mitochondrial energy metabolism. Biochem Soc Trans 25:944–949.

Bouma GJ, Muizelaar JP. 1992. Cerebral blood flow, cerebral blood volume, and cerebrovascular reactivity after severe head injury. J Neurotrauma 1(Suppl):333–348.

Brown GC, Borutaite V. 2002. Nitric oxide inhibition of mitochondrial respiration and its role in cell death. Free Radic Biol Med 33:1440–1450.

Brown GC, Cooper CE. 1994. Nanomolar concentrations of nitric oxide reversibly inhibit synaptosomal respiration by competing with oxygen at cytochrome oxidase. FEBS Lett 356:295–298.

Bruce AJ, Boling W, Kindy MS, Peschon J, Kraemer PJ, Carpenter MK, Holtsberg FW, Mattson MP. 1996. Altered neuronal and microglial responses to excitotoxic and ischemic brain injury in mice lacking TNF receptors. Nat Med 2:788–794.

Chao CC, Hu S, Ehrlich L, Peterson PK. 1995a. Interleukin-1 and tumor necrosis factor-alpha synergistically mediate neurotoxicity: involvement of nitric oxide and of *N*-methyl-D-aspartate receptors. Brain Behav Immun 9:355–365.

Chao CC, Hu S, Peterson PK. 1995b. Modulation of human microglial cell superoxide production by cytokines. J Leukoc Biol 58:65–70.

Chao CC, Hu S, Sheng WS, Bu D, Bukrinsky MI, Peterson PK. 1996. Cytokine-stimulated astrocytes damage human neurons via a nitric oxide mechanism. Glia 16:276–284.

Choi DW. 1996. Ischemia-induced neuronal apoptosis. Curr Opin Neurobiol 6:667–672.

Clark RS, Kochanek PM, Schwarz MA, Schiding JK, Turner DS, Chen M, Carlos TM, Watkins SC. 1996. Inducible nitric oxide synthase expression in cerebrovascular smooth muscle and neutrophils after traumatic brain injury in immature rats. Pediatr Res 39:784–790.

Cleeter MW, Cooper JM, Darley-Usmar VM, Moncada S, Schapira AH. 1994. Reversible inhibition of cytochrome c oxidase, the terminal enzyme of the mitochondrial respiratory chain, by nitric oxide. Implications for neurodegenerative diseases. FEBS Lett 345:50–54.

De Visscher G, Springett R, Delpy DT, Van Reempts J, Borgers M, van Rossem K. 2002. Nitric oxide does not inhibit cerebral cytochrome oxidase in vivo or in the reactive hyperemic phase after brief anoxia in the adult rat. J Cereb Blood Flow Metab 22:515–519.

Demerle-Pallardy C, Lonchampt MO, Chabrier PE, Braquet P. 1993. Nitric oxide synthase induction in glial cells: effect on neuronal survival. Life Sci 52:1883–1890.

Desmond DW. 1996. Vascular dementia: a construct in evolution. Cerebrovasc Brain Metab Rev 8:296–325.

Eddleston M, Mucke L. 1993. Molecular profile of reactive astrocytes—implications for their role in neurologic disease. Neuroscience 54:15–36.

Endoh M, Maiese K, Wagner J. 1994. Expression of the inducible form of nitric oxide synthase by reactive astrocytes after transient global ischemia. Brain Res 651:92–100.

Ganter S, Northoff H, Mannel D, Gebicke-Harter PJ. 1992. Growth control of cultured microglia. J Neurosci Res 33:218–230.

Gebicke-Haerter PJ, Bauer J, Schobert A, Northoff H. 1989. Lipopolysaccharide-free conditions in primary astrocyte cultures allow growth and isolation of microglial cells. J Neurosci 9:183–194.

Hewett SJ, Csernansky CA, Choi DW. 1994. Selective potentiation of NMDA-induced neuronal injury following induction of astrocytic iNOS. Neuron 13:487–494.

Hu J, Akama KT, Krafft GA, Chromy BA, Van Eldik LJ. 1998. Amyloid-beta peptide activates cultured astrocytes: morphological alterations, cytokine induction and nitric oxide release. Brain Res 785:195–206.

Iadecola C, Zhang F, Xu X. 1995. Inhibition of inducible nitric oxide synthase ameliorates cerebral ischemic damage. Am J Physiol 268:286–292.

Jendroska K, Poewe W, Daniel SE, Pluess J, Iwerssen-Schmidt H, Paulsen J, Barthel S, Schelosky L, Cervos-Navarro J, DeArmond SJ. 1995. Ischemic stress induces deposition of amyloid beta immunoreactivity in human brain. Acta Neuropathol (Berl) 90:461–466.

Juurlink BH, Sweeney MI. 1997. Mechanisms that result in damage during and following cerebral ischemia. Neurosci Biobehav Rev 21:121–128.

Kawase M, Kinouchi H, Kato I, Akabane A, Kondo T, Arai S, Fujimura M, Okamoto H, Yoshimoto T. 1996. Inducible nitric oxide synthase following hypoxia in rat cultured glial cells. Brain Res 738:319–322.

Kim HS, Lee SH, Kim SS, Kim YK, Jeong SJ, Ma J, Han DH, Cho BK, Suh YH. 1998. Post-ischemic changes in the expression of Alzheimer's APP isoforms in rat cerebral cortex. Neuroreport 9:533–537.

Kreutzberg GW. 1996. Microglia: a sensor for pathological events in the CNS. Trends Neurosci 19:312–318.

Kyrkanides S, O'Banion MK, Whiteley PE, Daeschner JC, Olschowka JA. 2001. Enhanced glial activation and expression of specific CNS inflammation-related molecules in aged versus young rats following cortical stab injury. J Neuroimmunol 119:269–277.

Lee VY, McClintock DS, Santore MT, Budinger GR, Chandel NS. 2002. Hypoxia sensitizes cells to nitric oxide-induced apoptosis. J Biol Chem 277:16067-16074.

Leist M, Volbracht C, Kuhnle S, Fava E, Ferrando-May E, Nicotera P. 1997. Caspase-mediated apoptosis in neuronal excitotoxicity triggered by nitric oxide. Mol Med 3:750–764.

Loihl AK, Murphy S. 1998. Expression of nitric oxide synthase-2 in glia associated with CNS pathology. Prog Brain Res 118:253–267.

Macko RF, Ameriso SF, Barndt R, Clough W, Weiner JM, Fisher M. 1996. Precipitants of brain infarction. Roles of preceding infection/inflammation and recent psychological stress. Stroke 27:1999–2004.

Mamchaoui K, Saumon G. 2000. A method for measuring the oxygen consumption of intact cell monolayers. Am J Physiol Lung Cell Mol Physiol 278:858–863.

Marty S, Dusart I, Peschanski M. 1991. Glial changes following an excitotoxic lesion in the CNS—I. Microglia/macrophages. Neuroscience 45:529–539.

McNaught KS, Brown GC. 1997. Nitric oxide causes release of glutamate from brain synaptosomes. Biochem Soc Trans 25:411.

Moncada S, Erusalimsky JD. 2002. Does nitric oxide modulate mitochondrial energy generation and apoptosis? Nat Rev Mol Cell Biol 3:214–220.

Munoz-Pinedo C, Ruiz-Ruiz C, Ruiz de Almodovar C, Palacios C, Lopez-Rivas A. 2003. Inhibition of glucose metabolism sensitizes tumor cells to death receptor-triggered apoptosis through enhancement of death-inducing signaling complex formation and apical procaspase-8 processing. J Biol Chem 278:12759–12768.

Pohorecki R, Becker GL, Reilly PJ, Landers DF. 1990. Ischemic brain injury in vitro: protective effects of NMDA receptor antagonists and calmidazolium. Brain Res 528:133–137.

Pulsinelli WA, Duffy TE. 1983. Regional energy balance in rat brain after transient forebrain ischemia. J Neurochem 40:1500–1503.

Sharshar T, Gray F, Lorin de la Grandmaison G, Hopkinson NS, Ross E, Dorandeu A, Orlikowski D, Raphael JC, Gajdos P, Annane D. 2003. Apoptosis of neurons in cardiovascular autonomic centres triggered by inducible nitric oxide synthase after death from septic shock. Lancet 362:1799–1805.

Simon RP, Swan JH, Griffiths T, Meldrum BS. 1984. Blockade of *N*-methyl-D-aspartate receptors may protect against ischemic damage in the brain. Science 226:850–852.

Sweeney MI, Yager JY, Walz W, Juurlink BH. 1995. Cellular mechanisms involved in brain ischemia. Can J Physiol Pharmacol 73:1525–1535.

Syrjanen J. 1993. Infection as a risk factor for cerebral infarction. Eur Heart J 14(Suppl):17–19.

Vernet D, Bonavera JJ, Swerdloff RS, Gonzalez-Cadavid NF, Wang C. 1998. Spontaneous expression of inducible nitric oxide synthase in the hypothalamus and other brain regions of aging rats. Endocrinology 139: 3254–3261.

Walton M, Connor B, Lawlor P, Young D, Sirimanne E, Gluckman P, Cole G, Dragunow M. 1999. Neuronal death and survival in two models of hypoxic-ischemic brain damage. Brain Res Brain Res Rev 29:137–168.

Woodward GE, Hudson MT. 1954. The effect of 2-desoxy-D-glucose on glycolysis and respiration of tumor and normal tissues. Cancer Res 14: 599–605.

Yager JY, Brucklacher RM, Vannucci RC. 1992. Cerebral energy metabolism during hypoxia-ischemia and early recovery in immature rats. Am J Physiol 262:672–677.

Yang XJ, Kow LM, Funabashi T, Mobbs CV. 1999. Hypothalamic glucose sensor: similarities to and differences from pancreatic beta-cell mechanisms. Diabetes 48:1763–1772.

Zhang F, Casey RM, Ross ME, Iadecola C. 1996. Aminoguanidine ameliorates and L-arginine worsens brain damage from intraluminal middle cerebral artery occlusion. Stroke 27:317–323.

Zhao X, Ross ME, Iadecola C. 2003. L-Arginine increases ischemic injury in wild-type mice but not in iNOS-deficient mice. Brain Res 966:308–311.