Journal of Neurochemistry
Raven Press, Ltd., New York

Nitric Oxide-Mediated Inhibition of the Mitochondrial Respiratory Chain in Cultured Astrocytes

J. P. Bolaños, S. Peuchen, S. J. R. Heales, J. M. Land, and J. B. Clark

Department of Neurochemistry, Institute of Neurology, London, England

Abstract: The Ca^{2+}-independent form of nitric oxide synthase was induced in rat neonatal astrocytes in primary culture by incubation with lipopolysaccharide (1 μg/ml) plus interferon-γ (100 U/ml), and the activities of the mitochondrial respiratory chain components were assessed. Incubation for 18 h produced 25% inhibition of cytochrome *c* oxidase activity. NADH–ubiquinone-1 reductase (complex I) and succinate–cytochrome *c* reductase (complex II–III) activities were not affected. Prolonged incubation for 36 h gave rise to a 56% reduction of cytochrome *c* oxidase activity and a 35% reduction in succinate–cytochrome *c* reductase activity, but NADH–ubiquinone-1 reductase activity was unchanged. Citrate synthase activity was not affected by any of these conditions. The inhibition of the activities of these mitochondrial respiratory chain complexes was prevented by incubation in the presence of the specific nitric oxide synthase inhibitor N^G-monomethyl-L-arginine. The lipopolysaccharide/interferon-γ treatment of the astrocytes produced an increase in glycolysis and lactate formation. These results suggest that inhibition of the mitochondrial respiratory chain after induction of astrocytic nitric oxide synthase may represent a mechanism for nitric oxide-mediated neurotoxicity. **Key Words:** Astrocytes—Lipopolysaccharide—Interferon-γ—Nitric oxide synthase—Nitric oxide—Mitochondrial respiratory chain.
J. Neurochem. **63,** 910–916 (1994).

There is increasing evidence to support the concept that defects in mitochondrial energy metabolism may underlie the pathology of neurodegenerative diseases such as Parkinson's disease or Alzheimer's disease (Beal et al., 1993). The exact mechanisms involved in the pathogenesis of these disorders are not known, but it is thought that there may be excessive production of oxygen-derived free radicals (Halliwell, 1992; Olanow, 1993). This notion is supported by in vitro work in which the components of the mitochondrial respiratory chain have been shown to be susceptible to damage by free radicals (Zhang et al., 1990).

Nitric oxide (NO) is a free radical that has several important messenger functions within the CNS. However, excessive production of NO appears to be neurotoxic (Dawson et al., 1991, 1993), but the mechanism of this toxicity is not known. Under certain conditions, NO may react with the superoxide anion (O_2^-) to form the peroxynitrite anion ($ONOO^-$), which is an extremely potent oxidising agent (Beckman et al., 1990; Radi et al., 1991). In view of its reactivity, $ONOO^-$ has been implicated as the agent responsible for NO-mediated neurotoxicity (Lipton et al., 1993), but the means whereby $ONOO^-$ brings about cell death is not known.

Within the brain, astrocytes may be a major source of NO, because these cells have the highest concentration of the NO precursor L-arginine (Aoki et al., 1991) and possess the inducible form of NO synthase (NOS; EC 1.14.13.39) (Galea et al., 1992; Simmons and Murphy, 1992, 1993; Murphy et al., 1993). The aim of this study was to investigate, using astrocytes in primary culture, the effects of the induction of NO synthesis on the mitochondrial respiratory chain.

MATERIALS AND METHODS

Materials

Earle's balanced salts solution (EBSS), lipopolysaccharide (LPS; from *Escherichia coli*), and interferon-γ (IFN-γ; mouse, recombinant) were obtained from Sigma Chemical Co. (Poole, U.K.). Minimal essential medium (MEM), foetal bovine serum, and tissue culture plastics were purchased from GIBCO BRL (Renfrewshire, U.K.). Ubiquinone-1 (CoQ_1) was a kind gift of the Eisai Chemical Co. (Tokyo, Japan). (6*R*)-5,6,7,8-Tetrahydro-L-biopterin was obtained from Dr. B. Schircks Laboratories (Jona, Switzerland). Cation exchange resin Dowex AG 50W-X8 (100–200 mesh, H^+ form) was purchased from Bio-Rad Laboratories (Richmond, CA, U.S.A.). This was converted to the Na^+ form by adding excess 1 *M* NaOH and then washing with distilled water

Received December 16, 1993; revised manuscript received January 26, 1994; accepted January 26, 1994.

Address correspondence and reprint requests to Dr. J. P. Bolaños at Department of Neurochemistry, Institute of Neurology, Queen Square, London WC1N 3BG, U.K.

Abbreviations used: CAT, catalase; CoQ_1, ubiquinone-1; EBSS, Earle's balanced salts solution; IFN-γ, interferon-γ; LPS, lipopolysaccharide; MEM, minimal essential medium; NMMA, N^G-monomethyl-L-arginine; NO, nitric oxide; NOS, nitric oxide synthase; SOD, superoxide dismutase.

until the pH of the resin was ≤9. L-[2,3,4,5-^{3}H]Arginine and L-[2,3,4,5-^{3}H]citrulline were obtained from Amersham International (Buckinghamshire, U.K.). Other substrates, enzymes, and coenzymes were purchased from Sigma or Boehringer Mannheim (Lewes, U.K.). Cytochrome *c* (Boehringer) was reduced with sodium ascorbate just before use and passed through Sephadex G-25M (PD-10 columns; Pharmacia LKB, Uppsala, Sweden) to remove the excess ascorbate.

Cell culture

Primary astrocyte cultures were prepared from neonatal (1–2 days old) Wistar rats as previously described (Tabernero et al., 1993) but with some modifications. In brief, animals were decapitated and their forebrains were removed. After removal of meninges and blood vessels, the tissue was dissociated by trituration in EBSS and filtered through a 280-μm (pore size) wire mesh. The cell suspension was washed, the cells were harvested by centrifugation (400 *g* for 10 min), the supernatant was discarded, and the pellet was resuspended in fresh EBSS. Subsequently the pellet was resuspended in EBSS containing 0.025% (wt/vol) trypsin (EC 3.4.21.4; type III; Sigma) and 200 μg/ml of deoxyribonuclease 5′-oligonucleotidohydrolase (EC 3.1.21.1; type II; Sigma) and incubated at 37°C for 15 min. The dissociated cells were washed three times in EBSS and once with a D-valine-based MEM containing Earle's salts (Cholewinski et al., 1989), and the suspension was filtered through a 104-μm (pore size) wire mesh. The cells were plated on tissue culture-treated plastic in a D-valine-based MEM supplemented with 10% (vol/vol) foetal bovine serum, 2 m*M* L-glutamine, and 1 m*M* L-malate and incubated at 37°C under a humidified atmosphere containing 5% CO_2/95% air. After 3 days, the culture medium was replaced and twice weekly thereafter. After 6 days the D-valine medium was replaced with a L-valine medium. Confluency was reached after 7 days of culture, when the cells were trypsinised and divided over a larger surface area.

After 14 days in culture the cells were removed from the plastic by trypsinisation, counted, and distributed into new flasks at densities of ~2×10^6 cells/25 cm^2 containing 5 ml of medium. Additions were made from concentrated solutions to the cell culture medium to give final concentrations of 0.01–1 μg/ml of LPS, 1–100 U/ml of IFN-γ, or LPS plus IFN-γ in combination (LPS/IFN-γ). Astrocytes were then incubated for 18 or 36 h at 37°C. Optionally, N^G-monomethyl-L-arginine (NMMA; 0.05–1 m*M*), superoxide dismutase (SOD; EC 1.15.1.1; 250 U/ml; Sigma) plus catalase (CAT; EC 1.11.1.6; 250 U/ml; Sigma), or cycloheximide (10 μg/ml) was also added. The culture medium of those cells incubated for 36 h was changed after the first 18 h with fresh medium containing the same additives. Parallel cultures in the absence of additions were carried out as controls. After the period of incubation the medium was collected, the cells were removed from the plastic by trypsinisation and washed twice in EBSS, and the cell pellet was frozen at −70°C until use (within 1 week). Culture medium was kept at 4°C until use (within the same day).

Preparation of astrocyte cytosol

Cells were homogenised in distilled water and centrifuged at 100,000 *g* for 60 min at 4°C. The supernatant was applied to a 0.5-ml column of cation exchange resin (Dowex AG 50W-X8, 100–200 mesh, Na^+ form) to remove the endogenous L-arginine. The resulting cytosol was frozen at −70°C until use for determination of NOS activity.

Enzyme activity determination

NOS activity in astrocyte cytosol was determined by measurement of formation of L-[^{3}H]citrulline from L-[^{3}H]-arginine, essentially as described by Bredt and Snyder (1989) with some modifications. In brief, 100 μl of cytosol (~30–50 μg of protein) was incubated at 37°C in a total volume of 200 μl of a 100 m*M* HEPES buffer (pH 7.5) containing 100 μ*M* L-[2,3,4,5-^{3}H]arginine (10 Ci/mol), 100 μ*M* β-NADPH, 1 m*M* magnesium acetate, 1 m*M* dithiothreitol, 1 m*M* $CaCl_2$, 10 μg/ml of calmodulin, 5 μ*M* FAD, 5 μ*M* FMN, and 50 μ*M* (6*R*)-5,6,7,8-tetrahydro-L-biopterin. Optionally, 1 m*M* EGTA or 1 m*M* NMMA was included. After 30 min, the incubations were terminated by addition of 50 μl of 1.53 *M* trichloroacetic acid, placed on ice, and mixed with 1 ml of 1.5 *M* HEPES, pH 6.0. One milliliter of the mixture was applied to 2 ml of cation exchange resin (Dowex AG 50W-X8, 100–200 mesh, Na^+ form), and L-[^{3}H]citrulline was specifically eluted with 4 × 1 ml of distilled water. L-[^{3}H]-Citrulline was quantified by liquid scintillation counting of the 5-ml flowthrough (50% efficiency). Parallel incubations, in which media were acidified with 50 μl of 1.53 *M* trichloroacetic acid before addition of cytosol, were used as blanks. Preliminary experiments showed that the amount of [^{3}H]-citrulline formed was linear with time throughout the 30-min incubation. The recovery of L-[^{3}H]citrulline, assessed by carrying out similar incubations except that L-[2,3,4,5-^{3}H]citrulline (50 μ*M*, 10 Ci/mol) was used instead of L-[2,3,4,5-^{3}H]arginine, was always ≥96%. The specific activity of L-arginine (dpm/mol) was used for the calculations. Cytosolic NOS activity is given as picomoles of L-citrulline formed per minute per milligram of protein.

Other enzyme activities were determined in the cell homogenates derived from the cell pellets (see above). The cell pellets were frozen and thawed three times to lyse cell membranes and resuspended at an approximate concentration of 1 mg of protein/ml of suspending medium (0.25 *M* sucrose and 2 m*M* HEPES, pH 7.2). Changes in absorbance were monitored using a Uvikon model 940 spectrophotometer (Kontron Instruments Ltd., Watford, U.K.). NADH–CoQ_1 reductase (complex I; EC 1.6.99.3) and succinate–cytochrome *c* reductase (complex II–III; EC 1.8.3.1) activities were determined as described by Schapira et al. (1990). Cytochrome *c* oxidase (complex IV; EC 1.9.3.1) activity was assayed by the method of Wharton and Tzagoloff (1967). Citrate synthase (EC 4.1.3.7) activity was measured according to the method of Shephard and Garland (1969). Lactate dehydrogenase (EC 1.1.1.27) activity was determined after the procedure of Vassault (1983). All these enzyme activities were expressed as nanomoles per minute per milligram of protein, except for cytochrome *c* oxidase, which was expressed as the first-order reaction constant (*k*/min/mg).

Metabolite content determination

Nitrates (NO_3^-) were reduced with washed acid cadmium powder (Davison and Woof, 1978) and then quantified as nitrites (NO_2^-) (designated $NO_2^- + NO_3^-$) by the colorimetric assay based on the Griess reaction (Green et al., 1982). Hydrogen peroxide was assayed by chemiluminescence as described by White and Clark (1989). Fresh culture media served as blanks for $NO_2^- + NO_3^-$ and H_2O_2 content determinations. L-Lactate was determined as described by Gutmann and Wahlefeld (1974), and D-glucose was quantified after the procedure of Bergmeyer et al. (1974).

Reduced glutathione (GSH) concentrations in astrocytes

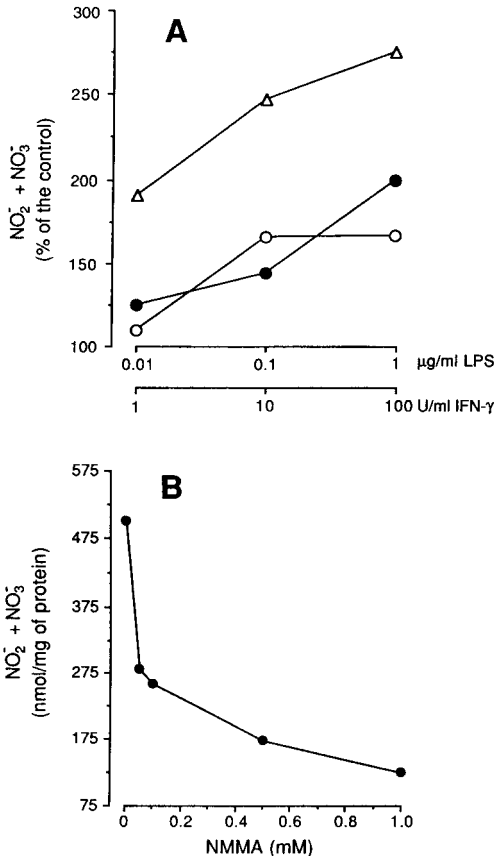

FIG. 1. A: Release of $NO_2^- + NO_3^-$ by astrocytes to the culture medium over 18 h of incubation with LPS alone (○), IFN-γ alone (●), or a combination of LPS plus IFN-γ (△). The control value was 73.1 nmol/mg of protein. **B:** Effect of increasing NMMA concentration on the LPS/IFN-γ (1 µg/ml plus 100 U/ml, respectively)-mediated $NO_2^- + NO_3^-$ release by astrocytes after 18 h of incubation. Results are expressed as means of determinations carried out in duplicate (SD ≤ 5%) in a representative experiment of a total of two.

were determined by HPLC and electrochemical detection (Riederer et al., 1989).

Protein content determination

Protein concentration was determined by the method of Lowry et al. (1951).

Statistical analysis

Determinations were performed in duplicate or in triplicate, and results are presented as mean ± SEM values for the number of culture preparations indicated in the legends. Statistical evaluation of results was assessed using Student's *t* test.

RESULTS

The production of $NO_2^- + NO_3^-$ by astrocytes in primary culture was assessed as a measure of NO synthesis (Ignarro et al., 1993) after 18 h of culture in the presence of increasing concentrations of LPS alone, IFN-γ alone, or in combination, and it was compared with the basal levels of $NO_2^- + NO_3^-$ of the untreated cells (Fig. 1A). LPS and IFN-γ progressively increased the $NO_2^- + NO_3^-$ production as compared with the control levels. The combination of LPS plus IFN-γ potentiated the $NO_2^- + NO_3^-$ release as compared with LPS and IFN-γ alone. In the presence of LPS (1 µg/ml) plus IFN-γ (100 U/ml) for 18 h, increasing concentrations of NMMA decreased the $NO_2^- + NO_3^-$ production by the astrocytes to 12% of maximum (Fig. 1B). Addition of NMMA to nontreated cells had no effect on $NO_2^- + NO_3^-$ release. Supplementation of L-arginine to the culture medium (up to 4 m*M*) did not increase the $NO_2^- + NO_3^-$ production further by treated astrocytes after 18 or 36 h. No L-arginine supplementation was made therefore, and 1 µg/ml of LPS plus 100 U/ml of IFN-γ (designated as LPS/IFN-γ) were the sole additions.

NOS was assayed in the cytosolic fraction of the cells after 18 or 36 h of treatment with LPS/IFN-γ. After 18 h of culture, NOS activity increased 96-fold in the absence and 73-fold in the presence of 1 m*M* EGTA in the reaction medium. No further increase in cytosolic NOS activity was found after 36 h in culture (Table 1). In all cases, the presence of 1 m*M* NMMA in the reaction medium inhibited NOS activity (~97% inhibition). The presence of the protein synthesis inhibitor cycloheximide in the LPS/IFN-γ-treated cells prevented the increase of cytosolic NOS activity (Table 1).

The increase in $NO_2^- + NO_3^-$ production after 18 and 36 h of LPS/IFN-γ treatment was accompanied by an increase in H_2O_2 production (Table 2). Both the increase in $NO_2^- + NO_3^-$ and H_2O_2 release were prevented by the presence of 1 m*M* NMMA in the

TABLE 1. *Induction of NOS activity by LPS/IFN-γ in neonatal astrocytes in primary culture*

Treatment	Cytosolic NOS (pmol of L-citrulline formed/min/mg of protein)		
	−EGTA	+EGTA	+NMMA, −EGTA
18 h			
Control	21.6 ± 0.3	25.7 ± 2.4	ND
LPS/IFN-γ	2,077 ± 28[a]	1,876 ± 8[a]	64.3 ± 2.8
LPS/IFN-γ + cycloheximide	43.8 ± 1.1	36.5 ± 0.9	ND
36 h			
Control	23.1 ± 2.0	24.0 ± 2.1	ND
LPS/IFN-γ	2,123 ± 28[a]	1,930 ± 27[a]	72.7 ± 3.1
LPS/IFN-γ + cycloheximide	30.1 ± 6.0	21.7 ± 3.3	ND

Astrocytes were incubated for 18 or 36 h in the absence (control) or presence of LPS plus IFN-γ (1 µg/ml plus 100 U/ml, respectively). Cycloheximide was added at a concentration of 10 µg/ml. NOS activity was determined in the cytosolic fraction in the absence or in the presence of 1 m*M* EGTA or 1 m*M* NMMA as described in Materials and Methods. Data are mean ± SEM values of triplicate determinations from three different culture preparations. ND, not detectable.

[a] $p < 0.001$, compared with the corresponding control group.

TABLE 2. *LPS/IFN-γ-mediated release of NO_2^- + NO_3^- and H_2O_2 by neonatal astrocytes in primary culture*

Condition	nmol/mg of protein	
	NO_2^- + NO_3^-	H_2O_2
18 h		
Control	51.9 ± 10.5	29.2 ± 2.3
LPS/IFN-γ	481.2 ± 17.0[a]	39.7 ± 1.9[b]
LPS/IFN-γ + NMMA	70.2 ± 11.8	22.5 ± 11.2
LPS/IFN-γ + SOD/CAT	219.5 ± 33.9[a]	ND
36 h		
Control	52.6 ± 11.9	31.1 ± 3.0
LPS/IFN-γ	865.6 ± 80.6[a]	49.3 ± 4.2[b]
LPS/IFN-γ + NMMA	105.6 ± 11.5[b]	21.6 ± 7.4
LPS/IFN-γ + SOD/CAT	303.5 ± 63.1[c]	ND

Astrocytes were incubated for 18 or 36 h in the absence (control) or presence of LPS plus IFN-γ (1 μg/ml plus 100 U/ml, respectively), NMMA (1 m*M*), or SOD plus CAT (250 U/ml each) as indicated. Culture medium was collected and used for NO_2^- + NO_3^- and H_2O_2 analysis within the same day as described in Materials and Methods. Data are mean ± SEM values of seven (NO_2^- + NO_3^-) or three (H_2O_2) different culture preparations. ND, not detectable.

[a] $p < 0.001$, [b] $p < 0.05$, [c] $p < 0.01$, compared with the corresponding control group.

culture medium. Supplementation of the medium with SOD plus CAT (250 U/ml each, thereafter designated as SOD/CAT) partially prevented the increase in NO_2^- + NO_3^- production. However, these reduced values were still statistically significantly higher than values for the control group (Table 2). SOD/CAT alone did not affect the basal NO_2^- + NO_3^- production (data not shown).

After LPS/IFN-γ treatment the cells were collected, and the activities of the individual components of the mitochondrial respiratory chain were studied. Table 3 shows that the treatment produced, after 18 h, a 25% inhibition of the activity of cytochrome *c* oxidase, without affecting the activities of NADH–CoQ_1 reductase or succinate–cytochrome *c* reductase. Citrate synthase activity was unchanged. After 36 h of treatment, a 56% inhibition of cytochrome *c* oxidase activity was found together with a 35% inhibition of succinate–cytochrome *c* reductase activity. No changes in NADH–CoQ_1 reductase or citrate synthase activities were found after 36 h of treatment. The presence of NMMA (1 m*M*) or SOD/CAT (250 U/ml each) in the culture medium prevented the inhibition of cytochrome *c* oxidase activity observed after 18 h and the inhibition of cytochrome *c* oxidase and succinate–cytochrome *c* reductase activities observed after 36 h.

In view of the fact that thiol-containing peptides are susceptible to oxidative damage by peroxynitrite (Radi et al., 1991), the GSH concentration in the astrocytes was determined after 18 h of LPS/IFN-γ treatment. As shown in Table 4, neither the LPS/IFN-γ-treated cells or those with LPS/IFN-γ plus 1 m*M* NMMA showed differences in the GSH concentration with respect to the control group.

To evaluate the cell death, lactate dehydrogenase activity was measured in the culture medium and in the cells after 18 and 36 h of LPS/IFN-γ treatment. Table 5 shows that neither LPS/IFN-γ or LPS/IFN-γ plus NMMA modified the intracellular lactate dehydrogenase activity or its release to the medium.

In view of the changes in the mitochondrial respiratory chain complexes, it was important to assess the glycolytic activity of the cells. Thus, the production of lactate and the consumption of glucose by astrocytes were determined after 18 and 36 h of LPS/IFN-γ treatment. The production of lactate was determined from the difference between the concentration of lactate in the cell-containing culture medium and that in the cell-free medium (~1.9 m*M*). The consumption of glucose was calculated from the difference between the con-

TABLE 3. *Effect of LPS/IFN-γ-mediated NOS induction on the activity of the mitochondrial respiratory chain complexes and citrate synthase in neonatal astrocytes in primary culture*

	NADH–CoQ_1 reductase (nmol/min/mg)	Succinate–cytochrome *c* reductase (nmol/min/mg)	Cytochrome *c* oxidase (*k*/min/mg)	Citrate synthase (nmol/min/mg)
18 h				
Control	65.5 ± 3.5	11.31 ± 1.41	2.12 ± 0.08	132.5 ± 13.0
LPS/IFN-γ	61.3 ± 4.7	10.70 ± 1.37	1.58 ± 0.12[a]	136.3 ± 9.5
LPS-IFN-γ + NMMA	63.5 ± 6.7	10.81 ± 2.91	2.03 ± 0.11	127.7 ± 9.1
LPS/IFN-γ + SOD/CAT	64.6 ± 10.9	12.15 ± 1.09	1.81 ± 0.14	130.4 ± 12.5
36 h				
Control	65.0 ± 5.2	11.78 ± 1.06	2.12 ± 0.10	131.3 ± 10.0
LPS/IFN-γ	62.4 ± 8.8	7.64 ± 1.10[b]	0.93 ± 0.05[c]	133.0 ± 9.0
LPS/IFN-γ + NMMA	58.9 ± 7.6	12.16 ± 2.07	2.08 ± 0.08	132.3 ± 7.8
LPS/IFN-γ + SOD/CAT	62.2 ± 5.1	11.02 ± 1.30	1.71 ± 0.17	135.3 ± 9.4

Astrocytes were incubated for 18 or 36 h in the absence (control) or in the presence of LPS plus IFN-γ (1 μg/ml plus 100 U/ml, respectively), NMMA (1 m*M*), or SOD plus CAT (250 U/ml each) as indicated. Cell homogenates were used for enzyme activity determinations as described in Materials and Methods. Data are mean ± SEM values of four to six different culture preparations.

[a] $p < 0.01$, [b] $p < 0.05$, [c] $p < 0.001$, compared with the corresponding control group.

TABLE 4. *GSH concentrations in neonatal astrocytes in primary culture after exposure to LPS/IFN-γ for 18 h*

	GSH concentration (nmol/mg of protein)
Control	26.5 ± 4.6
LPS/IFN-γ	33.0 ± 6.9
LPS/IFN-γ + NMMA	30.5 ± 5.1

Astrocytes were incubated for 18 h in the absence (control) or presence of LPS plus IFN-γ (1 μg/ml plus 100 U/ml, respectively). NMMA was added at the concentration of 1 m*M*. GSH was quantified in the cells by HPLC as described in Materials and Methods. Data are mean ± SEM values of three different culture preparations.

centration of glucose in the cell-free culture medium (~5.5 m*M*) and that in the cell-containing medium. As shown in Table 6, lactate production was increased by LPS/IFN-γ treatment, which was accompanied by a parallel increase in glucose consumption. This effect was observed after either 18 or 36 h of treatment. Both NMMA or SOD/CAT reduced the LPS/IFN-γ-induced increases in lactate production and glucose consumption.

DISCUSSION

Astrocytes possess both the constitutive (Murphy et al., 1990) and the inducible (Galea et al., 1992; Simmons and Murphy, 1992) form of NOS. The presence of LPS plus IFN-γ potentiated the NO_2^- + NO_3^- release by astrocytes incubated in the presence of LPS and IFN-γ alone (Fig. 1A). This was accompanied by induction of a cytosolic, Ca^{2+}-independent NOS activity (Table 1) that is ~20-fold higher than that found by Galea et al. (1992) after treatment with LPS alone. This potentiation agrees with the notion that cytokines may mediate the induction of NOS activity by LPS (Murphy et al., 1993; Simmons and Murphy, 1993).

TABLE 5. *Lactate dehydrogenase activity released by neonatal astrocytes in primary culture after exposure to LPS/IFN-γ*

	Lactate dehydrogenase activity (nmol/min/mg of protein)		
	Intracellular	Extracellular	Released (%)
18 h			
Control	1,182 ± 130	153.6 ± 7.1	13.0 ± 1.4
LPS/IFN-γ	1,069 ± 145	155.0 ± 19.1	14.5 ± 1.6
LPS/IFN-γ + NMMA	1,086 ± 138	137.9 ± 13.9	12.7 ± 1.1
36 h			
Control	1,044 ± 110	157.7 ± 11.4	15.1 ± 1.6
LPS/IFN-γ	1,154 ± 132	161.5 ± 23.4	14.0 ± 1.3
LPS/IFN-γ + NMMA	1,139 ± 125	168.5 ± 0.9	14.8 ± 1.4

Astrocytes were incubated for 18 or 36 h in the absence (control) or presence of LPS plus IFN-γ (1 μg/ml plus 100 U/ml, respectively). NMMA was added at a concentration of 1 m*M*. Lactate dehydrogenase activity was determined in the cell homogenate (intracellular) and in the culture medium (extracellular) as described in Materials and Methods. Data are mean ± SEM values of five different culture preparations.

TABLE 6. *Lactate produced and glucose consumed by neonatal astrocytes in primary culture after exposure to LPS/IFN-γ*

	μmol/mg of protein	
	Lactate produced	Glucose consumed
18 h		
Control	25.9 ± 1.9	14.9 ± 1.4
LPS/IFN-γ	53.0 ± 3.3[a]	28.8 ± 1.9[a]
LPS/IFN-γ + NMMA	32.9 ± 1.7	19.3 ± 2.4
LPS/IFN-γ + SOD/CAT	35.7 ± 3.1	18.7 ± 3.0
36 h		
Control	27.3 ± 2.3	15.5 ± 1.8
LPS/IFN-γ	70.1 ± 6.3[a]	34.2 ± 2.4[a]
LPS/IFN-γ + NMMA	31.7 ± 3.0	16.8 ± 2.6
LPS/IFN-γ + SOD/CAT	46.8 ± 7.2	23.7 ± 5.8

Astrocytes were incubated for 18 or 36 h in the absence (control) or presence of LPS plus IFN-γ (1 μg/ml plus 100 U/ml, respectively), NMMA (1 m*M*), or SOD plus CAT (250 U/ml each) as indicated. Culture medium was collected, and the amounts of L-lactate produced and D-glucose consumed were analysed as described in Materials and Methods. Data are mean ± SEM values of three different culture preparations.

[a] $p < 0.01$, compared with the corresponding control group.

LPS/IFN-γ treatment for 18 h on astrocytes in culture caused a marked decrease in the activity of cytochrome *c* oxidase without affecting NADH–CoQ_1 reductase, succinate–cytochrome *c* reductase, or citrate synthase (Table 3). Only after a longer period of treatment (36 h) was an inhibition of succinate–cytochrome *c* reductase observed. These effects were completely prevented by the specific NOS inhibitor NMMA, suggesting that NOS activity is necessary for inhibition of these enzymes.

Our results show that NO_2^- + NO_3^- release by LPS/IFN-γ treatment was accompanied by an enhanced H_2O_2 production (Table 2). These findings are consistent with the observation that NOS also produces O_2^- and/or H_2O_2 in addition to NO (Heinzel et al., 1992; Pou et al., 1992). Because H_2O_2 is formed by dismutation of O_2^-, astrocytes may produce O_2^- and hence synthesis of $ONOO^-$ can occur. Peroxynitrite is an unstable species at physiological pH, protonating to form peroxynitrous acid (ONOOH), which spontaneously decomposes to the nitrogen dioxide (NO_2) radical and the hydroxyl (OH) radical in 20–30% yield; the remaining ONOOH isomerises to nitrate (NO_3^-) (Beckman et al., 1990). It is difficult to assess which of the oxidant compounds cited above is responsible for the inhibition of cytochrome *c* oxidase and succinate–cytochrome *c* reductase activities in astrocytes.

However, the scavenging of O_2^- by SOD and CAT partially prevented both the NO_2^- + NO_3^- content increase (Table 2) and the inhibition of the activities of these enzymes (Table 3), suggesting that synthesis of $ONOO^-$ does occur under our conditions.

Benzi et al. (1991) reported that cytochrome *c* oxidase, succinate–cytochrome *c* reductase, and NADH–CoQ_1 reductase are sequentially inhibited by experimental deficiency in the thiol group-containing compound GSH. Because peroxynitrite has been implicated in the oxidation of tissue sulphydryls (Radi et al., 1991), GSH oxidation by $ONOO^-$ might be anticipated. However, the GSH concentration in the astrocytes was not modified by LPS/IFN-γ treatment (Table 4), suggesting that the inhibition of the cytochrome *c* oxidase and succinate–cytochrome *c* reductase activities was not secondary to a GSH deficiency. Alternatively, it has been reported that cytochrome *c* oxidase activity is specifically inhibited by peroxidation of cardiolipin during ischaemia/reperfusion injury in muscle (Soussi et al., 1990). Because $ONOO^-$ and/or its derivative NO_2 are potent activators of lipid peroxidation (Beckman et al., 1990; Radi et al., 1991), it is possible that the mechanism whereby the mitochondrial succinate–cytochrome *c* reductase and cytochrome *c* oxidase are inhibited involves a peroxynitrite-mediated loss of cell cardiolipin. It must be stated, however, that modulation on gene transcription and/or destabilising mRNA cannot be ruled out (Peunova and Enikolopov, 1993).

The cytotoxic activity of LPS/IFN-γ-activated macrophages on tumour cells is believed to occur by inhibition of mitochondrial respiration (Hibbs et al., 1988; Stuehr and Nathan, 1989). These authors reported inhibition of aconitase, NADH–CoQ_1 reductase, and succinate–cytochrome *c* reductase in the target tumour cells. However, under our conditions, NADH–CoQ_1 reductase was not affected in the LPS/IFN-γ-treated astrocytes. This apparent difference with respect to the action of NO or its derivatives may be due to a difference in susceptibility of the target cells. Alternatively, differences in the methodology may explain this discrepancy. The previous authors have measured the oxygen consumption of permeabilised tumour cells in an attempt to provide information on integrated mitochondrial function, whereas the present study deals with the individual analysis of the respiratory chain components.

The physiological significance of the NO-mediated inhibition of mitochondrial respiratory complexes in astrocytes remains to be established. Levels of cytokines may be elevated in neural and nonneural cells in pathological states such as multiple sclerosis or encephalitis (Merrill, 1987), and it is known that brain ischaemia activates microglia to produce cytokines (Banati et al., 1993). Some of these cytokines may induce NOS in astrocytes (Simmons and Murphy, 1993), suggesting that astrocyte-derived NO may be responsible for some of the phenomena observed during such pathological states (Murphy et al., 1993). In addition, the pathogenesis of some neurodegenerative diseases is thought to involve glutamate receptor stimulation (Meldrum and Garthwaite, 1990), and NO has been proposed to mediate glutamate neurotoxicity (Dawson et al., 1991, 1993). Because the neuronal activation that is concomitant with the excitatory amino acid release requires a significant input of energy for the restoration of the resting potential (Beal et al., 1993), it is tempting to speculate that neurones may be particularly vulnerable target cells for NO-mediated mitochondrial respiratory complex inhibition. Such speculation would be consistent with observations of reduced NADH–CoQ_1 reductase activity seen in postmortem brain of cases of Parkinson's disease (Schapira et al., 1990) and cytochrome *c* oxidase in Alzheimer's disease cases (Kish et al., 1992). However, in this context it is significant that the induction of NOS in astrocytes does not affect their survival (Table 5) because the astrocytes appear to have the flexibility to switch to an increased glycolysis (Table 6) to maintain their energy homeostasis. This agrees with previous studies showing that simulation of brain ischaemia by inhibition of ATP synthesis with antimycin (Pauwels et al., 1985) or dinitrophenol (Walz and Mukerji, 1990) induces a marked increase in lactate production by astrocytes but not by neurones. This effect may be compensatory to mitochondrial respiratory chain inhibition, because the resulting increased glycolysis is able to maintain the cellular ATP levels (Pauwels et al., 1985).

In conclusion, the results reported in this study suggest that LPS/IFN-γ-mediated NOS induction in astrocytes in primary culture causes marked inhibition of cytochrome *c* oxidase as a first event and, as a second, inhibition of succinate–cytochrome *c* reductase. However, these events occurred without apparent cell death. The astrocyte-derived NO, or its related oxidant metabolites, may diffuse to the neighbouring neurones, representing a mechanism of targeting NO-mediated neurotoxicity.

Acknowledgment: J.P.B. is a Postdoctoral Research Fellow funded by the Human Capital and Mobility Programme of the European Community. S.P. is funded by the Brain Research Trust and S.J.R.H. by the Worshipful Company of Pewterers. The technical assistance of Mrs. J. Choudhury is gratefully acknowledged.

REFERENCES

Aoki E., Semba R., Mikoshiba K., and Kashiwamata S. (1991) Predominant localization in glial cells of free L-arginine. Immunocytochemical evidence. *Brain Res.* **547,** 190–192.

Banati R. B., Gehrmann J., Schubert P., and Kreutzberg G. W. (1993) Cytotoxicity of microglia. *Glia* **7,** 111–118.

Beal M. F., Hyman B. T., and Koroshetz W. (1993) Do defects in mitochondrial energy metabolism underlie the pathology of neurodegenerative diseases? *Trends Neurosci.* **16,** 125–131.

Beckman J. S., Beckman T. W., Chen J., Marshall P. A., and Freeman B. A. (1990) Apparent hydroxyl radical production by

peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc. Natl. Acad. Sci. USA* **87,** 1620–1624.

Benzi G., Curti D., Pastoris O., Marzatico F., Villa R. F., and Dagani F. (1991) Sequential damage in mitochondrial complexes by peroxydative stress. *Neurochem. Res.* **16,** 1295–1302.

Bergmeyer H. U., Bernt E., Schmidt F., and Stork H. (1974) D-Glucose. Determination with hexokinase and glucose-6-phosphate dehydrogenase, in *Methods of Enzymatic Analysis, Vol. 3.* (Bergmeyer H. U., ed), pp. 1196–1201. Verlag Chemie GmbH, Weinheim.

Bredt D. S. and Snyder S. H. (1989) Nitric oxide mediates glutamate-linked enhancement of cGMP levels in the cerebellum. *Proc. Natl. Acad. Sci. USA* **86,** 9030–9033.

Cholewinski A. J., Reid J. C., McDermott A. M., and Wilkin G. P. (1989) Purification of astroglial-cell cultures from rat spinal cord. The use of D-valine to inhibit fibroblast growth. *Neurochem. Int.* **15,** 365–369.

Davison W. and Woof C. (1978) Comparison of different forms of cadmium as reducing agents for the batch determination of nitrate. *Analyst* **103,** 403–406.

Dawson V. L., Dawson T. M., London E. D., Bredt D. S., and Snyder S. H. (1991) Nitric oxide mediates glutamate neurotoxicity in primary cortical cultures. *Proc. Natl. Acad. Sci. USA* **88,** 6368–6371.

Dawson V. L., Dawson T. M., Bartley D. A., Uhl G. R., and Snyder S. H. (1993) Mechanisms of nitric oxide-mediated neurotoxicity in primary brain cultures. *J. Neurosci.* **13,** 2651–2661.

Galea E., Feinstein D. L., and Reis D. J. (1992) Induction of calcium-independent nitric oxide synthase activity in primary rat glial cultures. *Proc. Natl. Acad. Sci. USA* **89,** 10945–10949.

Green L. C., Wagner D. A., Glogowski J., Skipper P. L., Wishnock J. S., and Tannenbaum S. R. (1982) Analysis of nitrate, nitrite, and [N-^{15}N]-labeled nitrate in biological fluids. *Anal. Biochem.* **126,** 131–138.

Gutmann I. and Wahlefeld A. W. (1974) L-(+)-Lactate. Determination with lactate dehydrogenase and NAD, in *Methods of Enzymatic Analysis, Vol. 3* (Bergmeyer H. U., ed), pp. 1464–1468. Verlag Chemie GmbH, Weinheim.

Halliwell B. (1992) Reactive oxygen species and the central nervous system. *J. Neurochem.* **59,** 1609–1623.

Heinzel B., John M., Klatt P., Böhme E., and Mayer B. (1992) Ca^{2+}/calmodulin-dependent formation of hydrogen peroxide by brain nitric oxide synthase. *Biochem. J.* **281,** 627–630.

Hibbs J. B. Jr., Taintor R. R., Vavrin Z., and Rachlin E. M. (1988) Nitric oxide: a cytotoxic activated macrophage effector molecule. *Biochem. Biophys. Res. Commun.* **157,** 87–94.

Ignarro L. J., Fukuto J. M., Griscavage J. M., Rogers N. E., and Byrns R. E. (1993) Oxidation of nitric oxide in aqueous solution to nitrite but not nitrate: comparison with enzymatically formed nitric oxide from L-arginine. *Proc. Natl. Acad. Sci. USA* **90,** 8103–8107.

Kish S. J., Bergeron C., Rajput A., Dozic S., Mastrogiacomo F., Chang L.-J., Wilson J. M., DiStefano L. M., and Nobrega J. N. (1992) Brain cytochrome oxidase in Alzheimer's disease. *J. Neurochem.* **59,** 776–779.

Lipton S. A., Choi Y.-B., Pan Z.-H., Lei S. Z., Chen H.-S. V., Sucher N. J., Loscalzo J., Singel D. J., and Stamler J. S. (1993) A redox-based mechanism for the neuroprotective and neurodestructive effects of nitric oxide and related nitroso-compounds. *Nature* **364,** 626–632.

Lowry O. H., Rosebrough N. J., Farr A. L., and Randall R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193,** 265–275.

Meldrum B. and Garthwaite J. (1990) Excitatory amino acid neurotoxicity and neurodegenerative disease. *Trends Pharmacol. Sci.* **11,** 379–387.

Merrill J. E. (1987) Macroglia: neural cells responsive to lymphokines and growth factors. *Immunol. Today* **8,** 146–150.

Murphy S., Minor R. L. Jr., Welk G., and Harrison D. G. (1990) Evidence for an astrocyte-derived vasorelaxing factor with properties similar to nitric oxide. *J. Neurochem.* **55,** 349–351.

Murphy S., Simmons M. L., Agulló L., García A., Feinstein D. L., Galea E., Reis D. J., Minc-Golomb D., and Schwartz J. P. (1993) Synthesis of nitric oxide in CNS glial cells. *Trends Neurosci.* **16,** 323–328.

Olanow C. W. (1993) A radical hypothesis for neurodegeneration. *Trends Neurosci.* **16,** 439–444.

Pauwels P. J., Opperdoes F. R., and Trouet A. (1985) Effects of antimycin, glucose deprivation, and serum on cultures of neurons, astrocytes, and neuroblastoma cells. *J. Neurochem.* **44,** 143–148.

Peunova N. and Enikolopov G. (1993) Amplification of calcium-induced gene transcription by nitric oxide in neuronal cells. *Nature* **364,** 450–453.

Pou S., Pou W. S., Bredt D. S., Snyder S. H., and Rosen G. M. (1992) Generation of superoxide by purified brain nitric oxide synthase. *J. Biol. Chem.* **267,** 24173–24176.

Radi R., Beckman J. S., Bush K. M., and Freeman B. A. (1991) Peroxynitrite oxidation of sulfhydryls. The cytotoxic potential of superoxide and nitric oxide. *J. Biol. Chem.* **266,** 4244–4250.

Riederer P., Sofic E., Rausch W.-D., Schimdt B., Reynolds G. P., Jellinger K., and Youdim M. B. H. (1989) Transition metals, ferritin, glutathione, and ascorbic acid in parkinsonian brains. *J. Neurochem.* **52,** 515–520.

Schapira A. H. V., Cooper J. M., Dexter D., Clark J. B., Jenner P., and Marsden C. D. (1990) Mitochondrial complex I deficiency in Parkinson's disease. *J. Neurochem.* **54,** 823–827.

Shephard J. A. and Garland P. B. (1969) Citrate synthase from rat liver. *Methods Enzymol.* **13,** 11–19.

Simmons M. L. and Murphy S. (1992) Induction of nitric oxide synthase in glial cells. *J. Neurochem.* **59,** 897–905.

Simmons M. L. and Murphy S. (1993) Cytokines regulate L-arginine-dependent cyclic-GMP production in rat glial cells. *Eur. J. Neurosci.* **5,** 825–831.

Soussi B., Idström J.-P., Scherstén T., and Bylund-Fellenius A.-C. (1990) Cytochrome c oxidase and cardiolipin alterations in response to skeletal muscle ischaemia and reperfusion. *Acta Physiol. Scand.* **138,** 107–114.

Stuehr D. J. and Nathan C. F. (1989) Nitric oxide. A macrophage product responsible for cytostasis and respiratory inhibition in tumor target cells. *J. Exp. Med.* **169,** 1543–1555.

Tabernero A., Bolaños J. P., and Medina J. M. (1993) Lipogenesis from lactate in rat neurons and astrocytes in primary culture. *Biochem. J.* **294,** 635–638.

Vassault A. (1983) L-Lactate dehydrogenase. UV method with pyruvate and NADH, in *Methods of Enzymatic Analysis, Vol. 3* (Bergmeyer J. and Graßl M., eds), pp. 118–126. Verlag Chemie GmbH, Weinheim.

Walz W. and Mukerji S. (1990) Simulation of aspects of ischaemia in cell culture: changes in lactate compartmentation. *Glia* **3,** 522–528.

Wharton D. C. and Tzagoloff A. (1967) Cytochrome oxidase from beef heart mitochondria. *Methods Enzymol.* **10,** 245–250.

White E. J. and Clark J. B. (1989) Menadione-treated synaptosomes as a model for postischaemic neuronal damage. *Biochem. J.* **253,** 425–433.

Zhang Y., Marcillat O., Giulivi C., Ernster L., and Davies K. J. A. (1990) The oxidative inactivation of mitochondrial electron transport chain components and ATPase. *J. Biol. Chem.* **265,** 16330–16336.