THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 33, Issue of August 16, pp. 29753–29759, 2002
Printed in U.S.A.

Peroxynitrite Stimulates L-Arginine Transport System y^+ in Glial Cells

A POTENTIAL MECHANISM FOR REPLENISHING NEURONAL L-ARGININE*

Received for publication, April 17, 2002, and in revised form, June 5, 2002
Published, JBC Papers in Press, June 10, 2002, DOI 10.1074/jbc.M203728200

Victoria Vega-Agapito‡§, Angeles Almeida‡¶, Maria Hatzoglou‖, and Juan P. Bolaños‡**

From the ‡Departamento de Bioquímica y Biología Molecular, Universidad de Salamanca, 37007 Salamanca, Spain, ¶Unidad de Investigación, Hospital Universitario de Salamanca, 37007 Salamanca, Spain, and the ‖Department of Nutrition, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106-4919

We have reported previously that peroxynitrite stimulates L-arginine release from astrocytes, but the mechanism responsible for such an effect remains elusive. To explore this issue, we studied the regulation of L-[^{3}H]arginine transport by either exogenous or endogenous peroxynitrite in glial cells. A 2-fold peroxynitrite-mediated stimulation of L-arginine release in C6 cells was found to be Na^+-independent, was prevented by 5 mM L-arginine and, although only in the presence of Na^+, was blocked by 5 mM L-alanine or L-leucine. Peroxynitrite-mediated stimulation of L-arginine uptake was trans-stimulated by 10 mM L-arginine and was inhibited in a dose-dependent fashion (k_i of ~40 μM) by the system y^+ inhibitor *N*-ethylmaleimide in C6 cells. Endogenous production of peroxynitrite in lipopolysaccharide-treated astrocytes triggered an increased L-arginine transport activity without affecting *Cat1* L-arginine transporter mRNA levels. However, Western blot analyses of peroxynitrite-treated astrocytes and C6 glial cells revealed a 3-nitrotyrosinated anti-Cat1-immunopositive band, strongly suggesting peroxynitrite-mediated Cat1 nitration. Furthermore, peroxynitrite stimulation of L-arginine release was abolished in fibroblast cells homozygous for a targeted inactivation of the *Cat1* gene. Finally, peroxynitrite-triggered L-arginine released from astrocytes was efficiently taken up by neurons in an insert-based co-culture system. These results strongly suggest that peroxynitrite-mediated activation of the Cat1 transporter in glial cells may serve as a mechanism focused to replenish L-arginine in the neighboring neurons.

The biosynthesis of the central nervous system physiological messenger nitric oxide ($^{\cdot}NO$)[1] (1, 2) requires L-arginine for the $^{\cdot}NO$ synthase (NOS)-catalyzed reaction (3). In neurons and astrocytes, stimulation of glutamate receptors leads to a Ca^{2+}-dependent activation of neuronal NOS (nNOS) (2, 4), although $^{\cdot}NO$ biosynthesis in neurons depends on a correct L-arginine supply (5–7), possibly provided from the glia (8).

Glial cells can be activated to endogenously produce $^{\cdot}NO$ through the induction of the inducible NOS isoform (iNOS) (9–13). Within the brain, free L-arginine is predominantly located in astrocytes (14), which produce large amounts of $^{\cdot}NO$ upon iNOS (15) and L-arginine transporter (7) induction. However, unlike in astrocytes, L-arginine content in neurons is very low and is limiting for nNOS activity (16, 17). Consistent with this, glial-derived L-arginine has been shown to be increased upon activation of ionotropic glutamate (non-*N*-methyl-D-aspartate) receptors (18) and peroxynitrite (19), suggesting a neuronal-astrocytic signaling transduction pathway focused to provide NOS substrate for the neurons. However, direct demonstration of such a pathway and the elucidation of the precise transport system involved remain elusive.

Plasma membrane L-arginine transport is brought about by two families of cationic amino acid transport proteins: Cat (cationic amino acid transporter) and Bat (broad scope amino acid transporter). The Cat family of transporters comprises three different genes encoding four isoform proteins (*Cat1*, *Cat2*, *Cat2a*, and *Cat3*) (20, 21), commonly referred to as the system y^+ (22), which is mostly selective for cationic amino acids, although it does show a weak interaction with neutral amino acids in the presence of Na^+ (23). Glial cells mainly, but not exclusively, express the high affinity (k_t for L-arginine = 40–250 μM) (24) 67-kDa Cat1 (constitutive) (25) and the 71.8-kDa Cat2 (inducible) (26) system y^+ proteins (7, 27). The *Bat* family of constitutive transporter proteins is found in systems $b^{o,+}$, $B^{o,+}$, and y^+L (y^+Lat1 and y^+Lat2), which are mainly expressed in kidney and intestine, except for y^+Lat2, which is expressed in astrocytes (28).

We have reported previously that the neurotoxic $^{\cdot}NO$ derivative, peroxynitrite anion ($ONOO^-$), specifically stimulates L-arginine release from astrocytes (19). Although the $ONOO^-$-mediated stimulatory effect was inhibited by L-lysine, hence suggesting the involvement of system y^+ (19), an in-depth study focused on elucidating the precise mechanism responsible for L-arginine transport activation has not yet been carried out. In view of the potential critical role of glial cells as neuronal L-arginine suppliers for $^{\cdot}NO$ biosynthesis (8, 18, 29), we were prompted to investigate the mechanism through which $ONOO^-$ modulates L-arginine transport across the glial cell plasma membrane as well as the potential relevance of such modulation for neuronal L-arginine uptake.

* This work was funded by grants from the Ministerio de Ciencia y Tecnología (Grant SAF2001-1961), the Junta de Castilla y León (Grant SA065/01) and Fundación Ramón Areces (to J. P. B.), and Grant RO1 DK53307-01 (to M. H.).

§ The recipient of a fellowship from the Fundación Miguel Casado San Jose.

** To whom correspondence must be addressed: Departamento de Bioquímica y Biología Molecular, Universidad de Salamanca, Edificio Departamental, Plaza Doctores de la Reina s/n, 37007 Salamanca, Spain. Tel.: 34-923-29-45-26; Fax: 34-923-29-45-79; E-mail: jbolanos@usal.es.

[1] The abbreviations used are: NO, nitric oxide; NOS, nitric oxide synthase; DETA-NO, 1-[2-(2-aminoethyl)-N-(2-ammonioethyl)amino] diazen-1-ium-1,2-diolate; AMT, 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine; Cat, cationic amino acid transporter; Bat, broad spectrum amino acid transporter; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; LPS, lipopolysaccharide; PBS, phosphate-buffered saline.

EXPERIMENTAL PROCEDURES

Materials—Peroxynitrite was synthesized and quantified spectrophotometrically ($\epsilon_{302} = 1{,}670\ M^{-1} \times cm^{-1}$) as described previously (30). Alkaline stock solutions, with an approximate $ONOO^-$ concentration of 0.3–0.4 M, were stable at −70 °C for at least 3–4 months. Dulbecco's modified Eagle's medium (DMEM), lipopolysaccharide (LPS), amino acids, and *N*-ethylmaleimide were obtained from Sigma. Fetal calf serum (FCS) was purchased from Roche Diagnostics. L-[2,3,4,5-^{3}H]Arginine and [α-^{32}P]dCTP were obtained from Amersham Biosciences. The iNOS inhibitor 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine (AMT) and the ˙NO donor 1-[2-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (DETA-NO) were purchased from Alexis Corp. (San Diego, CA). Plastic tissue culture dishes were purchased from Nunc (Roskilde, Denmark). Cell culture inserts (Millicell-PCF; 4.2 cm^2 of effective membrane area; 0.4-mm membrane pore size) were obtained from Millipore (Bedford, MA). Hybond® nitrocellulose membrane was purchased from Amersham Biosciences. Anti-Cat1 antiserum was a generous gift from Dr. M. Kilberg (Department of Biochemistry and Molecular Biology, University of Florida College of Medicine, Gainesville, FL). Anti-3-Nitrotyrosine antibody was kindly provided by Dr. J. Beckman (Department of Anesthesiology, University of Alabama at Birmingham, AL). Anti-rabbit or anti-mouse secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Other substrates, enzymes, and coenzymes were purchased from Sigma, Roche Diagnostics or Merck.

Cell Culture—Astroglia-rich primary cultures derived from neonatal 1-day-old Wistar rats were prepared as described previously (31). Cell suspensions were plated in DMEM supplemented with 10% (v/v) fetal calf serum at a density of 1.25×10^5 cells/cm^2 in 150-cm^2 flasks. Cells were maintained in a humidified incubator under an atmosphere of 5% CO_2/95% air at 37 °C with a change of medium twice a week. After 12–14 days, astrocytes reached confluence, and cells were collected by trypsinization, reseeded in DMEM/10% FCS at a density of 2.5×10^5 cells/cm^2 either in 6-well plates or in cell culture inserts as described previously (32), and used after 24 h.

Neuronal-rich primary cultures were prepared from fetal (embryonic day 17) rats as described previously (33) and grown at a density of 2.5×10^5 cells/cm^2 in poly-D-lysine-coated dishes in DMEM/10% FCS. Forty-eight h after plating, the medium was replaced with DMEM supplemented with 5% horse serum and 20 mM D-glucose. On day 4 of culture, cytosine arabinoside (10 μM) was added to prevent non-neuronal proliferation, and neurons were used on day 8. For astrocytic-neuronal co-cultures, astrocyte-containing inserts were placed on the top of 8-day-old neuronal cultures and bathed with Hanks' buffer.

C6 glioma cells (passage number 48) were kindly provided by Prof. B. Hamprecht (Tübingen, Germany) and were maintained in DMEM/10% FCS. Cells were passaged twice per week and were seeded at a density of 1.5×10^5 cells/cm^2 24 h before experiments. Mouse fibroblast cell line K047, which is homozygous for inactivation of the *Cat1* gene (*Cat1*$^{-/-}$) (34), as well as the wild type were maintained in DMEM/10% FCS/1% non-essential amino acids. Cells were passaged twice per week and were seeded at a density of 1.5×10^5 cells/cm^2 24 h before experiments.

L-Arginine Release Experiments—Release experiments were carried out essentially as described previously (19). Briefly, 24 h after reseeding, the culture medium was removed, and cells were washed once with prewarmed (~37 °C) Hanks' buffer (5.26 mM KCl, 0.43 mM KH_2PO_4, 132.4 mM NaCl, 4.09 mM $NaHCO_3$, 0.33 mM Na_2HPO_4, 5.44 mM glucose, 2 mM $CaCl_2$, and 20 mM HEPES, pH 7.4) or Na^+-free Hanks' buffer (NaCl was replaced by 132.4 mM choline chloride). Cells were then incubated in fresh Hanks' buffer (with or without Na^+) containing 1 μCi/ml L-[2,3,4,5-^{3}H]arginine (50 μM) at 37 °C for 30 min. Extensive washing was performed until a constant basal L-[^{3}H]arginine release was found. Then, $ONOO^-$ (100 μM) was added, and the radioactivity released from the cells was measured after 5 min. When appropriate, all incubations were carried out in the presence of 5 mM L-arginine, L-alanine, or L-leucine. The final buffer pH varied between 7.3 and 7.4. Control cells were treated in the same way except that the $ONOO^-$ solution was degraded previously in Hanks' buffer at 37 °C for 15 min before being added to the cells. In some experiments, astrocytes were preincubated for 24 h with LPS (1 μg/ml) alone, LPS in combination with AMT (50 μM), or DETA-NO alone (0.1 mM, which continuously releases 0.28 μM ˙NO for ~24 h as measured with an ˙NO electrode; ISO-NO, World Precision Instruments). In these experiments, L-arginine release experiments were carried out as above except that exogenous peroxynitrite was not included. Results were expressed as percentages of L-arginine release obtained in each treatment as compared with controls (degraded $ONOO^-$-treated cells), which were arbitrarily given a value of 100%.

L-Arginine Uptake Experiments—Uptake experiments were carried out as described previously (5, 6). Briefly, 24 h after reseeding, the culture medium was removed, and cells were washed once with prewarmed Na^+-free Hanks' buffer. Cells were preincubated in Na^+-free Hanks' buffer containing 50 μM L-arginine at 37 °C for 5 min. For trans-stimulation studies, cells were preincubated in Na^+-free Hanks' buffer containing 10 mM L-arginine at 37 °C for 30 min. Uptake experiments were performed in fresh Na^+-free Hanks' buffer containing 0.25 μCi/ml L-[2,3,4,5-^{3}H]-arginine (50 μM). In experiments focused on inhibiting the y^+ L-arginine transporter, all incubations were carried out in the presence of *N*-ethylmaleimide at the indicated concentrations. Peroxynitrite additions (100 μM) and the appropriate controls were done as in the release experiments, and cells were incubated at 37 °C. After 5 min, the buffer was aspirated, and cells were rapidly washed three times with ice-cold Hanks' buffer. Cells were then lysed with 0.5 ml of 10 mM NaOH/0.1% Triton X-100, and the radioactivity present in the cell lysates was measured. In some experiments, astrocytes were preincubated for 24 h with LPS (1 μg/ml) alone, LPS in combination with AMT (50 μM), or DETA-NO alone (0.1 mM). In these experiments, L-arginine uptake experiments were carried out as above except that exogenous peroxynitrite was not included. Blanks were obtained from cells briefly (~2 s) exposed to L-[^{3}H]arginine medium on ice (~0–4 °C) whose radioactivity was subtracted from sample values. Results were expressed as percentages of L-arginine uptake obtained in each treatment as compared with the controls (degraded $ONOO^-$-treated cells), which were arbitrarily given a value of 100%.

Glial Release/Neuronal Uptake of L-Arginine in Co-culture—To study the neuronal uptake of L-arginine released from $ONOO^-$-stimulated astrocytes, a co-culture system was used (32). Astrocytes seeded in cell culture inserts were loaded with 5 μCi/ml L-[2,3,4,5-^{3}H]arginine (50 μM) in Hanks' buffer at 37 °C for 30 min. After extensive washing, astrocyte-containing inserts were placed on the top of 8-day-old neuronal cultures and bathed with Hanks' buffer. Peroxynitrite (100 μM) was added, and the co-culture system was further incubated at 37 °C for 5 min. After this incubation, inserts were removed, and neurons washed and lysed with 0.5 ml of 10 mM NaOH/0.1% Triton X-100 to determine the radioactivity present in the neuronal lysates. Blanks were performed as described above, and results were expressed as pmol of L-arginine taken up/min/mg of protein. Controls were carried out using degraded $ONOO^-$. In some experiments, neurons were depleted of L-arginine by incubating these cells in DMEM containing arginase (2 units/ml; Sigma) for 24 h as described previously (35) before the co-incubation with astrocytes.

Western Blotting—Cells were scraped off the plastic dishes with lysis buffer (12.5 mM Na_2HPO_4, 116 mM NaCl, 0.5 M EDTA, 1% (by volume) Triton X-100, 0.1% (w/v) sodium dodecylsulfate, 100 μM *N*-α-p-tosyl-L-lysine chloromethylketone, 100 μM phenylmethylsulfonyl fluoride, 1 mM phenanthroline, 10 μg/ml pepstatin A, 100 μM *N*-tosyl-L-phenylalanine chloromethylketone, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml soybean trypsin inhibitor, pH 7) and 100 μg of protein from each sample, extemporarily determined following Bradford (36), using ovalbumin as standard, and the BenchMark™ prestained protein ladder (Invitrogen) were electrophoresed on 8% acrylamide gel (MiniProtan®, Bio-Rad) and transferred to a Hybond® nitrocellulose membrane (Amersham Biosciences). Membranes were blocked with 5% (w/v) low fat milk Tween Tris buffer solution (20 mM Tris, 500 mM NaCl, 0.1% (w/v) Tween 20 (pH 7.5)) for 1 h and then incubated in the presence of the appropriate antibody (either anti-nitrotyrosine at 1:100 dilution or anti-Cat1 at 1:2000 dilution) at 4 °C overnight. After washing, membranes were further incubated in 2% (w/v) low fat milk Tween Tris buffer solution for 45 min at room temperature in the presence of the appropriate (anti-rabbit or anti-mouse) IgG-horseradish peroxidase secondary antibodies (at 1:40,000 or 1:1,000 dilution, respectively) and immediately incubated with the luminol chemiluminescence reagent (Santa Cruz Biotechnology) for 1 min before exposure to Hyperfilm™ chemiluminescence film for 5 min.

Northern Blotting—Northern blotting analysis was carried out in total RNA samples isolated from the cells by the guanidinium isothiocyanate method as described previously (37). The samples were electrophoresed (22 μg of RNA/line) on a 1% (w/v) agarose-formaldehyde gel. After transfer to a GeneScreen Plus membrane (PerkinElmer Life Sciences) and cross-linking with ultraviolet irradiation (UV Stratalinker, Model 2400, Genetic Research Instruments, Essex, UK), membranes were hybridized for 18 h at 65 °C in the presence of the appropriate random-primed [α-^{32}P]dCTP-radiolabelled cDNA probes and exposed to Kodak XAR-5 film. As cDNA probes, we used either a 0.9-kb

iNOS cDNA fragment (a generous gift of Dr. Elena Galea, University of Illinois, Chicago, IL) or a 0.9-kb *Cat1* cDNA fragment (generously provided by Dr. Manuel Palacín, University of Barcelona, Barcelona, Spain).

Immunocytochemistry—Immunocytochemistry was carried out on astrocytes grown on glass coverslips, which were fixed for 30 min in PBS containing 4% paraformaldehyde, rinsed with PBS, and permeabilized for 10 min with methanol at −20 °C. Cells were then incubated at room temperature in PBS containing blocking serum (10% normal goat serum) plus anti-3-nitrotyrosine antibody for 3 h. After washing with PBS, cells were incubated in PBS containing the secondary antibody (anti-mouse-IgG-fluorescein isothiocyanate) for 2 h. Finally, coverslips were washed and developed using the SlowFade® light antifade kit (Molecular Probes, Eugene, OR) for fluorescence microphotographs.

Statistical Analysis—Data are expressed as mean ± S.E. values for the number of culture preparations indicated in the figure legends. Statistical significance was evaluated by one-way analysis of variance followed by the least significant difference multiple range test. $p < 0.05$ was considered significant.

RESULTS

Peroxynitrite Stimulates L-Arginine Release in an Na^+-independent Fashion—To elucidate the mechanism involved in $ONOO^-$-mediated L-arginine release (19), we first studied the Na^+ dependence on this effect in C6 glial cells. Cells were loaded with L-[^{3}H]arginine and incubated either in the absence (Na^+-free, choline chloride-containing Hanks' buffer) or in the presence (Hanks' buffer) of Na^+ at 37 °C for 25 min and then exposed to 100 μM $ONOO^-$ or to degraded $ONOO^-$. After 5 min, L-arginine release was determined as reported previously (19). As shown in Fig. 1, $ONOO^-$ increased L-arginine release by about 2-fold both in the presence (Fig. 1*A*) and in the absence (Fig. 1*B*) of Na^+. Supplementation of excess (5 mM) L-arginine, L-alanine, or L-leucine partially or fully prevented $ONOO^-$-stimulated L-arginine release in the presence of Na^+ (Fig. 1*A*). However, only L-arginine, but not L-alanine or L-leucine supplementation, was able to prevent $ONOO^-$-mediated L-arginine release in the absence of Na^+ (Fig. 1*B*).

Trans-stimulation of $ONOO^-$-mediated Activation of L-arginine Uptake and Inhibition by the System y^+-specific Inhibitor N-Ethylmaleimide—To further investigate the transport system involved in the $ONOO^-$-mediated activation of L-arginine release in glial cells, we tested whether the release was trans-stimulated by L-arginine. To simplify the experimental design, L-arginine uptake instead of efflux was measured in the absence or presence of a large excess (10 mM) of extracellular L-arginine. Fig. 1*C* shows that $ONOO^-$ significantly stimulated (by ~1.6-fold) L-arginine uptake in the absence of 10 mM L-arginine. Moreover, in the presence of 10 mM L-arginine, $ONOO^-$-mediated stimulation of L-arginine uptake was further increased by a factor of ~1.4. Peroxynitrite-mediated stimulation of L-arginine uptake was dose dependently abolished by the system y^+-specific inhibitor *N*-ethylmaleimide (38) (Fig. 1*D*). Polynomial ($n = 2$) transformation of the data (not shown) yielded a k_i of ~40 μM for *N*-ethylmaleimide.

Peroxynitrite Fails to stimulate L-arginine Release in $Cat1^{-/-}$ Fibroblasts—In view of the possibility that system y^+ might be involved in $ONOO^-$-mediated stimulation of L-arginine transport, we were prompted to investigate the effect of $ONOO^-$ in cells lacking this type of transporter system. For this purpose, we used $Cat1^{-/-}$ fibroblast cells, which functionally lack this type of transporter (34), and the effect was compared with those observed in wild-type fibroblast cells, C6, and astrocytic cells, which constitutively express the Cat1 transporter (7, 27). We found that the stimulation caused by $ONOO^-$ on L-arginine release from wild-type fibroblast cells was very similar to that found in C6 cells and astrocytes (Fig. 2). However, this effect was fully abolished in homozygous ($Cat1^{-/-}$) *Cat1* knock-out fibroblast cells (Fig. 2).

Peroxynitrite-mediated 3-Nitrotyrosination of Cat1 in Glial Cells—The functional protein modification by $ONOO^-$-mediated nitration of 3-nitrotyrosine residues (3-nitrotyrosination) is now widely documented (39, 40). In view of the rapid effect brought about by $ONOO^-$ on L-arginine transport activity, we wondered whether Cat1 3-nitrotyrosination might be taking place under our own conditions. A 5-min incubation of intact C6 or astrocytic cells in the presence of 100 μM $ONOO^-$ revealed intense protein 3-nitrotyrosination, as judged by Western blotting using monoclonal anti-3-nitrotyrosine antibody in both cell types (Fig. 3). Interestingly, among other 3-nitrotyrosinated bands, in both C6 cells and astrocytes, a protein was also found to be anti-Cat1-immunopositive, as judged by parallel Western blotting using an antiserum raised against Cat1 protein (Fig. 3). Either immunoprecipitation with anti-Cat1 and Western blotting with anti-3-nitrotyrosine or immunoprecipitation with anti-3-nitrotyrosine and Western blotting with anti-Cat1 were not successful, probably due to the very low level of nitrated Cat1 protein.

Endogenous Peroxynitrite, but Not Nitric Oxide Formation, Stimulates L-Arginine Transport Activity in Astrocytes—In

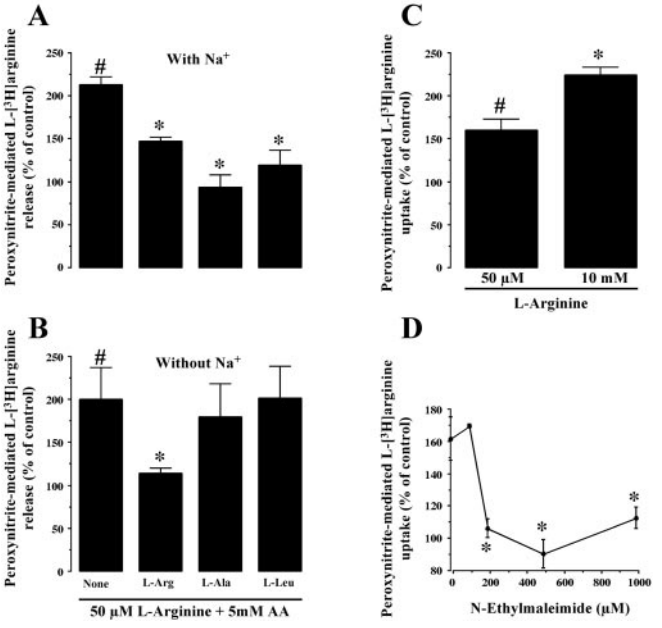

FIG. 1. **Peroxynitrite stimulates a trans-stimulable, Na^+-independent L-arginine transport activity.** C6 glial cells were preloaded with 1 μCi/ml L-[2,3,4,5-^{3}H]arginine (50 μM) in either Hanks' buffer (*A*) or Na^+-free Hanks' buffer (*B*) (NaCl was replaced by 132.4 mM choline chloride) at 37 °C for 30 min. After extensive washing, $ONOO^-$ (100 μM) was added, and L-[^{3}H]arginine released was measured 5 min later. Where indicated, all incubations were performed in the presence of 5 mM L-arginine, L-alanine, or L-leucine. For trans-stimulation experiments (*C*), cells were preincubated in Na^+-free Hanks' buffer containing either 50 μM or 10 mM L-arginine at 37 °C for 30 min. For *N*-ethylmaleimide experiments (*D*), all incubations were carried out in the presence of this inhibitor at the indicated concentrations. Uptake experiments were performed in Na^+-free Hanks' buffer containing 0.25 μCi/ml L-[2,3,4,5-^{3}H]-arginine (50 μM). Peroxynitrite (100 μM) was added, and cells were incubated at 37 °C for 5 min. L[^{3}H]Arginine retained by the cells was measured in the cell lysates as described under "Experimental Procedures." Uptake blanks were obtained from cells briefly (~2 s) exposed to L-[^{3}H]arginine medium on ice (~0–4 °C), whose radioactivity was subtracted from sample values. Results are expressed as percentages of L-arginine release/uptake as compared with that released/taken up by control cells (*i.e.* degraded $ONOO^-$-treated cells), which were arbitrarily given a value of 100%. Control release values were 352 ± 23 and 324 ± 23 cpm/10^3 cells with and without Na^+, respectively. Control uptake values were 79 ± 12 pmol/min/mg of protein. In all cases, data are mean ± S.E. values from three separate experiments. #, $p < 0.05$ as compared with the control group. *, $p < 0.05$ as compared with the *None* (*A* and *B*), 50 μM L-arginine (*C*), or 0 mM *N*-ethylmaleimide (*D*) groups, respectively.

view of the evidence showing L-arginine transport activity stimulation by exogenous $ONOO^-$ and to elucidate the possible physiological relevance of this phenomenon, we were prompted to investigate the possible role of endogenous $ONOO^-$ formation on L-arginine transport activity. Accordingly, we used astrocytes in primary culture incubated with LPS (1 μg/ml), an endotoxin that is well known to induce iNOS in these cells (9, 10) and to stimulate iNOS-dependent $ONOO^-$ formation (41). We have corroborated iNOS induction (Fig. 4*D*) as well as functional iNOS activity by measuring LPS-mediated, AMT-(50 μM) inhibitable nitrite released to the culture medium (results not shown) (42). Furthermore, endogenous $ONOO^-$ formation was also confirmed by immunocytochemical evidence for LPS-mediated 3-nitrotyrosine formation in these cells (Fig. 5*B*). Since $ONOO^-$ synthesis requires $^{\cdot}NO$ reaction with $O_2^{\cdot-}$ (43), to prevent $ONOO^-$ formation, $^{\cdot}NO$ synthesis was inhibited in the LPS-treated cells by the iNOS-specific inhibitor AMT, thus preventing cellular 3-nitrotyrosination (Fig. 5*C*). Both negative control (*i.e.* degraded $ONOO^-$) and positive control (authentic $ONOO^-$) 3-nitrotyrosinated cells were carried out in parallel to confirm the specificity of 3-nitrotyrosination (Fig. 5, *D* and *E*). Immunoprecipitation of LPS-treated astrocytes with anti-3-nitrotyrosine followed by Western blotting failed to detect any Cat1-nitrated protein (not shown), a result that could be due to the low level of Cat1 nitration in this system.

As shown in Fig. 4*C*, LPS-treated cells showed enhanced L-arginine uptake activity in an L-arginine concentration-dependent fashion. At 50 μM extracellular L-arginine concentration, LPS-treated cells showed a 2.8-fold enhancement in the rate of L-arginine uptake (Fig. 4*A*) and a 1.5-fold increase in the rate of L-arginine release (Fig. 4*B*). Inhibition of iNOS activity with AMT (50 μM) significantly, but not fully, prevented LPS-mediated activation of L-arginine transport activity (Fig. 4, *A* and *B*). To elucidate the possible role for $^{\cdot}NO$ on L-arginine transport activity, we used DETA-NO (0.1 mM), a compound widely used as a chemical source of $^{\cdot}NO$ (44). DETA-NO (0.1 mM) was shown to continuously release 0.28 μM $^{\cdot}NO$ for about 24 h in Hanks' buffer at 37 °C as measured by an $^{\cdot}NO$-sensitive electrode. This steady-state $^{\cdot}NO$ concentration is closely similar to that observed in LPS-treated astrocytes (15). Incubation of astrocytes with DETA-NO (0.1 mM) during 24 h did not change the rates of L-arginine uptake or release (Fig. 4, *A* and *B*). A degraded solution of DETA-NO was used as control, showing no alteration in these parameters when compared with untreated astrocytes (not shown).

Peroxynitrite-mediated L-Arginine Release from Astrocytes Is Taken up by Co-cultured Neurons—To further elucidate the potential physiological relevance of $ONOO^-$-mediated activation of L-arginine transport in glial cells, we investigated whether, upon an $ONOO^-$ stimuli, astrocytic-released L-arginine could be taken up by neighboring neurons. Accordingly, L-[^{3}H]arginine-preloaded insert-seeded astrocytes were co-incubated with neurons, and either degraded or active $ONOO^-$ (0.1 mM) was added. After 5 min, astrocyte-containing inserts were removed, and the radioactivity present in neuronal lysates was measured. As shown in Fig. 6, $ONOO^-$ treatment significantly increased L-[^{3}H]arginine uptake by neurons. However, L-[^{3}H]arginine uptake was unmodified by $ONOO^-$ treatment to neurons cultured alone (results not shown). Furthermore, the effect of $ONOO^-$ was enhanced when neurons were L-arginine-depleted prior to the co-culture with astrocytes (Fig. 6).

DISCUSSION

The studies carried out by Grima *et al.* (8, 18) first reported glutamate-stimulated L-arginine release from glial cells, possibly by acting on non-*N*-methyl-D-aspartate receptors. These authors suggested that this would be a neuronal-astrocytic signaling transduction pathway whose job would be to provide L-arginine for NOS activity within neighboring neurons (8, 18). In fact, sustained $^{\cdot}NO$ production in astrocytes (7) as well as in macrophages (45) has been shown to be dependent of an efficient L-arginine (Cat2) transporter activity. Our later work in cultured astroglial cells showed that exogenously added peroxynitrite anion stimulated L-arginine release in a dose-dependent (from 50–1000 μM) and selective way (19), a protein carrier-mediated effect that was not mimicked by $^{\cdot}NO$ nor by other oxygen-derived free radicals such as H_2O_2 or $O_2^{\cdot-}$. Given the potential pathophysiological relevance of this possible neuronal-astroglial intercellular communication, the first aim of the present work was to elucidate the molecular mechanism leading to $ONOO^-$-mediated activation of L-arginine transport in glial cells.

Despite the apparent complexity of the cationic amino acid

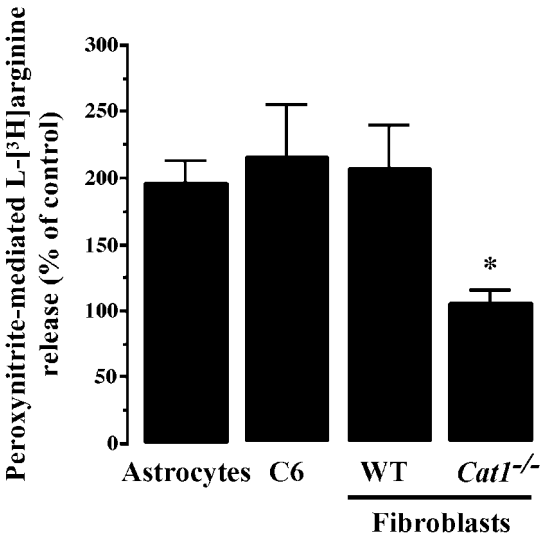

FIG. 2. **Peroxynitrite fails to stimulate L-arginine release in *Cat1* knock-out fibroblast cells.** C6 glial cells, astrocytes or fibroblast (wild-type (*WT*)$^-$ or *Cat1*$^{-/-}$) cells were preloaded with 1 μCi/ml L-[2,3,4,5-^{3}H]arginine (50 μM) in Hanks' buffer at 37 °C for 30 min. After extensive washing, $ONOO^-$ (100 μM) was added, and the L-[^{3}H]arginine released was measured 5 min later. Results are expressed as percentages of L-arginine release as compared with that released by the appropriate control cells (*i.e.* degraded $ONOO^-$-treated cells), which were arbitrarily given a value of 100%. Control release values were 401 ± 11, 352 ± 23, 293 ± 10, and 285 ± 15 cpm/10^3 cells for astrocytes, C6, wild-type (*WT*) fibroblasts, and *Cat1*$^{-/-}$-fibroblasts, respectively. Data are mean ± S.E. values from three separate experiments. *, $p < 0.05$ as compared with the wild-type (*WT*) fibroblasts group.

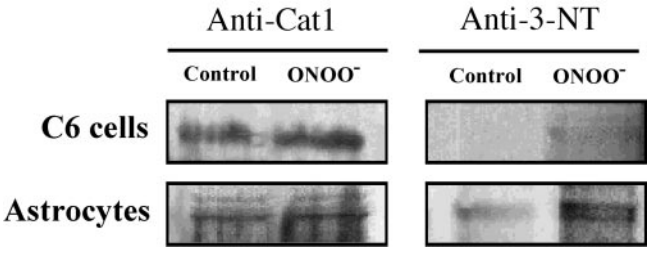

FIG. 3. **Peroxynitrite-mediated 3-nitrotyrosination (*3-NT*) of Cat1 in glial cells.** C6 glial cells or astrocytes were exposed to $ONOO^-$ (100 μM) for 5 min, and 100 μg of protein from each sample and the BenchMark™ prestained protein ladder (Invitrogen) were subjected to Western blotting. Anti-3-nitrotyrosine monoclonal antibody and anti-Cat1 antisera were used to detect protein nitration or Cat1, respectively. Control cells received equivalent amounts of degraded $ONOO^-$. The figure shows one representative experiment out of three.

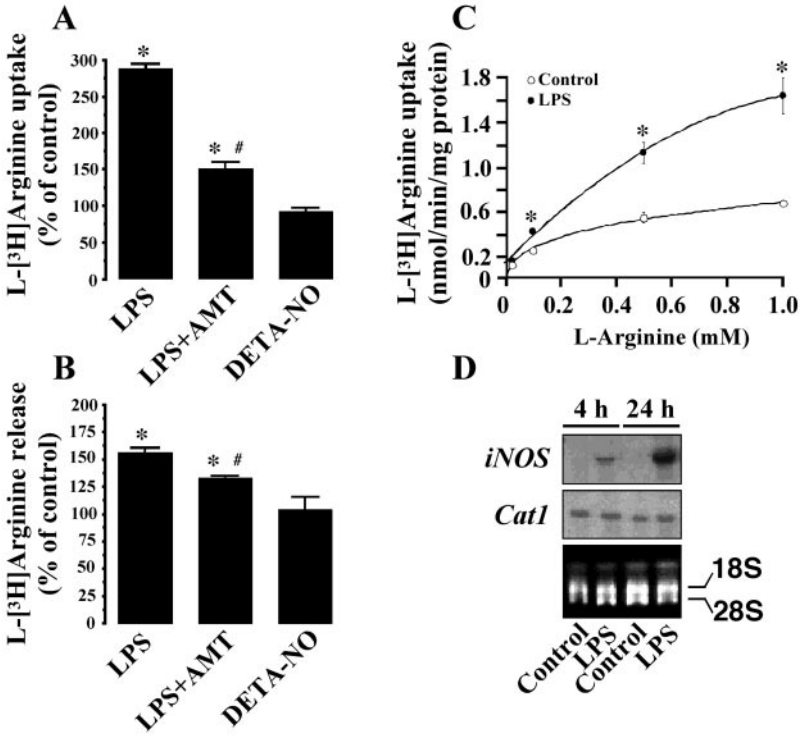

FIG. 4. **Endogenously produced peroxynitrite stimulates L-arginine transport activity.** To induce nitric oxide synthase (iNOS) activity, astrocytes were incubated in the absence or in the presence of LPS (1 μg/ml) for 24 h prior to the release/uptake experiments. In some experiments, the specific iNOS inhibitor AMT (50 μM) was also included. Where indicated, cells were incubated only in the presence of the nitric oxide donor DETA-NO (0.1 mM). Following this 24-h incubation, cells were washed, and uptake experiments (*A* and *C*) were performed in Na^+-free Hanks' buffer containing 0.25 μCi/ml L-[2,3,4,5-^{3}H]-arginine (ranging between 0.05 mM, as in *panel A*, up to 1 mM, as in *panel C*) at 37 °C for 5 min. L-[^{3}H]Arginine retained by the cells was measured in the cell lysates as described under "Experimental Procedures." Uptake blanks were obtained from cells briefly (~2 s) exposed to L-[^{3}H]arginine medium on ice (~0–4 °C), whose radioactivity was subtracted from sample values. For release experiments (*B*), cells were preloaded with 1 μCi/ml L-[2,3,4,5-^{3}H]arginine (50 μM) in Hanks' buffer at 37 °C for 30 min. After extensive washing every 5 min, basal L-[^{3}H]arginine released during a 5-min period was measured at 30 min. Northern blotting analyses (*D*) were carried out in 22 μg of total RNA/lane extracted from astrocytes incubated either in the absence (*Control*) or in the presence of LPS (1 μg/ml) for 4 or 24 h using the appropriate *iNOS* and *Cat1* ^{32}P-labeled cDNA probes. Results are expressed as percentages of L-arginine release/uptake as compared with that released/taken up by control cells (*i.e.* untreated cells), which were arbitrarily given a value of 100%. Control uptake values were 942 ± 285 pmol/min/mg of protein. Control release values were 401 ± 11 cpm/10^3 cells. In all cases, data are mean ± S.E. values from three separate experiments. *, $p < 0.05$ as compared with the control group. #, $p < 0.05$ as compared with the LPS group.

transport systems found in the brain (*i.e.* the Cat and Bat proteins), these can be identified on the basis of their interactions with inorganic ions, particularly with Na^+ (22). Thus, to elucidate the Na^+ dependence in the $ONOO^-$-mediated stimulation of L-arginine release, we incubated C6 glial cells either in the presence or in the absence of Na^+, and the effect of $ONOO^-$ (100 μM) was evaluated. Our results showed that there was a ~2-fold stimulation of L-arginine release, which was found to be independent of extracellular Na^+. Since several Na^+-independent cationic amino acid transporter systems can operate in glial cells (25, 28), we next investigated the specificity of the $ONOO^-$-sensitive transport activity. In particular, system y^+ for cationic amino acids appears to be a major Na^+-independent carrier system in glial cells, although (and only in the presence of Na^+) it does show a weak interaction with neutral amino acids (6, 7). Our results revealed that only in the presence of Na^+ was $ONOO^-$-mediated L-arginine stimulation abolished by both excess cationic (L-arginine and L-lysine) (19) and neutral (L-alanine and L-leucine) amino acids. By contrast, in the absence of Na^+, only L-arginine, but not L-alanine or L-leucine, was able to abolish $ONOO^-$-mediated L-arginine release. These results are consistent with the notion that system y^+ could be a potential target of $ONOO^-$.

The possibility that system y^+ might be involved in the $ONOO^-$-mediated stimulation of L-arginine transport was further corroborated by two sets of data, *i.e.* 1) the specific system y^+ inhibitor, *N*-ethylmaleimide (38), dose dependently (k_i of ~40 μM) abolished the $ONOO^-$-mediated stimulation of L-arginine uptake; and 2) preloading of cells with L-arginine (10 mM) stimulated $ONOO^-$-mediated L-arginine uptake. Since system y^+ has been shown previously to be subject to trans-stimulation (46), these data further confirm that $ONOO^-$ may cause the activation of system y^+.

The potency with which $ONOO^-$ stimulated L-arginine release from wild-type fibroblast cells was very similar to that found for C6 cells and astrocytes, which constitutively express the Cat1 transporter (7, 27). However, this effect was fully abolished in homozygous ($Cat1^{-/-}$) *Cat1* knock-out fibroblast cells, which have been shown to be functionally compensated by the brain-specific L-arginine transporter Cat3 (47). These results strongly suggest that the $ONOO^-$-mediated activation of Cat1 protein would be involved in the mechanism leading to system y^+ activation in glial cells.

It is well known that $ONOO^-$ causes protein sulfhydryl nitrosylation and tyrosine nitration as well as lipid peroxidation (39, 40). It could be speculated that $ONOO^-$-stimulated L-arginine release would be due to a possible modification in the system y^+ L-arginine transporter Cat1. Interestingly, $ONOO^-$ induced protein nitration in both C6 cells and astrocytes, as judged by the observed enhancement in 3-nitrotyrosine immunoreactivity. Noticeably, and in particular, $ONOO^-$ nitrated an anti-Cat1-immunopositive band, strongly suggesting $ONOO^-$-mediated Cat1 3-nitrotyrosination. Unfortunately, and possibly due to a low level of nitrated Cat1

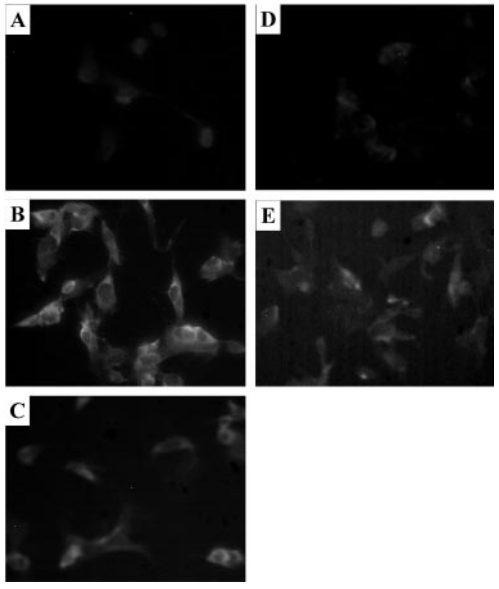

FIG. 5. **Immunocytochemical evidence for peroxynitrite production by LPS-treated astrocytes.** Astrocytes were incubated either in the absence (*A*) or in the presence of LPS (1 μg/ml) alone (*B*) or in combination with AMT (50 μM) (*C*) for 24 h. In parallel, cells were incubated with either degraded (*D*) or active (*E*) peroxynitrite for 5 min. Peroxynitrite production/release was judged by immunocytochemically evidenced 3-nitrotyrosination in the cells using an anti-3-nitrotyrosine antibody, as described under "Experimental Procedures."

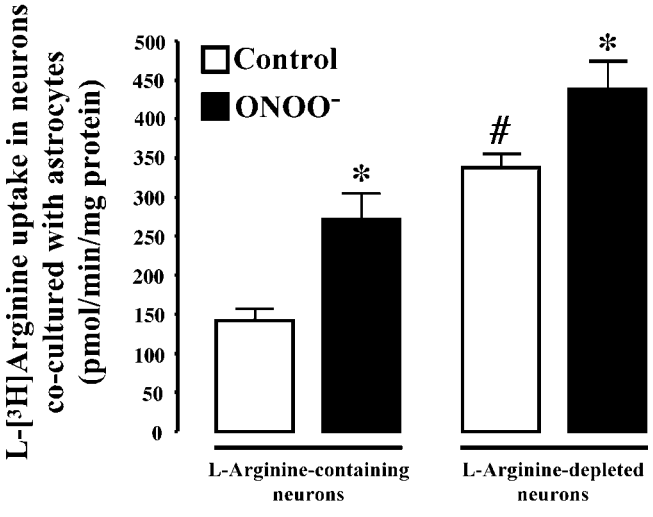

FIG. 6. **Peroxynitrite-mediated neuronal uptake of astrocytic-released L-arginine.** To remove endogenous L-arginine, neurons were preincubated in DMEM containing 2 units/ml arginase at 37 °C for 24 h (L-arginine-depleted neurons). L-Arginine-containing neurons were incubated in the absence of arginase. Insert-seeded astrocytes were preloaded with 5 μCi/ml L-[2,3,4,5-^{3}H]arginine (50 μM) in Hanks' buffer at 37 °C for 30 min. After extensive washing of both neuronal and astrocytic cells, the inserts were placed over the neuronal cultures and bathed with fresh Hanks' buffer. Either degraded (*Control*) or active $ONOO^-$ (100 μM) was immediately added to this co-culture system and further incubated at 37 °C for 5 min. After insert removal, L-[^{3}H]arginine retained by the neurons was measured in the cell lysates as described under "Experimental Procedures." Data are mean ± S.E. values from three separate experiments. *, $p < 0.05$ as compared with the corresponding control group. #, $p < 0.05$ as compared with the corresponding L-arginine-containing group.

protein, Cat1 3-nitrotyrosination could not be shown by immunoprecipitation experiments. Nevertheless, a tempting possibility is that this putative Cat1 nitration might be a contributing cause of the functional modification of transporter activity, although this remains to be further elucidated.

To investigate the possible physiological role for $ONOO^-$-mediated stimulation of L-arginine transport activity, we carried out two sets of experiments, *i.e.* to modulate L-arginine transport activity by endogenously produced $ONOO^-$ in astrocytes and to investigate L-arginine trafficking from astrocytes to neurons upon an $ONOO^-$ stimuli. Thus, our results showed that incubation of astrocytes with LPS caused iNOS activity-dependent 3-nitrotyrosination, suggesting $ONOO^-$ formation in these cells (41). Furthermore, $ONOO^-$-producing astrocytes showed increased L-arginine transport, consistent with former results obtained by Schmidlin and Wiesinger (35). In this study, inhibition of iNOS activity by AMT partially prevented LPS-mediated increase in transport activity (see also Ref. 35). As indicated by *Cat1* mRNA levels, there was no modification in *Cat1* expression in LPS-treated cells (see also Ref. 7). In addition, $^{\cdot}NO$ cannot be responsible for the AMT-inhibitable LPS-mediated increase in transport activity because exposure of cells to $^{\cdot}NO$ under conditions closely similar to those obtained with LPS treatment (*i.e.* using 0.1 mM DETA-NO for 24 h) failed to alter L-arginine transport activity. Therefore, since both $^{\cdot}NO$ and $O_2^{\cdot-}$ are necessary for endogenous $ONOO^-$ synthesis (41), our data are consistent with the iNOS-dependent Cat1 nitration and activation by $ONOO^-$. Whether induction of the other system y^+, Cat2, by LPS treatment would be responsible for the iNOS activity-independent increase in L-arginine transport is unknown. However, this would be a plausible hypothesis since Stevens *et al.* (7) have shown that $^{\cdot}NO$ biosynthesis in astrocytes upon LPS treatment is dependent on induced alternatively spliced *Cat2* encoding L-arginine transport in astrocytes.

Finally, we have studied the possible trafficking of L-arginine between astrocytes and neurons. Thus, whereas $ONOO^-$ failed to increase L-arginine uptake in neurons when cultured alone, using a co-culture system L-arginine released from $ONOO^-$-treated astrocytes was efficiently taken up by neurons. Furthermore, $ONOO^-$-mediated neuronal uptake of astrocytic-released L-arginine increased when neuronal L-arginine was depleted previously, thus highlighting a potential physiological relevance of this phenomenon under L-arginine-starving conditions.

In conclusion, our results show that both exogenous and endogenous $ONOO^-$ stimulates L-arginine transport in glial cells, possibly as a direct effect on the y^+ L-arginine transporter system. Thus, when the NOS substrate L-arginine becomes a limiting factor for $^{\cdot}NO$ synthesis, $ONOO^-$ formation in neurons (17) would diffuse to astrocytes, thereby representing a possible intercellular signal designed to activate L-arginine release. In fact, here we show, for the first time, that L-arginine released from astrocytes is efficiently taken up by neurons in co-culture. Whether the reported glutamate-mediated increase in L-arginine release (8, 18) is brought about by endogenous $ONOO^-$ production remains obscure, but our previous (19) and present results are indeed compatible with such a hypothesis. This mechanism would replenish cytosolic L-arginine to facilitate physiological $^{\cdot}NO$ instead of neurotoxic $ONOO^-$ in neurons. If so, such a neuronal-glial interaction could help to prevent the propagation of neuronal death upon excess glutamate-receptor activation.

REFERENCES

1. Palmer, R. M. J., Ferrige, A. G. & Moncada, S. (1987) *Nature* **327,** 524–526
2. Garthwaite, J., Charles, S. L. & Chess-Williams, R. (1988) *Nature* **336,** 385–387
3. Knowles, R. G., Palacios, M., Palmer, R. M. J. & Moncada, S. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 5159–5162
4. Agulló, L. & García, A. (1992) *Biochem. Biophys. Res. Commun.* **182,** 1362–1368
5. Westergaard, N., Beart, P. M. & Schousboe, A. (1993) *J. Neurochem.* **61,** 364–367
6. Schmidlin, A. & Wiesinger, H. (1994) *Glia* **11,** 262–268

7. Stevens, B. R., Kakuda, D. K., Yu, K., Waters, M., Vo, C. B. & Raizada, M. K. (1996) *J. Biol. Chem.* **271,** 24017–24022
8. Grima, G., Cuénod, M., Pfeiffer, S., Mayer, B. & Do, K. Q. (1998) *J. Neurochem.* **71,** 2139–2144
9. Simmons, M. L. & Murphy, S. (1992) *J. Neurochem.* **59,** 897–905
10. Galea, E., Feinstein, D. L. & Reis, D. J. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 10945–10949
11. Galea, E., Reis, D. J. & Feinstein, D. L. (1994) *J. Neurosci. Res.* **37,** 406–414
12. Corradin, S. B., Mauël, J., Donini, S. D., Quattrocchi, E. & Ricciardi-Castagnoli, P. (1993) *Glia* **7,** 255–262
13. Merrill, J. E., Murphy, S. P., Mitrovic, B., Mackenzie-Graham, A., Dopp, J. C., Ding, M., Griscavage, J., Ignarro, L. J. & Lowenstein, C. J. (1997) *J. Neurosci. Res.* **48,** 372–384
14. Aoki, E., Semba, R., Mikoshiba, K. & Kashiwamata, S. (1991) *Brain Res.* **547,** 190–192
15. Brown, G. C., Bolaños, J. P., Heales, S. J. R. & Clark, J. B. (1995) *Neurosci. Lett.* **193,** 201–204
16. Garthwaite, J., Garthwaite, G., Palmer, R. M. J. & Moncada, S. (1989) *Eur. J. Pharmacol.* **172,** 413–416
17. Culcasi, M., Lafon-Cazal, M., Pietri, S. & Bockaert, J. (1994) *J. Biol. Chem.* **269,** 12589–12593
18. Grima, G., Benz, B. & Do, K. Q. (1997) *Eur. J. Neurosci.* **9,** 2248–2258
19. Vega-Agapito, V., Almeida, A., Heales, S. J. R., Medina, J. M. & Bolaños, J. P. (1999) *J. Neurochem.* **73,** 1446–1452
20. MacLeod, C. L., Finley, K. D. & Kakuda, D. K. (1994) *J. Exp. Biol.* **196,** 109–121
21. Aulak, K. S., Liu, J., Wu, J., Hyatt, S. L., Puppi, M., Henning, S. J. & Hatzoglou, M. (1996) *J. Biol. Chem.* **271,** 29799–29806
22. Devés, R. & Boyd, C. A. R. (1998) *Physiol. Rev.* **78,** 487–545
23. White, M. F., Gazzola, G. C. & Christensen, H. N. (1982) *J. Biol. Chem.* **257,** 4443–4449
24. Malandro, M. S. & Kilberg, M. S. (1996) *Annu. Rev. Biochem.* **65,** 305–336
25. Stoll, J., Wadhwani, K. C. & Smith, Q. R. (1993) *J. Neurochem.* **60,** 1956–1959
26. MacLeod, C. L., Finley, K. D., Kakuda, D. K., Kozak, C. & Wilkinson, M. (1990) *Mol. Cell. Biol.* **10,** 3663–3674
27. Kilberg, M. S., Stevens, B. R. & Novak, D. A. (1993) *Annu. Rev. Nutr.* **13,** 137–165
28. Broër, A., Carsten, A., Wagner, Lang, F. & Broer, S. (2000) *Biochem. J.* **349,** 787–795
29. Wiesinger, H. (2001) *Progr. Neurobiol.* (*N. Y.*) **64,** 365–391
30. Hughes, M. N. & Nicklin, H. G. (1968) *J. Chem. Soc. Abstr.* 450–452
31. Bolaños, J. P., Peuchen, S., Land, J. M., Clark, J. B. & Heales, S. J. R. (1997) *Brain Res. Protoc.* **1,** 258–262
32. Bolaños, J. P., Heales, S. J. R., Peuchen, S., Barker, J. E., Land, J. M. & Clark, J. B. (1996) *Free Radic. Biol. Med.* **21,** 995–1001
33. Delgado-Esteban, M., Almeida, A. & Bolaños, J. P. (2000) *J. Neurochem.* **75,** 1618–1624
34. Perkins, C. P., Mar, V., Shutter, J. R., del Castillo, J., Danilenko, D. M., Medlock, E. S., Ponting, I. L., Graham, M., Stark, K. L., Zuo, Y., Cunningham, J. M. & Bosselman, R. A. (1997) *Genes Dev.* **11,** 914–925
35. Schmidlin, A. & Wiesinger, H. (1995) *J. Neurochem* **65,** 590–594
36. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
37. Chomczynski, P. & Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159
38. Devés, R., Angelo, S. & Chavez, P. (1993) *J. Physiol.* **468,** 753–766
39. Radi, R., Beckman, J. S., Bush, K. M. & Freeman, B. A. (1991) *J. Biol. Chem.* **266,** 4244–4250
40. Estévez, A. G., Spear, N., Manuel, S. M., Barbeito, L., Radi, R. & Beckman, J. S. (1998) *Prog. Brain Res.* **118,** 269–280
41. Xia, Y., Roman, I. J., Masters, B. S. & Zweier, J. L. (1998) *J. Biol. Chem.* **273,** 22635–22639
42. Cidad, P., García-Nogales, P., Almeida, A. & Bolaños, J. P. (2001) *J. Neurochem.* **79,** 17–24
43. Beckman, J. S., Beckman, T. W., Chen, J., Marshall, P. A. & Freeman, B. A. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 1620–1624
44. Almeida, A., Almeida, J., Bolaños, J. P. & Moncada, S. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 15294–15299
45. Nicholson, B., Manner, C. K., Kleeman, J. & MacLeod, C. L. (2001) *J. Biol. Chem.* **276,** 15881–15885
46. White, M. F. & Christensen, H. N. (1982) *J. Biol. Chem.* **257,** 4450–4457
47. Nicholson, B., Sawamura, T., Masaki, T. & MacLeod, C. L. (1998) *J. Biol. Chem.* **273,** 14663–14666