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Metabotropic glutamate receptor 3 activation prevents nitric oxide-induced death in cultured rat astrocytes

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Abstract

Altered glial function may contribute to the initiation or progression of neuronal death in neurodegenerative diseases. Thus, modulation of astrocyte death may be essential for preventing pathological processes in the CNS. In recent years, metabotropic glutamate receptor (mGluR) activation has emerged as a key target for neuroprotection. We investigated the effect of subtype 3 mGluR (mGluR3) activation on nitric oxide (NO)-induced astroglial death. A mGluR3 selective agonist, LY379268, reduced inducible NO synthase expression and NO release induced by bacterial lipopolysaccharide and interferon-γ in cultured rat astrocytes. In turn, a NO donor (diethylenetriamine/NO) induced *apoptotic-like* death in cultured astrocytes, which showed apoptotic morphology and DNA fragmentation, but no caspase 3 activation. LY379268 prevented astrocyte death induced by NO exposure, which correlates with a reduction in: phosphatidylserine externalization, p53 and Bax activation and mitochondrial permeability. The reported effects of LY379268 were prevented by the mGluR3 antagonist (*s*)-α-ethylglutamic acid. All together, these findings show the protective effect of mGluR3 activation on astroglial death and provide further evidence of a role of these receptors in preventing CNS injury triggered by several inflammatory processes associated with dysregulated NO production.

Keywords: apoptosis, astrocytes, metabotropic glutamate receptors 3, nitric oxide.

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Astrocytes, the most abundant glial cell type in the CNS, perform a wide range of adaptive functions involved in the control of brain homeostasis (Chen and Swanson 2003). They release neurotrophins and several antioxidant factors that contribute to neuronal survival (Müller *et al.* 1995; Wilson 1997; Kirchhoff *et al.* 2001). They also participate in the blood–brain barrier (Hamby *et al.* 2006), accumulate glycogen as an energetic reservoir (Dringen *et al.* 1993) and modulate the concentration of ions, metabolites, and neurotransmitters thereby regulating synaptic transmission (Araque *et al.* 2001). Thus, impairment of astrocyte functions may critically influence neuronal survival and contribute to the pathogenesis of many acute and chronic neurodegenerative disorders (Takuma *et al.* 2004). In this regard, regulation of astrocyte apoptosis may be essential in pathological processes of the CNS.

Cytokines (Ehrlich *et al.* 1999; Saas *et al.* 1999; Pahan *et al.* 2000), bacterial lipopolysaccharide (LPS) (Suk *et al.* 2001), and nitric oxide (NO) (Kim *et al.* 2001) can induce astrocyte death. We previously showed that LPS and interferon-γ (IFN-γ) treatment increases NO release and induces caspase 3-mediated death of cultured astrocytes by modifying the ratio Bax/Bcl-2 (Caruso *et al.* 2007). An increment in NO release in the CNS is associated with inhibition of mitochondrial functions, fast release of glutamate from astrocytes and neurons and excitotoxic neuronal death (Bal-Price and Brown 2001). However, the mechanism underlying induction of astrocyte death by NO is not fully understood. In cerebrocortical murine astrocytes, NO induces cell death with several features of apoptosis but no caspase activation (Yung *et al.* 2004), although other authors previously described caspase-dependent astroglial death induced by NO (Takuma *et al.* 1999; Suk *et al.* 2001).

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Abbreviations used: AIF, apoptosis-inducing factor; DETA, diethylenetriamine; EGLU, (*s*)-α-ethylglutamic acid; FBS, fetal bovine serum; IFN-γ, interferon-γ; iNOS, inducible NO synthase; L-CCG-I, (2*S*, 1′*S*, 2′*S*)-2-(carboxycyclopropyl)glycine; LPS, lipopolysaccharide; mGluR, metabotropic glutamate receptor; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; NO, nitric oxide; OD, optical density; PBS, phosphate-buffered saline; PI, propidium iodide; PS, phosphatidylserine; TdT, deoxynucleotidyl transferase.

Metabotropic glutamate receptors (mGluR) form a family of G protein-coupled receptors and have been implicated in neuroplasticity associated with normal brain functions as well as in a variety of nervous system disorders. It is now clear that mGluR also play an important role in neuroprotection (Harrison *et al.* 2008; Vernon *et al.* 2008; Berent-Spillson and Russell 2007; Zhou *et al.* 2006; Baskys *et al.* 2005; among others). Eight mGluR subtypes have been cloned to date and are classified into groups I (mGluR1 and 5), II (mGluR2 and 3) and III (mGluR4, 6, 7, and 8). Groups II and III are negatively coupled to adenylyl cyclase, thereby inhibiting cAMP formation and cAMP-dependent protein kinase-A activation (for review, see Schoepp *et al.* 1999).

Several reports show mGluR expression in glial cells. Of the group II mGluR, the mGluR3 subtype was found in astrocytes by immunohistochemical and *in situ* hybridization studies (Mudo *et al.* 2007) where it may play protective roles. In fact, agonists of group II mGluR are more effective against excitotoxic death in mixed neuron–glia cultures than in pure neuronal cultures (Kingston *et al.* 1999). Activation of glial mGluR3 protects neurons from oxidative injury (Berent-Spillson and Russell 2007). Activation of group II mGluR stimulates the release of neuroprotective factors such as brain derived neurotrophic factor and transforming growth factor-β from astrocytes (Bruno *et al.* 1998), which in turn promote cell survival and proliferation of the astrocytes themselves (Albrecht *et al.* 2002). Ciccarelli *et al.* (2007) recently demonstrated that a synthetic mGluR3 agonist, (−)2-oxa-4-aminocyclo-[3.1.0]hexane-4,6-dicarboxylic acid (LY379268), protects cultured astrocytes against apoptotic death induced by oxygen/glucose deprivation.

In this study, we investigated whether mGluR3 activation could have a protective role against astrocyte death induced by NO and examined the molecular mechanisms involved in its modulatory action. Our results demonstrate that mGluR3 activation decreases NO production in LPS/IFN-γ-treated astrocytes and protects cultured astrocytes against cell death induced by NO exposure. This protective effect correlates with reduced expression and activation of p53 and Bcl-2 family proteins.

Experimental procedures

Materials

(−)2-Oxa-4-aminobicyclo[3.1.0]hexane-4,6-dicarboxylic acid (LY379268) was kindly provided by Eli Lilly (Indianapolis, IN, USA) and (2*S*, 1′*S*, 2′*S*)-2-(carboxycyclopropyl)glycine (L-CCG-I) and (*s*)-α-ethylglutamic acid (EGLU) was purchased from Tocris Bioscience (Ellisville, MO, USA).

Interferon-γ was purchased from Boehringer Ingelheim (Buenos Aires, Argentina). Diethylenetriamine NO adduct (DETA/NO), LPS (*Escherichia coli*, serotype O127:B8), and anti-β-actin antibody were purchased from Sigma-Aldrich Corporation (St Louis, MO, USA). Fetal bovine serum (FBS) was obtained from PAA laboratories GmBH (Pasching, Austria). Dulbecco's modified Eagle's medium/F-12, antibiotics, antimycotics, and all RT-PCR reagents were purchased from Invitrogen Life technologies (Carlsbad, CA, USA), unless otherwise specified. Anti-Bcl-2, anti-Bax, and anti-apoptosis-inducing factor (AIF) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-inducible NO synthase (iNOS) and anti-cytochrome *c* antibodies, Annexin V-FITC, and propidium iodide (PI) were purchased from BD Biosciences (San José, CA, USA). Anti-glial fibrillary acidic protein, anti-mGluR2/3, and biotinylated donkey anti-mouse and anti-rabbit antibodies were obtained from Chemicon International Inc. (Temecula, CA, USA). Anti-p53 and anti-phospho-p53 antibodies were obtained from Cell Signalling Technology Inc. (Beverly, MA, USA). iNOS and β-actin primers were purchased from Transgenomics Inc. (Omaha, NE, USA). Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) reactives were obtained from Roche Diagnostics (Mannheim, Germany). All other media and supplements were obtained from Sigma-Aldrich Corporation unless otherwise specified.

Cell culture

Astrocyte primary cultures were carried out as previously described (Caruso *et al.* 2007). Briefly, cerebral hemispheres of 1- to 2-day-old postnatal Wistar rat pups were dissected, freed from meninges, and cut into small fragments. The tissue was disrupted by triturating it through a needle in Dulbecco's modified Eagle's medium/F-12 medium containing 10% FBS, 50 μg/mL streptomycin, 0.125 μg/mL amphotericin, and 50 U penicillin. Then, cells were seeded in 75 cm^2 poly-L-lysine coated culture flasks. Cultured cells were kept at 37°C in 5% CO_2. After astrocytes reached confluence, they were separated from microglia and oligodendrocytes by shaking the flasks for 24 h in an orbital shaker at 2.68 *g*. Cells were tripsinized, subcultured, and after 2–3 days of stabilization, incubated with the drugs in minimal essential medium containing 2% FBS, 2 mM L-glutamine, 50 μg/mL streptomycin, 0.125 μg/mL amphotericin, and 50 U penicillin.

Cultures were routinely over 95% pure astrocytes, when assessed by glial fibrillary acidic protein immunostaining as previously described (Caruso *et al.* 2007).

All experimental procedures were approved by the Committee on Ethics of the School of Medicine (UBA) and were carried out in accordance with the guidelines of the NIH Guide for the Care and Use of Laboratory Animals.

Treatments

Astrocytes were incubated with LPS 1 μg/mL and IFN-γ 50 ng/mL for 24 h, or with DETA/NO 1 mM for 48 h in presence or absence of LY379268 100 μM (highly selective group II mGluR agonist, considerably more mGluR3 selective; Imre 2007) and EGLU 300 μM (highly selective group II mGluR antagonist). When treated with the antagonist, astrocytes were pre-incubated with EGLU 300 μM alone for 30 min and then treated with the other drugs. Control cells were grown in minimal essential medium containing 2% FBS, 2 mM L-glutamine, 50 μg/mL streptomycin, 0.125 μg/mL amphotericin, and 50 U penicillin.

cAMP determination

Intracellular cAMP concentration was determined by employing an enzyme immunoassay kit (Assay Designs, Inc., Ann Arbor, MI,

USA), following the manufacturer's instructions; 4×10^4 cells were treated with forskolin (10 μM) in presence or absence of LY379268 (10^{-8}–10^{-3} M) for 20 min. After removing the culture medium, cells were incubated with 0.1 M HCl for 20 min to allow cell lysis. The cell lysate was centrifuged at 600 *g* for 10 min and the supernatants were dried down prior to reconstitution on Assay Buffer. Samples were incubated with alkaline phosphatase conjugated with cAMP and with a rabbit polyclonal antibody anti-cAMP. p-nitrophenyl phosphate substrate was added and optical density (OD) measured in a microplate spectrophotometer (BioRad Laboratories, Hercules, CA, USA) at 405 nm with correction at 595 nm. cAMP concentration was determined from a cAMP standard curve and expressed as pmol/mL. Assay controls included: blank wells, non-specific-binding wells, and total activity wells. Assay sensitivity was 0.3 pmol/mL.

cGMP determination

Intracellular cGMP concentration was determined with an enzyme immunoassay kit (Assay Designs, Inc.) following the manufacturer's instructions for the acetylated version; 3×10^5 cells were pre-treated with the phosphodiesterase inhibitor isobutyl-methylxanthine (IBMX) (1 mM) for 30 min followed by incubation with DETA/NO and IBMX in presence or absence of LY379268 (10^{-6}–10^{-4} M) for 20 min. After removing the culture medium, cells were incubated with 0.1 M HCl for 30 min to allow cell lysis. The cell lysate was centrifuged at 500 *g* for 15 min and the supernatants were acetylated prior to incubation with alkaline phosphatase conjugated with cGMP and with a rabbit polyclonal antibody anti-cGMP overnight at 4°C. After washing, p-nitrophenyl phosphate substrate was added and OD was measured at 405 nm with correction at 595 nm. cGMP concentration was determined from a cGMP standard curve and expressed as pmol/mL. Assay controls included: blank wells, non-specific-binding wells, and total activity wells. Assay sensitivity was 0.08 pmol/mL.

RT–PCR assay

Total RNA from cultured astrocytes was extracted using TRIZOL reagent (Invitrogen Life technologies) according to the manufacturer's protocol; 5 μg of total RNA was treated with 1 U Dnase (Promega Corporation, Madison, WI, USA) at 37°C for 10 min and reverse transcribed as described before (Caruso *et al.* 2007). Amplification was performed with 2 μL of cDNA as template as previously described (Caruso *et al.* 2007); 20 μL of each reaction were analyzed on 2% agarose gels and stained with ethidium bromide. RT-PCR products were analyzed using SCION Image software (Scion Corporation, Frederick, MD, USA). Results were normalized to the internal control β-actin. Values were expressed relative to the respective treatment with LPS + IFN-γ. Experiments always included non-reverse transcribed RNA samples as negative controls.

Nitric oxide release assay

Synthesis of NO by astrocytes was determined by measuring the release of nitrite (a stable product of NO with molecular oxygen) to the culture medium by the Griess method. Briefly, 100 μL of culture supernatant were incubated with 50 μL 1% sulfanilic acid (in H_3PO_4 5%) for 5 min and then with 50 μL of *N*-(1-naphtyl)ethylenediamine dihydrochloride 0.1% for 15 min at 20–25°C. OD was measured in a microplate spectrophotometer at 595 nm. Nitrite concentrations were calculated from a sodium nitrite standard curve. Data were expressed relative to the respective control, which was considered as 100%.

Metabolic activity assay

Metabolic activity of viable cells was measured by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, cells (5×10^4) were washed and incubated for 4 h in 100 μL Krebs buffer plus 50 μg of MTT reagent dissolved in 10 μL phosphate-buffered saline (PBS) at 37°C. Formazan crystals obtained from MTT reduction were dissolved in 100 μL 0.04 M HCl in isopropanol and OD was measured in a microplate spectrophotometer at 595 nm.

Phosphatidylserine externalization measurement

Translocation of phosphatidylserine (PS) from the inner to the outer side of the plasma membrane was assessed with a FITC-conjugated-annexin V apoptosis detection kit (BD Biosciences) according to the manufacturer's instructions. After incubation with the drugs, astrocytes (2–3×10^5 cells) were washed with PBS, tripsinized, and centrifuged. The pellet was diluted in Annexin V Binding Buffer and astrocytes were double stained with annexin V and PI. Then, stained cells were analyzed by flow cytometry. Cells positive for annexin V and negative for PI were scored as apoptotic-like. The following controls were performed: unstained cells, annexin V-only cells and PI-only cells. Data were analyzed with the WINMDI Software (Scripps Research Institute, San Diego, CA, USA).

Microscopic determination of DNA fragmentation by the terminal TdT-mediated dUTP nick end labeling assay

Cells were fixed with 4% formaldehyde in PBS, pH 7.4, for 30 min and permeabilized by microwave irradiation. DNA strand breaks were labeled with digoxigenin-dUTP using terminal TdT (0.18 U/μL) as previously described (Caruso *et al.* 2004). The incorporation of nucleotides into the 3′-OH end of damaged DNA was detected with an anti-digoxigenin-fluorescein antibody. Slides were mounted with mounting medium for fluorescence (Vectashield, Vector Laboratories Inc., Burlingame, CA, USA) containing 4′,6-diamido-2-phenylindole dihydrochloride for DNA staining and visualized in a fluorescence microscope (Axiophot, Carl Zeiss, Jena, Germany). Negative control slides were incubated in absence of TdT.

Caspase 3 activity assay

Caspase 3 activity was measured using a colorimetric kit (Assay Designs, Inc.). Astrocytes (1×10^6) were homogenized in lysis buffer and centrifuged (13 148 *g*, 20 min). The supernatant was added to the reaction mixture containing Ac-DEVD-*p*-nitroaniline, the colorimetric reaction substrate, and incubated at 37°C for 4 h. OD was measured in a microplate spectrophotometer at 415 nm. Caspase 3 activity was expressed as units per mL.

Western blot analysis

Astrocytes (1×10^6) were homogenized in lysis buffer as previously described (Caruso *et al.* 2007). For western blot of phospho-p53, MgCl 5 mM, dithiothreitol 1 mM and phosphatase inhibitors NaF 50 mM, and Na_3VO_4 1 mM were added to the lysis buffer. Following sonication and centrifugation at 9660 *g* for 30 min, the

supernatant was assayed by immunoblot. Protein concentration in the samples was determined by the Bradford method (BioRad Laboratories) using bovine serum albumin as standard; 30–50 μg of protein were size-fractionated in a sodium dodecyl sulfate–polyacrylamide gel, then electrotransferred to a polyvinylidene difluoride membrane. Blots were blocked for 2 h in 5% non-fat dry milk–Tris-buffered saline–0.1% Tween 20 and incubated overnight with appropriate primary antibodies in 5% milk–Tris-buffered saline–0.1% Tween 20 at 4°C (anti-Bax and anti-Bcl-2: 1 : 200, anti-iNOS: 1 : 10 000, anti-p53: 1 : 1000, anti-phospho-p53: 1 : 500, anti-mGluR2/3: 1 : 1000, and anti-AIF: 1 : 100). This was followed by 1 h incubation with the respective biotinylated secondary antibody and 1 h incubation with Streptavidin-peroxidase (Chemicon International Inc.). Immunoreactivity was detected by enhanced chemiluminescence (ECL plus; Amersham Biosciences, GE Healthcare, Piscataway, NJ, USA). Bands were analyzed using SCION Image software. Results were normalized to the internal control β-actin. Values were expressed relative to respective controls (or to respective treatment with LPS/IFN-γ for iNOS determination).

Immunocytochemistry for cytochrome *c*

After drug treatment, astrocytes (2×10^4 cells/mL) were incubated for 30 min at 37°C with 100 nM MitoTracker Red CMX (Invitrogen Life technologies, Carlsbad, CA, USA) diluted in the culture medium. Then, cells were fixed in PBS–formaldehyde 4% for 30 min at 4°C. Cells were permeabilized in PBS-Triton X 100 0.1% and incubated in blocking buffer containing 3% FBS, 3% donkey serum, and 0.2 mL/mL avidin solution for 1 h at 20–25°C. Subsequently, cells were incubated overnight at 4°C with primary monoclonal antibody against native cytochrome *c* (1 : 50 dilution in blocking buffer containing 3% FBS, 1% donkey serum, and 0.2 mL/mL biotin solution). After rinsing, slides were incubated with biotin-conjugated donkey anti-mouse secondary antibodies (1 : 600 in blocking solution) for 1 h at 20–25°C. Then, cells were incubated with FITC-conjugated Avidin (1 : 600 in 10 mM HEPES, pH 7.9) for 20 min at 20–25°C and coverslipped with mounting medium (Vectashield, Vector Laboratories). Staining was visualized in a fluorescence microscope. Negative control slides were incubated with blocking buffer instead of primary antibody. A punctuate/nuclear rounding pattern of staining for cytochrome *c*, which colocalizes with MitoTracker Red CMX staining, was considered alive/healthy cells, whereas cells showing a more diffuse pattern and absence of colocalization with the mitochondrial marker were considered dying cells.

Statistical analysis

Data were expressed as mean ± SEM and analyzed by one sample *t*-test, unpaired *t*-test, or one-way ANOVA followed by Dunnett's or Bonferroni's multiple comparisons test. The number of apoptotic cells identified by the TUNEL method was expressed as the percentage of apoptotic cells of the total number of cells counted for each specific condition. Differences between proportions were analyzed by the chi-squared test with 95% confidence, and confidence intervals for odds ratios were computed.

Differences with a $p < 0.05$ were considered statistically significant. All experiments were performed at least twice.

Results

Inhibition of cAMP accumulation by mGluR3 activation

As group II mGluR activation diminishes cAMP production (Wroblewska *et al.* 1998), we determined the effect of the highly selective mGluR3 agonist, LY379268, on cAMP accumulation in cultured rat astrocytes. cAMP production

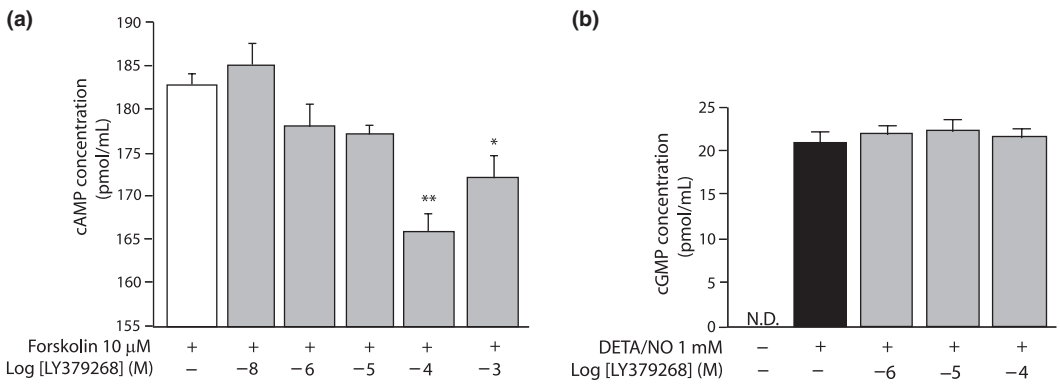

Fig. 1 Inhibition of cAMP accumulation by mGluR3 activation. (a) Cultured rat astrocytes were incubated with forskolin (10 μM) in presence or absence of mGluR3 agonist LY379268 (10^{-8}–10^{-3} M) for 20 min. Astrocytes were lysed and intracellular cAMP concentration was quantified using an enzyme immunoassay kit (Assay Designs, Inc.). cAMP concentration was expressed as pmol/mL. Bars represent the mean ± SEM of four determinations per group of one representative experiment of two independent ones; $^*p < 0.05$ and $^{**}p < 0.01$ versus forskolin; one-way ANOVA, followed by Dunnett's test for multiple comparisons. (b) Astrocytes were pre-incubated with phosphodiesterase inhibitor, isobutyl-methylxanthine 1 mM for 30 min, before incubation with the NO donor, DETA/NO 1 mM in presence or absence of LY379268 (10^{-6}–10^{-4} M), for 20 min. Intracellular cGMP levels were quantified using a direct enzyme immunoassay kit (Assay Designs, Inc.) following the manufacturer's instructions for the overnight acetylated protocol. cGMP concentration was expressed as pmol/mL. Bars represent the mean ± SEM of five determinations per group of one representative experiment of two independent ones. N.D., non-detectable levels.

was strongly stimulated by forskolin 10 μM (control: 27.93 ± 2.21 pmol/mL; forskolin: 182.92 ± 1.26 pmol/mL, $p < 0.001$ versus control). LY379268 inhibited forskolin-stimulated cAMP accumulation in a dose-dependent manner (Fig. 1a). This effect was maximal with LY379268 100 μM. Therefore, all further experiments were performed using LY379268 100 μM. Wroblewska *et al.* (2006) also showed that several agonists of group II mGluR reduce cGMP levels stimulated by a NO donor, sodium nitroprusside, in cerebellar astrocytes. In our conditions, LY379268 (10^{-6}–10^{-4} M) did not modify DETA/NO-induced cGMP production (Fig. 1b), indicating that this second messenger is not involved in the signaling cascade triggered by group II mGluR activation.

mGluR3 activation reduced LPS/IFN-γ-induced iNOS expression and NO release and prevented LPS/IFN-γ-induced cell death in cultured rat astrocytes

To investigate the effect of LY379268 on iNOS expression and NO production induced by a proinflammatory agent, primary astrocyte cultures were treated for 24 h with LPS (1 μg/mL) plus IFN-γ (50 ng/mL) and/or LY379268 (100 μM). No basal iNOS expression was detected in either control or LY379268-treated astrocytes, as assayed by RT-PCR and western blot (Fig. 2a and b). iNOS gene and protein expression were induced by LPS/IFN-γ, but the presence of the mGluR3 agonist significantly reduced iNOS mRNA and protein levels (Fig. 2a and b). Pre-incubation with the

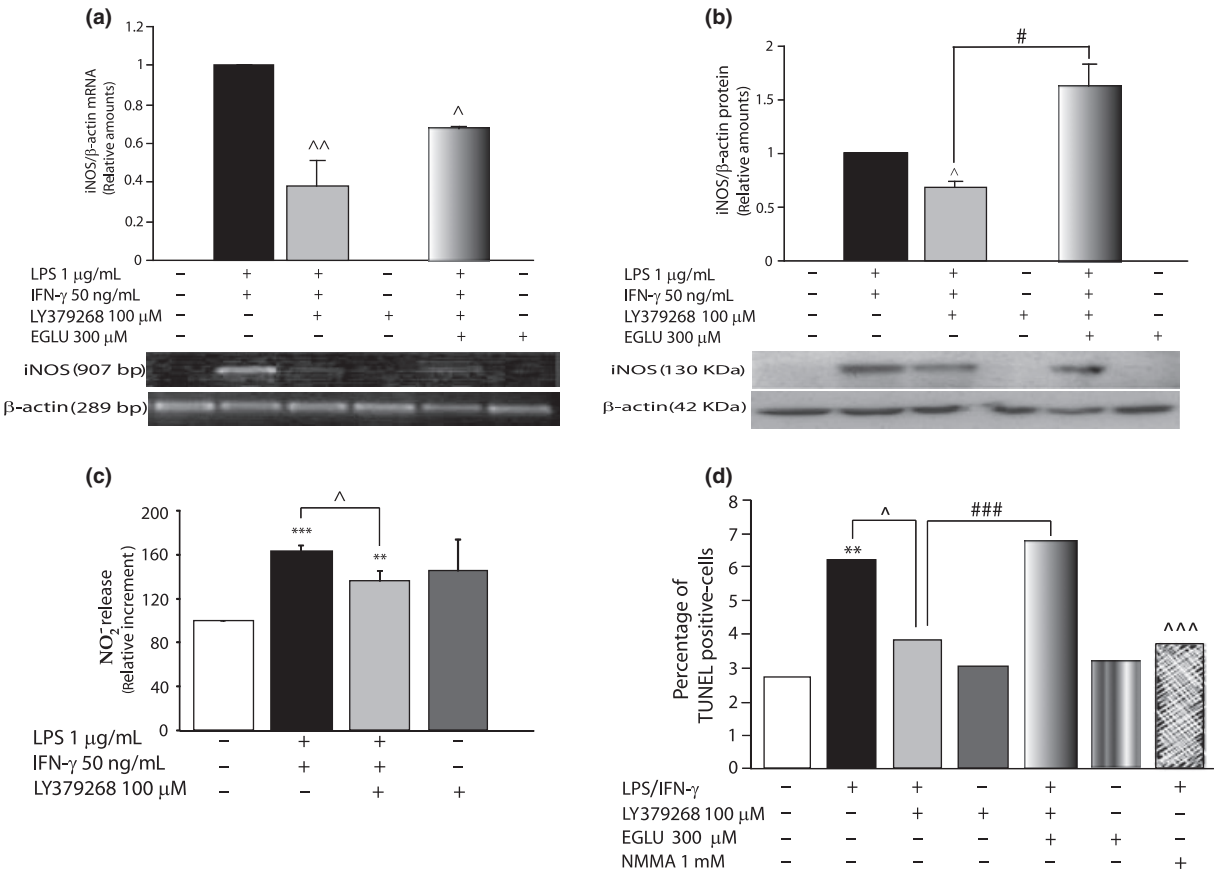

Fig. 2 mGluR3 activation by the agonist LY379268 reduces LPS/IFN-γ-induced iNOS expression, NO production, and DNA fragmentation in cultured rat astrocytes. Astrocytes were incubated with LPS (1 μg/mL) plus IFN-γ (50 ng/mL) and/or LY379268 (100 μM) for 24 h. When treated with the mGluR3 antagonist EGLU, astrocytes were pre-incubated with EGLU (300 μM) for 30 min before LPS/IFN-γ + LY379268 + EGLU treatment. iNOS mRNA levels were determined by semi-quantitative RT-PCR (a) and iNOS protein levels by western blot (b). Values of OD from obtained bands were normalized to the internal control β-actin, and data were expressed relative to LPS/IFN-γ group. (c) NO production was estimated by quantification of nitrites (NO_2^-) accumulated in the culture medium 24 h after treatments, by the Griess method. NO_2^- concentration was expressed as relative percentages compared with the control group. Bars represent the mean ± SEM of at least three independent experiments; ^$p < 0.05$ and ^^$p < 0.01$ versus LPS/IFN-γ; $^{\#}p < 0.05$ versus LPS/IFN-γ + LY379268; **$p < 0.01$ and ***$p < 0.001$ versus control, one sample *t*-test and Student's unpaired *t*-test. (d) DNA fragmentation of astrocytes was determined by the TUNEL method after 24 h drugs exposure. The non-selective NOS inhibitor N^G-methyl-L-arginine (NMMA) (1 mM) was co-incubated with LPS/IFN-γ. Bars represent percentage of TUNEL-positive cells of two independent experiments; **$p < 0.01$ versus control; ^$p < 0.05$ and ^^^$p < 0.001$ versus LPS/IFN-γ; $^{\#\#\#}p < 0.001$ versus LPS/IFN-γ + LY379268; χ^2 test.

mGluR3 selective antagonist EGLU (300 μM) diminished the inhibitory effect of LY379268 on iNOS mRNA levels and reverted the action of LY379268 on iNOS protein levels (Fig. 2a and b). EGLU alone did not modify iNOS expression. Accordingly, LY379268 attenuated LPS/IFN-γ-stimulated NO_2^- release from astrocytes, whereas it had no effect *per se* on NO_2^- release (Fig. 2c).

As we had previously shown that LPS/IFN-γ induced astrocyte apoptosis (Caruso *et al.* 2007), we studied whether LY379268 could also modulate this effect. LY379268 prevented DNA fragmentation induced by LPS/IFN-γ, as measured by the TUNEL method (Fig. 2d). Pre-incubation with EGLU reverted the effect of LY379268, while neither LY379268 nor EGLU had any effect *per se* on DNA fragmentation.

To determine whether NO release mediates LPS/IFN-γ-induced cell death, TUNEL assays in the presence of the non-selective NOS inhibitor N^G-methyl-L-arginine (1 mM) were performed. N^G-methyl-L-arginine reverted, though not completely, the increase in the percentage of TUNEL-positive astrocytes induced by LPS/IFN-γ (Fig. 2d), suggesting that NO may be the major, but not the only, mediator of LPS/IFN-γ-induced astrocyte apoptosis.

mGluR2/3 expression was induced by LPS/IFN-γ and reduced by LY379268 *per se*

As group II mGluR levels change in response to different cerebral injuries (Geurts *et al.* 2003; Ferraguti *et al.* 2001; among others), mGluR2/3 expression was analyzed in our experimental conditions. The inflammatory stimulus LPS/IFN-γ significantly raised mGluR2/3 protein levels in cultured rat astrocytes, whereas the selective agonist LY379268 *per se* diminished mGluR2/3 protein levels (Fig. 3).

Group II mGluR agonists prevented cell death caused by NO

As NO was proposed as the principal mediator of cell damage after LPS/IFN-γ challenge, we determined the effect of group II mGluR agonists on NO-induced astrocyte death. Astrocytes were treated with a NO donor, DETA/NO (1 mM), for 48 h and their metabolic activity was determined. DETA/NO significantly reduced cell viability, whereas LY379268 (100 μM) prevented this effect (Fig. 4). Moreover, when astrocytes were pre-incubated with EGLU (300 μM), the effect of LY379268 was blocked (Fig. 4); however, neither LY379268 nor EGLU modified cell viability *per se* (Fig. 4). DNA fragmentation was also evaluated by the TUNEL method. TUNEL-positive astrocytes showed typical apoptotic morphology when treated with DETA/NO: condensed nuclei and, in some cases, apoptotic bodies (Fig. 5a). Moreover, the percentage of TUNEL-positive astrocytes was significantly incremented by DETA/NO (Fig. 5c), whereas in combination with LY379268 this percentage was significantly reduced only at 10 and 100 μM (Fig. 5b), indicating that LY379268 prevents NO-induced DNA degradation in a dose-dependent manner. Moreover, LY379268 failed to decrease the percentage of

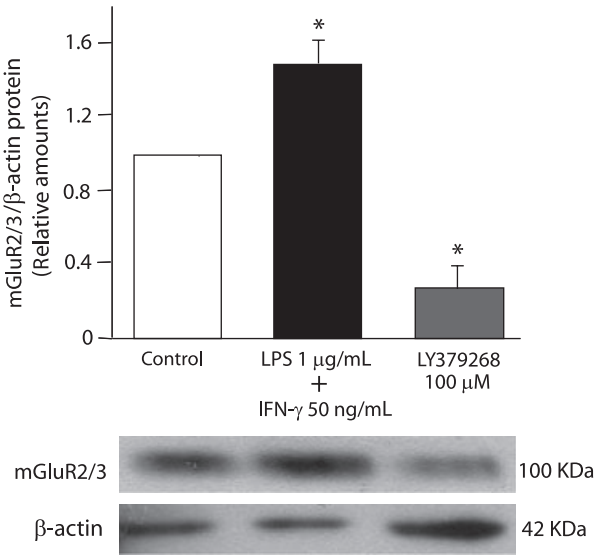

Fig. 3 mGluR2/3 protein levels are induced by LPS/IFN-γ and reduced by LY379268. Astrocytes were incubated with LPS (1 μg/mL) plus IFN-γ (50 ng/mL) or with LY379268 (100 μM) for 24 h. Total cellular proteins were extracted and 30 μg of protein extracts were used to perform sodium dodecyl sulfate–polyacrylamide gel electrophoresis as described in Experimental procedures. Values of OD from obtained bands were normalized to the internal control β-actin, and data expressed relative to the control group. Bars represent the mean ± SEM of three independent experiments; $^*p < 0.05$ versus control, one sample *t*-test.

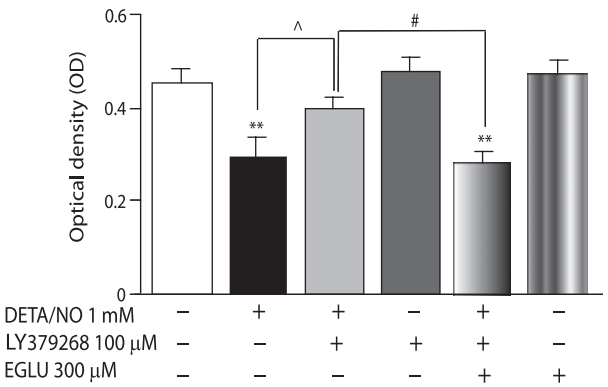

Fig. 4 Selective group II mGluR activation increases astrocyte viability reduced by NO. Astrocytes were exposed to a NO donor (DETA/NO; 1 mM) in presence or absence of LY379268 100 μM. When treated with EGLU, astrocytes were pre-incubated with EGLU 300 μM 30 min before DETA/NO + LY379268 + EGLU treatment. Cell viability was determined by the MTT method. Plots show the mean ± SEM of eight determinations per group, of one representative experiment of two independent ones; $^{**}p < 0.01$ versus control; $^{\wedge}p < 0.05$ versus DETA/NO; $^{\#}p < 0.05$ versus DETA/NO + LY379268; one-way ANOVA, followed by Bonferroni's test for multiple comparisons.

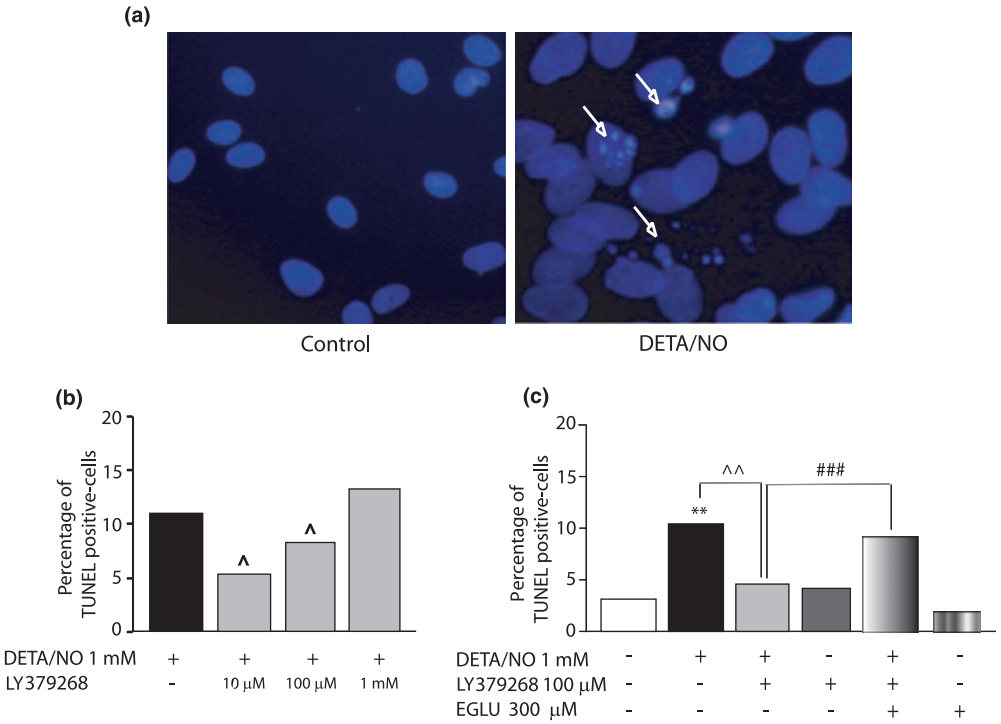

Fig. 5 LY379268 prevents DNA fragmentation induced by NO in astrocytes. Fragmentation of astrocytes DNA was determined by the TUNEL method as an index of cell death. (a) Control (left) and DETA/NO-treated astrocytes (right) were stained with the nuclear marker 4′,6-diamido-2-phenylindole dihydrochloride. Note that astrocyte nuclei become smaller and more condensed after DETA/NO exposure and observe some apoptotic bodies (arrows). (b) Astrocytes were exposed to a NO donor (DETA/NO; 1 mM) in presence or absence of LY379268 (10, 100, and 1000 μM) for 48 h and the number of TUNEL-positive cells was determined. Bars represent the percentage of TUNEL-positive cells. Odds ratios for proportions of dead cells were computed and confidence intervals for odds ratios analyzed with 95% confidence; ^$p < 0.05$ versus DETA/NO group. (c) Cultured astrocytes were incubated with DETA/NO and/or LY379268 (100 μM) and/or EGLU (300 μM) for 48 h. Bars represent the percentage of TUNEL-positive cells; **$p < 0.01$ versus control; ^^$p < 0.01$ versus DETA/NO; ###$p < 0.001$ versus DETA/NO + LY379268; χ^2 test.

TUNEL-positive astrocytes induced by NO when cells were pre-incubated with EGLU (Fig. 5c). Treatment with the agonist or antagonist alone had no effect on DNA degradation (Fig. 5c).

To further prove the protective effect of group II mGluR, we tested the action of another potent agonist of these receptors, L-CCG-I. We found that L-CCG-I at 100 μM and 1 mM blocked the reduction of cell viability caused by NO, the higher concentration being more effective (control: 0.42 ± 0.0098; DETA/NO 1 mM: 0.35 ± 0.0098, $p < 0.01$ versus control; DETA/NO + L-CCG-I 100 μM: 0.39 ± 0.0037, $p < 0.01$ versus DETA/NO; DETA/NO + L-CCG-I 1 mM: 0.42 ± 0.0088, $p < 0.001$ versus DETA/NO).

We also studied the action of glutamate, the endogenous agonist of mGluR. We observed no effect of glutamate (10^{-6}–10^{-4} M) on astrocyte viability, either alone or in combination with the NO donor, at any concentration tested (data not shown), suggesting that activation of other glutamate receptor types may be responsible for this absence of effect.

LY379268 reduced phosphatidylserine exposure induced by NO

Translocation of PS from the inner to the outer side of the plasma membrane has been proposed as a typical feature of apoptotic processes. To investigate the action of LY379268 on this mechanism, we used the PS-binding protein, FITC-annexin V, to confirm the presence of externalized PS, and PI to detect dead cells with permeable plasma membranes. After obtaining the cell population distribution based on both FITC (Fig. 6a, *x*-axis) and PI (Fig. 6a, *y*-axis) fluorescence, we determined the number of annexin V-positive/PI-negative cells scored as apoptotic cells. DETA/NO significantly increased the percentage of apoptotic astrocytes, whereas co-treatment with LY379268 reduced this proportion (Fig. 6a and b). When pre-incubated with EGLU, the percentage of apoptotic astrocytes was similar to that observed in the DETA/NO group (Fig. 6a and b). Neither LY379268 nor EGLU *per se* had any effect on this percentage (Fig. 6a and b).

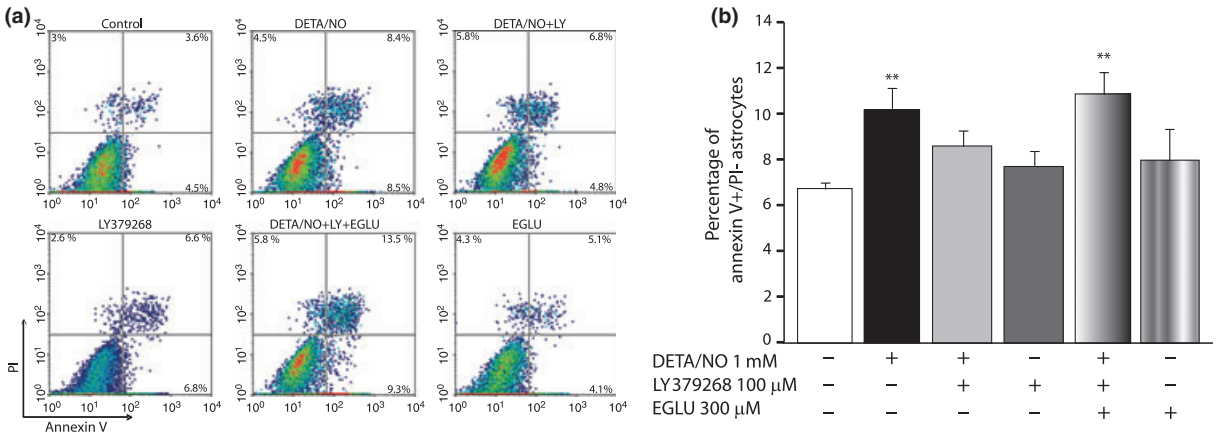

Fig. 6 LY379268 reduces phosphatidylserine externalization induced by the NO donor. The FITC-annexin V/PI staining profile of cultured astrocytes exposed for 48 h to DETA/NO (1 mM) in presence or absence of LY379268 (100 μM) and EGLU (300 μM) was studied by flow cytometry. (a) Density plots showing the proportion of healthy astrocytes (annexin V^-/PI^-, lower-left quadrant), apoptotic astrocytes (annexin V^+/PI^-, lower-right quadrant), late apoptotic plus necrotic astrocytes (annexin V^+/PI^+, upper-right quadrant), and necrotic plus damaged astrocytes (annexin V^-/PI^+, upper-left quadrant), from one representative experiment. Numbers at the corners of each plot represent percentages of cells belonging to each quadrant determined with the WinMDI Software. (b) Bars represent the mean ± SEM of percentages of apoptotic astrocytes from four independent experiments performed in triplicate; $^{**}p < 0.01$ versus control; one-way ANOVA, followed by Dunnett's test for multiple comparisons.

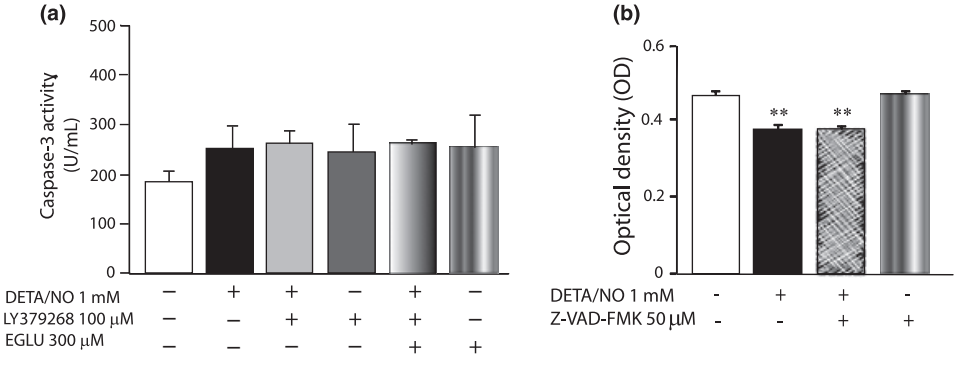

Fig. 7 Caspase 3 activity is not modified by DETA/NO or LY379268. (a) Astrocytes were incubated with DETA/NO (1 mM) in presence or absence of LY379268 (100 μM) and/or EGLU (300 μM) for 16 h and caspase 3 activity was assessed with a colorimetric kit (Assay Design, Inc.). Enzymatic activity was expressed as units per mL (U/mL). Bars represent the mean ± SEM of two independent experiments performed in duplicate. (b) Astrocytes were exposed to DETA/NO in presence or absence of a broad spectrum caspase inhibitor, Z-VAD-FMK (50 μM), for 48 h and cell viability was assessed by the MTT method. Bars represent the mean ± SEM of eight determinations per group of one representative experiment; $^{**}p < 0.01$ versus control; one-way ANOVA, followed by Bonferroni's test for multiple comparisons.

Caspase 3 activity was modified neither by DETA/NO nor LY379268

To determine whether apoptosis is the type of cell death induced by NO in astrocytes, we assayed caspase 3 activity. No modification in this enzyme's activity was detected in any of the treatments tested (Fig. 7a). Moreover, a broad spectrum caspase inhibitor, Z-VAD-FMK 50 μM, did not modify the reduction of astrocyte cell viability produced by DETA/NO (Fig. 7b). These results suggest that NO induces an *apoptotic-like* death in astrocytes which is independent of caspases activation.

LY379268 inhibited p53 and Bax activation and modulated Bcl-2 expression

To test whether the expression of pro- and antiapoptotic members of the Bcl-2 family is involved in the protective action of mGluR3 agonists, the effect of LY379268 on proapoptotic (Bax) and antiapoptotic (Bcl-2) protein levels in astrocytes was determined. DETA/NO decreased Bcl-2 levels (Fig. 8a) whereas it failed to significantly modify Bax protein levels (data not shown). LY379268 had no effect on Bcl-2 levels *per se* but attenuated the NO-stimulated decrease of Bcl-2 protein levels (Fig. 8a).

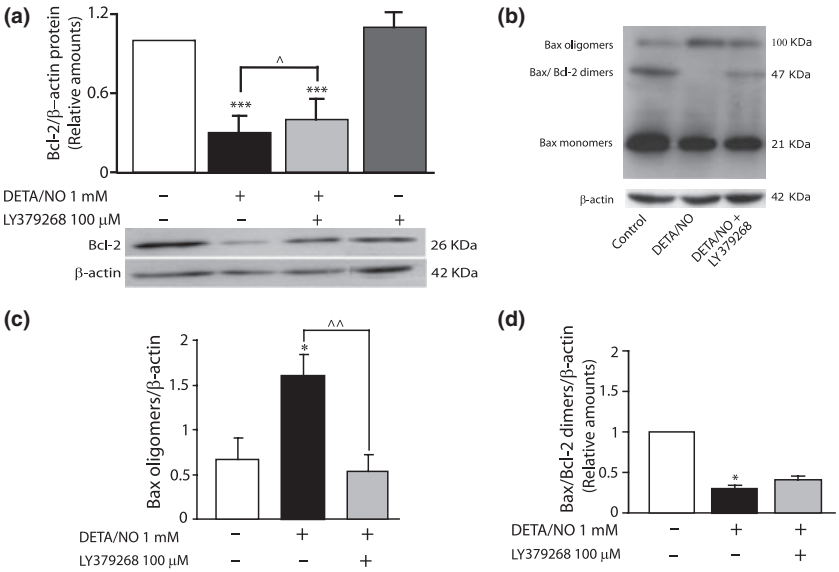

Fig. 8 mGluR3 stimulation attenuates the reduction in Bcl-2 protein levels caused by NO and reverts NO-induced Bax activation. Astrocytes were exposed to DETA/NO (1 mM) in presence or absence of LY379268 (100 μM) for 48 h. Total cellular proteins were extracted and 30 μg of protein extracts were used to perform sodium dodecyl sulfate–polyacrylamide gel electrophoresis as described in Experimental procedures. (a) Immunoblotting for Bcl-2. Values of OD from obtained bands were normalized to the internal control β-actin, and data were expressed relative to the control group. Bars represent the mean ± SEM of three independent experiments; ***$p < 0.001$ versus control; ^$p < 0.05$ versus DETA/NO, one sample *t*-test and Student's unpaired *t*-test. (b) Immunoblotting for Bax. Note the presence of the specific band for monomeric Bax (21 kDa) and additional bands for putative Bax/Bcl-2 dimers (47 kDa) and Bax oligomers (100 kDa). Values of OD from Bax oligomer bands (c) or Bax/Bcl-2 dimer bands (d) were normalized to the internal control β-actin. Bars represent the mean ± SEM of three independent experiments. *$p < 0.05$ versus control; ^^$p < 0.01$ versus DETA/NO, one sample *t*-test and Student's unpaired *t*-test.

Despite the reducing conditions in which we ran the western blot assays, we detected other bands besides the specific ones. These bands may correspond to Bax/Bcl-2 heterodimers (47 kDa, an inactive form of Bax) and Bax oligomers (100 kDa, an active proapoptotic complex) (Fig. 8b) as previously described by immunoprecipitation or gel filtration chromatography (Antonsson *et al.* 2001; Mikhailov *et al.* 2001; Dlugosz *et al.* 2006; Billen *et al.* 2008). Accordingly, sodium dodecyl sulfate-resistant Bax oligomers have been described (Seye *et al.* 2003); 100 kDa bands were detected only with anti-Bax antibody, while 47 kDa bands were observed after incubation with both anti-Bax and anti-Bcl-2 antibodies, thus reinforcing our hypothesis. DETA/NO increased levels of putative Bax oligomers and LY379268 reduced this effect (Fig. 8c), whereas Bax/Bcl-2 dimers were reduced by DETA/NO and the mGluR3 agonist attenuated this reduction (Fig. 8d). LY379268 had no effect *per se* on Bax oligomers or Bax/Bcl-2 dimers levels (data not shown). These results indicate that NO shifts the balance between pro- and antiapoptotic proteins of the Bcl-2 family toward astrocyte death and induces Bax activation, whereas LY379268 prevents these effects.

As the tumor suppressor protein p53 is able not only to stimulate Bax transcription but also to induce Bax oligomerization (Bradley Jacobs *et al.* 2006), p53 expression was analyzed. DETA/NO induced an increase in total p53 protein levels whereas LY379268 reduced this increment (Fig. 9a). At the same time, DETA/NO treatment significantly increased the active form of p53 (phospho-p53) whereas LY379268 reduced this increment (Fig. 9a and b), suggesting that mGluR3 stimulation prevents not only p53 transcription but also its post-translational activation. The agonist had no effect *per se* on p53 and phospho-p53 protein levels (Fig. 9a and b).

mGluR3 activation prevented mitochondrial membrane permeabilization induced by DETA/NO

To evaluate loss of mitochondrial membrane integrity, an event induced by Bax activation, a double immunocytochemistry for cytochrome *c* and MitoTracker Red (a mitochondrial marker) was performed and protein expression of AIF was analyzed.

A punctuate staining pattern for cytochrome *c* and colocalization of cytochrome *c* and MitoTracker Red were observed in control astrocytes (Fig. 10a–d), whereas treatment with DETA/NO reduced the proportion of cells showing colocalization and produced a diffuse cytochrome *c* staining pattern (Fig. 10e–h). The release of cytochrome *c*

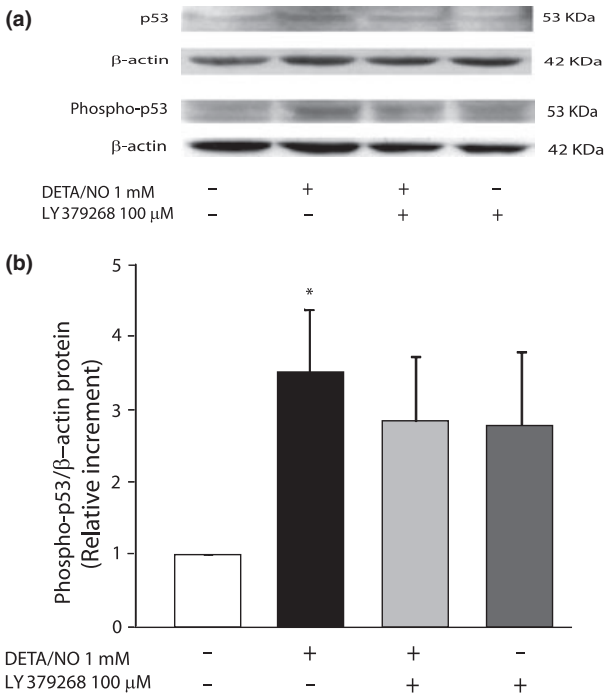

Fig. 9 mGluR3 stimulation reduces NO-induced p53 activation. Cultured astrocytes were treated with DETA/NO (1 mM) in presence or absence of LY379268 (100 μM) for 48 h. Total cellular proteins were extracted and 30 μg of protein extracts used to perform sodium dodecyl sulfate–polyacrylamide gel electrophoresis for total p53 and its active form, phospho-p53. (a) Representative immunoblots for total p53 (top) and phospho-p53 (bottom). β-Actin was assessed as an internal control. (b) Values of OD from active phospho-p53 bands were normalized to β-actin and data expressed relative to the control group. Bars represent the mean ± SEM of three independent experiments; $^*p < 0.05$ versus control, one sample *t*-test.

to the cytosol was less evident in the presence of LY379268 (Fig. 10i–l).

Apoptosis-inducing factor protein expression was analyzed by western blot employing an antibody that recognizes its apoptogenic form (57 kDa). DETA/NO augmented 57 kDa AIF levels (Fig. 11), implying that mitochondria-released AIF may be responsible for the caspase-independent astrocyte death induced by NO. In the presence of LY379268, AIF protein levels were reduced (Fig. 11), which would correlate with the prevention of *apoptotic-like* astrocyte death by mGluR3 activation.

Discussion

The present results demonstrate that mGluR3 activation by their selective agonist LY379268 inhibits iNOS expression and NO release induced by LPS/IFN-γ and that it prevents caspase-independent cell death promoted by NO in cultured astrocytes. This protective action correlates with the modulation of Bcl-2 expression and Bax and p53 activation and with the reduction of cytochrome *c* and AIF release to the cytosol and DNA fragmentation. Thus, it is conceivable that the protective role of mGluR3 activation is carried out by modulating mitochondrial permeability induced by active Bax complexes and thus preventing AIF-mediated DNA fragmentation.

Growing evidence on the expression and protective functions of mGluR in astrocytes reveals the importance of these receptors in the modulation of excitotoxicity, neurodegeneration and psychiatric disorders which involve impairment of astroglial function. Of the group II mGluR, mGluR3 (but not mGluR2) are expressed in astrocytes *in vitro* and *in vivo* (Petralia *et al.* 1996; Ohishi *et al.* 1998; Mudo *et al.* 2007), and their activation protects neurons from glucose-induced oxidative injury (Berent-Spillson and Russell 2007) and NMDA toxicity (Corti *et al.* 2007). Recently, Ciccarelli *et al.* (2007) demonstrated that LY379268, via mGluR3, protects cultured astrocytes against apoptotic death induced by oxygen/glucose deprivation.

In this study, we showed that LY379268 decreased cAMP accumulation induced by forskolin in primary cultured astrocytes. However, whether this cAMP reduction is responsible for the protective actions of mGluR3 remains to be studied. As another possible protective mechanism, Wroblewska *et al.* (2006) have postulated the NO second messenger, cGMP, as a target of mGluR action. However, in our experimental system LY379268 failed to modify NO-induced cGMP levels.

We showed here that LY379268 diminished mGluR2/3 expression in astrocytes, suggesting the involvement of autoregulatory mechanisms in the control of group II mGluR activity. In this regard, it has been demonstrated that mGluR3 are subjected to kinases-mediated homologous desensitization in human embryonic kidney 293 cells (Iaccovelli *et al.* 2009), which may act as a feedback mechanism protecting against receptor over-stimulation (Dhami and Ferguson 2006). On the other hand, the up-regulation of mGluR2/3 protein levels by LPS/IFN-γ reported here indicates that changes in the expression of these receptors may be of relevance to glial response after an inflammatory challenge. In fact, modifications of mGluR expression were observed in reactive astrocytes in multiple sclerosis lesions (Geurts *et al.* 2003) and in brain injury induced by kainate (Ferraguti *et al.* 2001).

Caruso *et al.* (2007) and Suk *et al.* (2001) have proposed that LPS/IFN-γ, through NO release, promotes caspase 3-mediated death of astrocytes. In this study, we showed that LY379268 not only reduces LPS/IFN-γ-induced iNOS expression and NO release and prevents NO-induced cell death, but also inhibits LPS/IFN-γ-induced astrocyte death. Although the reduction in NO release by LY379268 was partial, the mGluR3 agonist produced a strong inhibitory effect on cell death. However, inhibition of NO synthesis did not completely abrogate LPS/IFN-γ-induced cell death, suggesting that induction of NO release is not the only

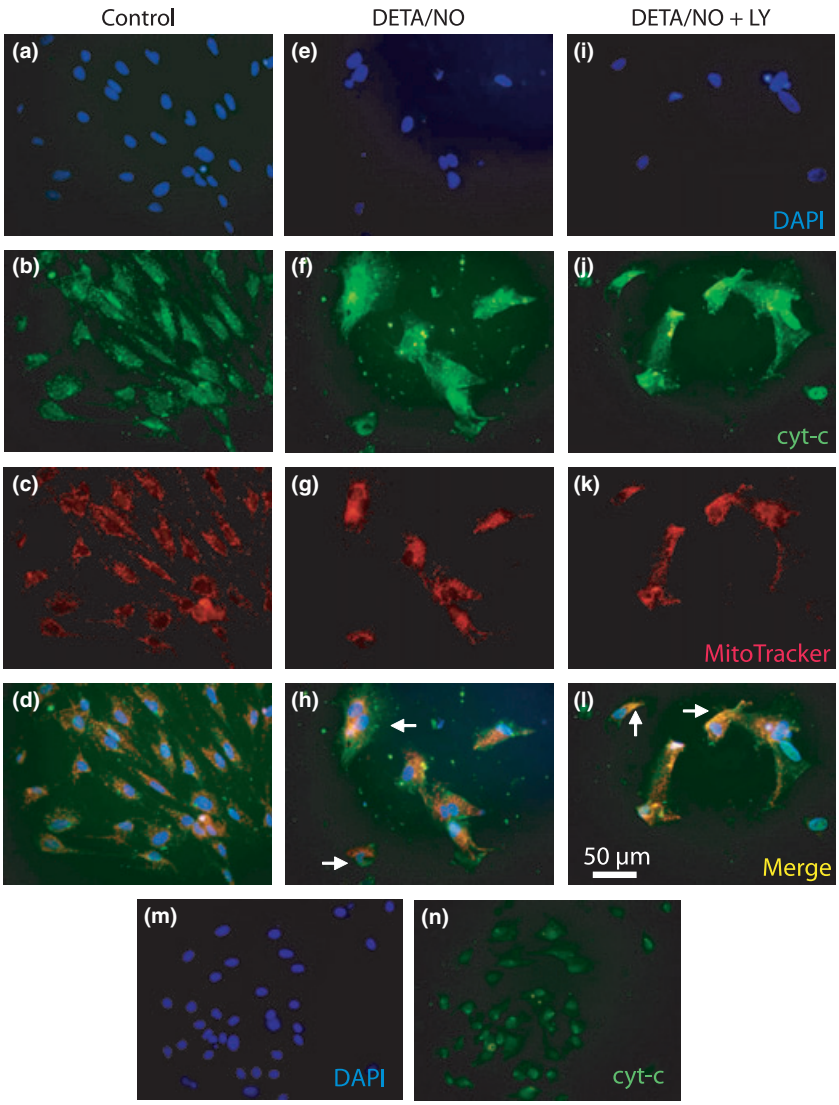

Fig. 10 LY379268 reduces cytochrome *c* release from mitochondria to the cytosol induced by DETA/NO. Astrocytes were exposed to DETA/NO (1 mM) in presence or absence of LY379268 (100 μM) for 48 h. Double immunofluorescence for cytochrome *c* (green, second line) and MitoTracker Red (a mitochondrial marker, red, third line) was performed as described in Experimental procedures. Nuclei were stained with 4′,6-diamido-2-phenylindole dihydrochloride (DAPI, blue, first line). Merged images are shown in the fourth line to illustrate colocalization of cytochrome *c* and mitochondria. (a–d) control group, (e–h) DETA/NO group, (i–l) DETA/NO + LY379268-treated astrocytes. Note punctuate staining pattern for cytochrome *c* and colocalization of cytochrome *c* and MitoTracker Red in control astrocytes, whereas in DETA/NO-treated cells this pattern becomes diffuse and a reduction in the proportion of cells showing colocalization is observed. The degree of colocalization is higher in DETA/NO + LY379268-treated cells. (m–n) negative controls of the immunofluorescence, carried out in absence of anti-cytochrome *c* antibody.

pathway by which LPS/IFN-γ causes astrocyte death. Moreover, as our study found no changes in caspase 3 activity in astrocytes during NO exposure and a wide spectrum inhibitor of caspases had no effect on NO-reduced cell viability, we suggest that NO does not provoke a classic apoptotic death, but instead an *apoptotic-like* death, as suggested by Yung *et al.* (2004) and Kawasaki *et al.* (2007). Therefore, it is possible that cell death induced by LPS/IFN-γ in astrocytes may proceed by two alternative pathways: one dependent on caspase 3 and the other mediated by NO and independent of caspase activation. Several cell death pathways independent of caspase activation have been described (Assuncao Guimaraes and Linden 2004), even in some forms of cell death induced by Bax (Jurgensmeier *et al.* 1998) as well as in cell death involving the activation of other proteases, such as calpain (Squier *et al.* 1994) and serine proteases (Assuncao Guimaraes and Linden 2004). However, discrepancies remain concerning the involvement of caspases in NO-induced astroglial death (Takuma *et al.* 1999; Suk *et al.* 2001; Yung *et al.* 2004; Kawasaki *et al.* 2007). These discrepancies may be explained either by the conversion of NO into several derivatives, which can mediate different responses depending on cell conditions (Chung *et al.* 2001), or by the ability of NO to inhibit caspase activity through nitrosylation and/or oxidation of its cysteine residues (for review, see Guix *et al.* 2005).

To elucidate the mechanisms involved in the protective effect of mGluR3 activation, we studied different mediators of the mitochondrial death pathway. NO was shown to induce astrocyte death through p53- and Bax-dependent pathways (Yung *et al.* 2004). However, we found no changes in monomeric Bax levels but a significant decrease in Bcl-2 protein levels after NO exposure, thus shifting the balance between pro- and antiapoptotic factors toward astrocyte

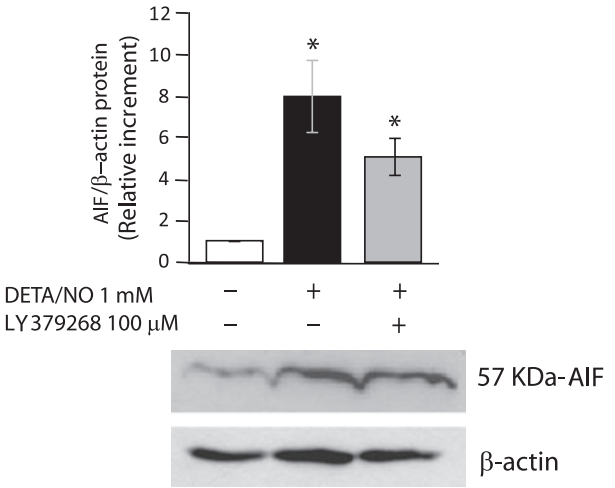

Fig. 11 DETA/NO induces apoptogenic AIF expression and LY379268 reduces this effect. Cultured astrocytes were treated with DETA/NO (1 mM) in presence or absence of LY379268 (100 μM) for 48 h. Total cellular proteins were extracted and 30 μg of protein extracts were used to perform western blots for AIF. Values of OD from AIF bands were normalized to β-actin and data expressed relative to the control group. Bars represent the mean ± SEM of two independent experiments; $^*p < 0.05$ versus control, one sample *t*-test.

death. This balance could be restored partly by LY379268, which raised NO-diminished Bcl-2 protein levels. A striking characteristic of several Bcl-2 family members is their propensity to form homo- and heterodimers (Gross *et al.* 1998). In contrast to oligomeric Bax, monomeric Bax can neither form channels in liposomes nor trigger cytochrome *c* release from isolated mitochondria (Antonsson *et al.* 2000). The proapoptotic functions of Bax at mitochondria are suppressed by its interaction with antiapoptotic Bcl-2 family members (Reed 2006). We demonstrated that LY379268 reduces NO-induced oligomerization of Bax and raises levels of protective heterodimers Bax/Bcl-2 which are decreased by NO. Therefore, our hypothesis is that, in addition to modulating levels of Bcl-2, pharmacological activation of mGluR3 modifies the conformational activation of Bax induced by NO exposure by modulating its homo- and hetero-oligomerization. Besides its transcriptional activity, p53 can also regulate the mitochondrial death pathway by modifying interaction between Bcl-2 family members (Bradley Jacobs *et al.* 2006). Thus, our results suggest that active, phosphorilated-p53 induced by NO may in turn decrease Bax oligomerization via a transcription-independent mechanism and that mGluR3 activation could prevent this effect by reducing p53 phosphorilation. Additional support for the involvement of interactions between Bcl-2 family members in NO-induced astroglial death emerged from the demonstration that LY379268 prevents the DETA/NO-increased cytochrome *c* release from mitochondria to the cytosol. These results indicate that the mGluR3 agonist might inhibit the detrimental effect of NO on permeability of the mitochondrial membrane mediated by active Bax complexes. This is consistent with the observation that LY379268 also reduced protein levels of mitochondrial-released AIF induced by NO exposure, further supporting that mGluR3 activation contributes to preserve mitochondrial membrane integrity. In pathological conditions, AIF undergoes a cleavage enabling release of 57 kDa AIF from the permeabilized mitochondria and its translocation into the nucleus (Modjtahedi *et al.* 2006), where it induces large scale DNA fragmentation (~50 kbp) and chromatin condensation, acting together with endonuclease G (Cande *et al.* 2002). Retinal endothelial cell apoptosis induced by a NO donor was recently shown to be independent of caspase activation and to correlate with AIF translocation to the nucleus (Leal *et al.* 2009). Hence, AIF might be responsible for the apoptotic features observed in our experimental system after NO exposure.

In summary, these results expand our understanding of mGluR3 mechanisms of neuroprotection and demonstrate that mGluR3 activation prevents astrocyte death induced by NO exposure. The current data suggest that development of selective mGluR3 ligands may provide a novel strategy for preventing brain inflammatory processes involving dysregulated NO production.

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