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Title page

Molecular signalling mediating the protective effect of A_1 adenosine and mGlu3 metabotropic glutamate receptor activation against apoptosis by oxygen/glucose deprivation in cultured astrocytes

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Running title page

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ASK1, apoptosis-signal-regulating kinase 1; CCPA, N^6-chlorocyclopentyl-ADO; DAPI, 4'-6-diamidino-2-phenylindole; DMEM, Dulbecco Modified Eagle's Medium; DMSO, dimethylsulfoxide; DPCPX, 1,3-dipropyl-8-cyclopentyl xanthine; ELISA, enzyme-linked immunoadsorbent assay; ERK1/2, extracellular signal-regulated kinases 1 and 2; FCS, fetal calf serum; HPR, horse radish peroxidase; JNK, c-Jun N-terminal kinase; LY294002, 2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride; LY341495, (2S,1'S,2'S)-2(9-xanthylmethyl)-2-(2'-carboxycyclo-propyl) glycine; LY379268, (-)2-oxa-4-aminocyclo-[3.1.0] hexane-4,6-dicarboxylic acid; MAPK, mitogen-activated protein kinase; mGluR(s), metabotropic glutamate receptor(s); MTS, 3-(4,5–dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; PBS, phosphate buffered saline; PI3K, phosphatidylinositol-3 kinase; OGD, oxygen glucose deprivation; PTX, pertussis toxin; PKB, protein kinase B; SDS, sodium dodecyl sulphate; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)-butadiene.

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Abstract

Astrocyte death may occur in neurodegenerative disorders, and complicates the outcome of brain ischemia, a condition associated with high extracellular levels of adenosine and glutamate. We show that pharmacological activation of A_1 adenosine and mGlu3 metabotropic glutamate receptors with N^6-chlorocyclopentyladenosine (CCPA) and (-)2-oxa-4-aminocyclo-[3.1.0]hexane-4,6-dicarboxylic acid (LY379268), respectively, protects cultured astrocytes against apoptosis induced by a 3-hour exposure to oxygen/glucose deprivation (OGD). Protection by CCPA and LY379268 was less than additive and was abrogated by receptor blockade with selective competitive antagonists or pertussis toxin. Both in control astrocytes and in astrocytes exposed to OGD, CCPA and LY379268 induced a rapid activation of the phosphatidylinositol-3-kinase (PI3K) and extracellular signal-regulated kinases 1 and 2 (ERK1/2)/mitogen-activated protein kinase (MAPK) pathways, which are known to support cell survival. In cultures exposed to OGD, CCPA and LY379268 reduced the activation of c-Jun N-terminal kinase (JNK) and p38/MAPK, reduced the levels of the pro-apoptotic protein Bad, increased the levels of the anti-apoptotic protein Bcl-X_L, and were highly protective against apoptotic death, as shown by nuclear DAPI staining and measurements of caspase 3 activity. All these effects were attenuated by treatment with U0126 and LY294002, which inhibit the MAPK and the PI3K pathways, respectively. These data suggest that pharmacological activation of A_1 and mGlu3 receptors protects astrocytes against hypoxic/ischemic damage by stimulating the PI3K and ERK1/2 MAPK pathways.

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INTRODUCTION

Astrocytes, the most abundant glial cell types in the brain, provide metabolic and trophic support to neurons by several mechanisms that include the clearance of ions and environmental toxins, the supply of energy substrates, and the production of trophic factors, and modulate synaptic activity (Volterra and Meldolesi, 2005). Impairments in these functions critically affect neuronal survival. Recent studies have shown that ischemic and inflammatory insults induce astrocyte apoptotic death and this contributes to the pathophysiology of acute and chronic neurodegenerative disorders (for a review see Takuma et al., 2004). Apoptotic astrocytes are found in Alzheimer's disease (Kobayashi et al., 2002), ischemic demyelinating lesions in vascular dementia (Tomimoto et al., 1997) and in the grey matter of fronto-temporal dementia (Martin et al., 2000). Moreover, astrocyte damage precedes neuronal death in the spinal cord of mice carrying mutations of type-1 SOD that are typically found in amyotrophic lateral sclerosis (Bruijn et al., 1997). Hence, membrane receptors that support glial cell survival may be targeted by drugs of potential use in the treatment of neurodegenerative disorders. We have focussed on some adenosine and glutamate receptors, which are known to be expressed by astrocytes and regulate several functions of glial cells, including the production of trophic factors (Bruno et al., 1998; Ciccarelli et al., 1999). Activation of A_1 adenosine receptors produces neuroprotective effects (Ribeiro et al., 2002) and protects human vascular endothelial cells against apoptosis induced by low concentrations of ethanol (Liu et al., 2002); however, whether these receptors regulate processes of death/survival in glial cells survival is unknown. In contrast, deleterious effects are induced by the activation of other adenosine receptor subtypes, and induction of astrocyte apoptosis by adenosine is mediated by A_3 receptors (Appel et al., 2001; Di Iorio et al., 2002).

Glutamate activates both ionotropic (AMPA, NMDA and kainate) and metabotropic (mGlu1 to -8) receptors. Astrocytes mainly express AMPA/kainate, mGlu3 and mGlu5 receptors, although other subtypes are occasionally found. Activation of glial mGlu3 receptors protects neighbour neurons in cultured cortical cells through a paracrine mechanism mediated by the production of transforming

growth-factor β (Bruno et al., 1998), and the presence of astrocytes is required for the neuroprotective effects of mGlu2/3 receptor agonists (D'Onofrio et al., 2001). The role of mGlu3 receptors in the regulation of glial cell survival is also unknown. It is noteworthy that A_1 and mGlu2/3 receptors are both linked to a Gi protein, and functionally interact in modulating glutamate release from nerve endings (Di Iorio et al., 1996).

The study of these two receptors is now facilitated by the availability of potent and selective ligands. A_1 receptors are activated by N^6-chlorocyclopentyl-ADO (CCPA) and antagonised by 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) (Jacobson and Gao, 2006), whereas mGlu3 receptors are activated by (-)2-oxa-4-aminocyclo-[3.1.0] hexane-4,6-dicarboxylic acid (LY379268) and antagonised by (2S,1'S,2'S)-2(9-xanthylmethyl)-2-(2'-carboxycyclo propyl)glycine (LY341495) (reviewed by Schoepp et al., 1999). Using these drugs, we now report that activation of both receptors protects cultured astrocytes against apoptotic death and that this effect is mediated by the activation of the phosphatidylinositol-3 kinase (PI3K) and extracellular signal-regulated kinase (ERK)1/2/mitogen-activated protein kinase (MAPK) pathways.

MATERIALS AND METHODS

Materials

Poly-D-lysine, L-leucine methyl ester and pertussis toxin (PTX) were supplied from Sigma (Sigma-Aldrich, Milan, Italy) whereas 2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride (LY294002), LY341495 and DPCPX were from Tocris (Bristol, UK). 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)-butadiene (U0126) was purchased from Calbiochem (San Diego, CA, USA), CCPA was from Research Biochemical Incorporated (Sigma-Aldrich). LY379268 was kindly provided by Eli Lilly (Indianapolis, IN, USA). Disposable materials for tissue cultures were supplied from NUNC (Mascia Brunelli, Milan, Italy). Culture medium, antibiotics and serum were from GibcoBRL (Life Technologies, Milan, Italy). All other chemicals were of analytical grade or the best commercially available.

Cell culture and treatments

Primary cultures of rat astrocytes were prepared from neonatal rats two to four days after birth as previously described (Di Iorio et al., 2004). Cerebral cortices were collected in growth medium (high glucose Dulbecco modified Eagle's Medium - DMEM - supplemented with 10% fetal calf serum – FCS - and 1% penicillin/streptomycin –10 000 units/ml penicillin G sodium and 10 000 µg/ml streptomycin sulphate in 0.85 % saline). Subsequently, the tissue was washed in phosphate buffered saline (PBS), cut in small fragments and digested with 0.025% trypsin/0.04% EDTA solution in PBS for 20 min at 37°C. The cells were then dissociated in 0.01% DNase solution in growth medium for 10 min at 37°C and centrifuged at 1000 rpm for 10 min. The pellet was re-suspended in growth medium, containing 5 mM L-leucine methyl ester to constrain microglia contamination. Cells, seeded on poly-D-lysine-coated T75 flasks, were grown in this medium for the first 24 h and were then maintained in an identical medium without leucine methyl ester. The medium was replaced every 3-4 days. At the 7th and then at the 13th day in vitro cells were shaken for 3 h at 80 rpm on a plate shaker to minimise attachment and hence microglial contamination of

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the cultures. Astrocytes were detached from the culture flasks by treatment (5-10 min at 37°C) with 0.025% trypsin/0.04% EDTA. Astrocytes were re-plated onto poly-D-lysine coated 100 mm dishes at a concentration of $2x10^6$ cells/dish for Western blot analyses or they were plated onto poly-D-lysine-coated round glass coverslips (Ø = 13 mm) at concentration of $3x10^4$ cell/coverslip and 96 multiwell plate at concentration of $1x10^4$ cells/well for the apoptosis/viability experiments. In experiments where enzyme inhibitors or receptor antagonists were tested, astrocytes were pre-treated with various agents for 30 minutes prior to the addition of CCPA or LY379268, except the ADP-ribosylating factor of the inhibitory guanosine nucleotide binding protein (Gi), PTX, which was added overnight (16 h). These treatments included the following: selective inhibitors of PI3K, LY294002, and MAPK kinase, U0126; the selective antagonists of the A_1 adenosine receptor, DPCPX, and of the mGlu2/3 receptor, LY341495.

Oxygen glucose deprivation (OGD) protocol

Apoptosis was induced in cultured astrocytes by exposing cells to a combined deprivation of oxygen and glucose (OGD). Cells, 24 h after replating, were serum starved for further 24 h. Then a glucose-free bicarbonate-buffered DMEM (Sigma-Aldrich), was added to the cultures, after a gentle cell washing with the same buffer. This medium was previously bubbled with 95% N_2/5% CO_2 at 3 l/min for 5 min and pre-warmed at 37°C. Hypoxia was induced by placing cells in a humidified, sealed chamber (Billups-Rothenberg, Del Mar, CA, USA) at 37 °C, which was flushed with 95% N_2/5% CO_2 for 5 min. In this condition all but 0.3% oxygen tension could be removed, as indicated by a PO_2 meter (Beckman oxygen analyser OM-11, Beckman Instruments, Milan, Italy). At the end of the OGD period (3 h), cultures were returned to standard condition for the indicated periods. In each experiment, cultures exposed to OGD were always compared with normoxic controls, supplied with DMEM containing glucose and maintained in standard incubation condition.

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Evaluation of apoptosis

DNA fragmentation was evaluated histochemically, by 4'-6-diamidino-2-phenylindole (DAPI) staining (Roche Molecular Biochemicals, Mannheim, Germany) and fluorescence microscopy. Cells were seeded onto poly-D-lysine-coated glass coverslips and part of them were exposed to OGD, after 24-h serum-starvation, in the presence or absence of CCPA or LY379268, for 3 hours in a glucose-free DMEM without FCS. Cells were then maintained in normal DMEM without serum, like control cells (not subjected to OGD) and subsequently astrocytes were fixed with 3.7% paraformaldehyde in PBS for 25 minutes at room temperature and then incubated with 70% ethanol for 15 minutes at room temperature. DAPI Antifade ES solution (0,125 µg/ml) was added for 5 minutes at room temperature to fixed astrocytes. Observations were carried out using a fluorescence microscope (Leica DMRXA2) (excitation 358 nm; emission at 461 nm). The number of apoptic (i.e. showing fragmented nuclei with condensed chromatin) and viable astrocytes was counted in five fixed fields/coverslip of up to five separate cultures. The percentage of apoptotic cells was calculated as follows: percent of apoptotic cells = (total number of cells with apoptotic nuclei/total number of counted cells) x 100.

MTS assay

The number of viable cells was determined using the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. Briefly, cell cultures ($1x10^4$ cells/well) were added with 20 µl of CellTiter 96® AQ_{ueous} One Solution Reagent containing a tetrazolium compound, 3- (4,5–dimethylthiazol-2-yl) -5- (3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), and an electron-coupling reagent (phenazine ethosulfate; PES). The plate was incubated at 37°C for 2 h in a humidified atmosphere. The reduction of MTS in the presence of cellular dehydrogenases yielded formazan crystals at the bottom of the plate. The absorbance was measured at 490 nm using a microtiter plate reader (Spectracount™, Packard Canberra, Meriden, CT, USA).

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Western blot analysis

Western blot analysis was used to detect phosphorylated extracellular signal-regulated kinases 1 and 2 (ERK1/2), p38 MAPK, c-Jun N-terminal kinase (JNK), Akt/protein kinase B (PKB), apoptosis signal-regulating kinase 1 (ASK1) and Bad protein as well as pro-caspase 3, Bad and Bcl-X_L protein content. Cultured astrocytes were serum-deprived for 24 h prior to pharmacological treatments (as reported in the figures). At the end of drug incubation, astrocytes were washed twice with ice cold PBS and then harvested at 4°C in a lysis buffer (25 mM Tris buffer, pH 7.4, containing 150 mM NaCl, 100 µM sodium orthovanadate, 1.5 mM $MgCl_2$, 1.0 mM EDTA, 1.0 mM EGTA, 1% Nonidet P40, 10% glycerol, 1 mM phenylmethylsulphonylfluoride, 5 µg/ml leupeptin, 5 µg/ml aprotinin). Cells were disrupted by sonication and then were centrifuged at 14,000 rpm for 5 min at 4°C. Aliquots (20 µl) were removed from the supernatants for the determination of protein concentration by the Bio-Rad method. Samples were diluted in sodium dodecyl sulphate (SDS)-bromophenol blue buffer and boiled for 5 min. Cell lysates were separated on 12% SDS-polyacrylamide gels and electrophoretically transferred to a polyvinylidene fluoride membrane (Bio-Rad Laboratories, Milan, Italy).

Membranes were incubated overnight at 4 °C with specific primary antibodies (polyclonal rabbit phospho-ERK1/2, phospho-Akt, phospho-p38, phospho-JNK, phospho-ASK1 (Ser83), and phospho-Bad (Ser112 or Ser136) (from Cell Signalling, New England Biolabs, Celbio, Pero, Italy) diluted 1:1000, or with polyclonal rabbit anti-caspase-3, anti-Bad or anti-Bcl-X_L (final dilution 1:200, Santa Cruz Biotechnogies, Santa Cruz, CA, USA). Membranes were then exposed to a secondary antibody for 1 h at room temperature (donkey anti-rabbit HPR-conjugated, Amersham Pharmacia Biotech, Milan, Italy). To confirm that equal amounts of protein were loaded in each lane, the membranes were incubated in stripping buffer (62.5 mM Tris-HCl, pH 6.7, 2% SDS and 100mM β-mercaptoethanol) at 50°C for 30 min to remove the primary/secondary antibody complex. The blots were then reprobed with non phosphorylated form of the antibodies mentioned above (dilution 1:1000, from Cell Signalling) or with goat poyclonal anti-β actin (dilution 1:100,

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from Santa Cruz Biotechnologies, incubation: 1h at room temperature). Membranes were then exposed to a secondary antibody for 1 h at room temperature (donkey anti-rabbit horse radish peroxidase (HPR)-conjugated from Amersham Pharmacia Biotech, or donkey anti-goat HPR-conjugated from Santa Cruz Biotechnologies, respectively, both diluted 1:2500), according to the manufacturer's instructions. Immunocomplexes were visualised using the enhancing chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech). Densitometric analysis was performed for the quantification of the immunoblots, using the Molecular Analyst System (Bio-Rad Laboratories) program.

Assay of caspase-3 activity

The activity of caspase-3 was determined using the Apo-ONE^{TM} Homogeneus Caspase 3/7 Assay according to manufacturer's instruction (Promega). Cell cultures ($1x10^4$ cell/well) were added with Homogeneous Caspase Reagent containing the fluorescent caspase substrate z-DEVD-R110 (rhodamine 110,bis (N-CBZ-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide) and were incubated for 4 hours. After shaking at 300 rpm for 30 seconds, the plate was incubated at room temperature for 30 min to 18 h. The fluorescence at different time points was measured at an excitation wavelength of 485±20 nm and an emission wavelength of 530±25 nm, using a microtiter plate reader (Fluorocount^{TM}, Packard Canberra). The values were expressed as the relative fluorescence units (RFLU) measured at each time point.

Measurements of extracellular adenosine and glutamate

To evaluate the endogenous adenosine and glutamate released from astrocytes, culture medium was replaced with Krebs solution containing (mM): 118.5 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 10 glucose and 25 $NaHCO_3$, equilibrated with 95% air/5% CO_2 at 37 °C (pH adjusted at 7.3-7.4). The cultures were maintained in Krebs solution for 60 min, in order to evaluate the purine and glutamate release in a steady-state condition, and then, this medium was renewed for further 60

min. Subsequently, some cultures were gently washed with glucose free bicarbonate-buffered Krebs, placed in the apparatus above described and incubated for 1 h under hypoxic-hypoglycemic conditions. The extracellular adenosine and glutamate levels in medium samples from cells under basal conditions and 1 hour after OGD exposure were measured by HPLC as described previously (Di Iorio et al., 1996). Adenine purine separation was carried out with a reverse-phase analytical column (LiChrospher 100 RP-18 5 µm, Merck). Elution was performed by applying a linear gradient from 100% solvent A (60 mM KH_2PO_4 and 5 mM tetrabutylammonium phosphate, pH 6.0) to 100% solvent B (30% methanol *plus* 70% solvent A) in 15 min at a flow rate of 1.5 ml/min. Adenosine was revealed by absorbance at 254 nm. To evaluate extracellular glutamate levels, samples were subjected to precolumn derivatisation with *o*-phthaldialdehyde/2-mercaptoethanol reagent. Glutamate was separated on a Waters Pico/TAG column with a linear gradient from 100% 100 mM potassium acetate (pH 7.1)/methanol (80:20) (solvent A) to 100% methanol/100 mM potassium acetate, pH 7.1 (80:20) (solvent B) and detected fluorimetrically.

Statistical analysis

All data are presented as the means±S.E.M. for a series of *n* experiments. Statistical analyses were performed by Prism3 software (GraphPad, San Diego, CA, USA), using unpaired Student's *t*-test or one-way analysis of variance (ANOVA) followed by Dunnett's *post hoc* comparison test. Group differences with $P<0.05$ were considered statistically significant. Dose- or time-response curves were calculated by using nonlinear regression (Prism3 software).

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RESULTS

Apoptosis induced by oxygen-glucose deprivation (OGD) in cultured astrocytes

Cultured astrocytes that had been maintained in serum-free medium for 24 hours were exposed to OGD for 3 hours. This treatment caused apoptotic cell death, which was detectable after 3-6 hours by combining microscopic analysis after DAPI nuclear staining (Fig. 1A), the MTS assay (Fig. 1B), and measurements of caspase 3 activity (Fig. 1C) and pro-caspase 3 levels by immunoblotting (Fig. 1D). The extent of apoptotic death reached a plateau between 12 and 24 hours following OGD exposure, when >50% of cell nuclei beared chromatin condensation or picnosis, MTS activity decreased by >50%, caspase 3 activity increased by about 3 fold, and pro-caspase 3 levels decreased by >80% (Fig. 1). OGD induced a sustained activation of the JNK and p38/MAPK pathways, which peaked between 30 min and 2 hours, and could still be detected after 3 hours (Fig. 2A and B). In contrast, OGD had no effect on the ERK1/2 MAPK pathway, and caused only a small and delayed stimulation of the PI3K pathway, as assessed by immunoblot analysis of phosphorylated-Erk1/2 and phosphorylated-Akt, respectively (Fig. 2 C-D). Stimulation of PI3K was associated to a transient increase in the levels of phosphorylated-(Ser83)/inactivated ASK1, which is a MAP kinase kinase kinase upstream to JNK and p38 MAPK in the death cascade (Sumbayev and Yasinska, 2005), and is under the inhibitory control of the PI3K pathway (Kim et al., 2001a). Finally, OGD altered the balance between pro- and anti-apoptotic members of the Bcl-2 family, causing a remarkable increase in the Bcl-2-associated death protein, BAD, without affecting the levels of the anti-apoptotic factor, Bcl-X_L (Fig. 2F).

Extracellular adenosine levels were 23 $\pm$ 6 nM under basal conditions and 62 $\pm$ 5 nM after 1 hour exposure to OGD. Glutamate levels were 0.35 $\pm$ 0.07 µM under basal conditions and 1.5 $\pm$ 0.22 µM after 1-hour exposure to OGD (means $\pm$ S.E.M., n = 4).

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Pharmacological activation of A_1 or mGlu3 receptors protects astrocytes against apoptosis by oxygen/glucose deprivation

The A_1 adenosine receptor agonist, CCPA (2.5-75 nM) and/or the mGlu2/3 receptor agonist, LY379268 (0.25-7.5 μM), were added to the cultures one hour prior to OGD exposure and maintained in the medium throughout OGD exposure (i.e. for the following 3 h). Both drugs reduced the percentage of apoptotic cells in a concentration-dependent manner (Figure 3A), with maximal anti-apoptotic effect at 30 nM CCPA and 5 μM LY379268. The calculated EC_{50} values were 15.4 ± 2.3 nM for CCPA and 2.23 ± 0.7 μM for LY379268. Similar results were obtained using the MTS assay (data not shown). Maximal concentrations of CCPA or LY379268 reduced the increase in caspase 3 activity by about 50%, as assessed 12 hours after OGD exposure (caspase 3 activity expressed as $RFLUx10^3$ = 70 ± 8.1 in untreated cultures, 35 ± 4.7 in cultures treated with 30 nM CCPA, and 33 ± 5.1 in cultures treated with 5 μM LY379268, respectively). The antiapoptotic effect of CCPA was largely attenuated by the A_1 receptor antagonist, DPCPX (100 nM), as well as by a pre-treatment with PTX (200 ng/ml). The antiapoptotic effect of LY379268 was instead sensitive to the preferential mGlu2/3 receptor antagonist, LY341495 (1 μM), as well as to PTX (Figure 3 B-C). We also combined CCPA and LY379268 at concentrations close to the IC_{25} or the IC_{50} values in cultures exposed to OGD. Protection was lower than that expected if the antiapoptotic effects of the two drugs were additive (Fig. 3D). This suggests that the activation of A_1 and mGlu2/3 receptors converges into a common, Gi-mediated, intracellular pathway, which is ultimately responsible for the antiapoptotic effect in cultured astrocytes challenged with OGD.

Signalling pathways and downstream effector molecules mediating the antiapoptotic effect of A_1 and mGlu3 receptors in cultured astrocytes

1) Effect of A_1 and mGlu3 receptor activation in control cultures

In control cultures, addition of CCPA (5-60 nM) induced a concentration-dependent activation of the PI3K pathway, which showed a rapid and long-lasting kinetics. The increase in Akt

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phosphorylation was detectable at 5 min, and reached a long plateau between 30 min and 4 hours (Fig. 4A-B). Stimulation was abrogated by DPCPX, PTX, as well as by the PI3K inhibitor, LY294002 (Fig. 4C). Stimulation of the PI3K pathway was associated with a parallel increase in ASK1 phosphorylation, which also peaked after 30 min (Fig. 4D). CCPA also stimulated ERK1/2 phosphorylation with a slightly different kinetics. Stimulation peaked after 5 min, remained stable up to 4 hours (Fig. 5A-B), and was sensitive to DPCPX, PTX, and to the MEK inhibitor, U0126 (Fig. 5C). Interestingly, stimulation of the ERK1/2 pathway by CCPA was also reduced by LY294002 (Fig. 5C), suggesting that the PI3K pathway precedes the MAPK pathway in the cascade of reactions triggered by A_1 receptors in astrocytes. Similar results were obtained with the mGlu2/3 receptor agonist, LY379268, which, however, stimulated both pathways with a different kinetics, as compared with CCPA. Phosphorylation of Akt/PI3K and ERK1/2 MAPK peaked after 60 min of exposure to LY379268, whereas phosphorylation of ASK1 increased linearly from 30 min to 3 h. The action of LY379268 was sensitive to the mGlu2/3 receptor antagonist, LY341495 (Fig. 4-5). Neither CCPA nor LY379268 had any effect on the JNK and the p38 MAPK pathways in control cultures (not shown). However, both drugs increased the levels of the phosphorylated/inactivated forms of the pro-apoptotic protein, Bad (Fig. 6A-B), at serine residues 136 and 112, which are phosphorylated by PI3K and ERK1/2, respectively (Datta et al., 1997; Scheid et al., 1999). As expected, phosphorylation at serine 136 was prevented by the PI3K inhibitor, LY294002, whereas phosphorylation at serine 122 was prevented by the MEK inhibitor, U0126 (Fig. 6A-B). CCPA and LY379268 also increased the levels of the anti-apoptotic protein, Bcl-X_L, as assessed by western blotting. This effect was abolished by LY294402 and attenuated by U0126 (Fig. 6C).

2. Effect of A_1 or mGlu3 receptor activation in cultures exposed to OGD

In cultures challenged with OGD, the two receptor agonists, CCPA and LY379268, retained the ability to stimulate the MAPK and PI3K pathways and attenuated the pathological activation of the JNK and p38 MAPK pathways (Fig. 7A-D). The two drugs could still activate Bad phosphorylation

(Fig. 8A-B), thus reducing the increase in Bad levels caused by OGD (Fig. 8C). CCPA and LY379268 could still enhance Bcl-X_L levels in cultures exposed to OGD (Fig. 8D). Both effects were largely attenuated by the PI3K inhibitor, LY294002, and the MEK inhibitor, U0126 (Fig. 8C-D). Finally, U0126 and LY294002 prevented the antiapoptotic effect of CCPA and LY379268 in cultures exposed to OGD, as assessed by DAPI staining and caspase 3 activity (Fig. 9A-B).

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DISCUSSION

Apoptosis is a phenotype of death common to virtually all cell types during development, senescence, and a variety of pathological conditions. In the CNS, most studies have focused on mechanisms regulating apoptotic death in neurons as potential targets for protective agents in neurodegenerative disorders. We have switched the attention on astrocytes because astrocyte death also occurs in neurodegenerative disorders and may have a profound impact on synaptic transmission and neuronal viability (Takuma et al., 2004). We challenged cultured astrocytes by OGD in an attempt to mimic cell damage occurring under hypoxic/ischemic conditions (Giffard and Swanson, 2005). A 3-hour exposure to OGD induced about 50% of cell death under our conditions. This extent of death allowed a reliable assessment of the underlying mechanisms and, at the same time, was considered optimal for the identification of protective strategies, which could have been obscured by a more severe insult.

OGD induced a number of processes that are causally related to apoptotic death, such as the activation of the p38 MAPK and JNK pathways, and an increase in the levels of Bad (Sumbayev and Yasinska, 2005; Chen et al., 2005). The increase in Bad levels was not apparently related to a reduction of protein phosphorylation. We rather observed a transient increase in Bad phosphorylation in cultures exposed to OGD, which may represent a compensatory mechanism aimed at avoiding excessive increases in Bad levels.

Our major finding was that pharmacological activation of A_1 or group-II mGlu receptors was highly protective against astrocyte death. Both types of receptors have an established protective function against neuronal damage in a variety of *in vitro* and *in vivo* models of neurodegenerative disorders (reviewed by Ribeiro et al., 2002; Bruno et al., 2001; Chong et al., 2003). At least a component of neuroprotection is mediated by A_1 and mGlu3 receptors present in astrocytes, which control the production of neurotrophic factors, such as transforming growth factor-β and nerve growth factor (Bruno et al., 1998; Ciccarelli et al., 1999; D'Onofrio et al., 2001). Thus,

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pharmacological activation of A_1 and mGlu3 receptors may provide a dual strategy of protection limiting the death of neurons and astrocytes at the same time.

CCPA and LY379268 have nanomolar affinity for A_1 and mGlu3 receptors (reviewed by Schoepp et al., 1999; Jacobson and Gao, 2006), respectively, but their intrinsic efficacy is not greater than that of adenosine and glutamate This suggests that A_1 and mGlu3 receptors present in astrocytes were not saturated by the relatively high amounts of extracellular adenosine and glutamate found during OGD. Whether glial receptors are also responsive to pharmacological agonists *in vivo* is unknown, and one should take into account that neurons release high amounts of purines and glutamate under hypoxic/ischemic conditions (Franke et al., 2006; Franceschini et al., 2006). However, it is noteworthy that at least glial mGlu3 receptors are not present on the surface of astrocytes facing the synaptic cleft (Shigemoto et al., 1996), and might not be accessible to synaptic glutamate.

A combined treatment with CCPA and LY379268, at the respective EC_{25} or EC_{50} values, was protective to a lower extent than that predicted if the two drugs were additive. Co-localisation and interactions between A_1 receptors and mGlu or other neurotransmitter receptors have been described (Torvinen et al., 2002; Ciruela et al., 2001). Because group-II mGlu receptor agonists to do not influence [^{3}H]CCPA binding to A_1 receptors on astrocyte membranes (Authors' unpublished observation), it is possible that group-II mGlu and A_1 receptors converge in activating mechanisms that are relevant to cytoprotection. They might recruit the same pool of Gi proteins, or converge in the activation of the MAPK or the PI3K pathways (see below). Thus, the combination between A_1 and mGlu2/3 receptor agonists may not be particularly helpful in *in vivo* models.

Searching for intracellular mechanisms that mediate the antiapoptotic effect of A_1 and mGlu3 receptors in cultured astrocytes we focused on the PI3K/Akt and the ERK/MAPK pathways Akt/PKB is a multifunctional mediator of PI3K-dependent signalling that promotes cell survival and exerts in models of neuronal or astrocyte death (Dudek et al., 1997; Di Iorio et al., 2004). ERK1/2 activation can also produce neuroprotection (Xia et al., 1995). Both intracellular pathways

are activated by A_1 receptor stimulation in non-neuronal (Germack and Dickenson, 2000) and neuronal cells (Angulo et al., 2003), as well as in brain tissue (Gervitz et al., 2002). Activation of mGlu2/3 receptors can also stimulate Akt/PKB and ERK1/2 in astrocytes and in brain tissue (D'Onofrio et al., 2001). The following observations demonstrate that A_1 and mGlu3 receptors protect cultured astrocytes through the activation of PI3K/Akt and ERK/MAPK pathways: (i) CPPA and LY379268 activated both pathways in a dose- and time-dependent manner; (ii) activation persisted in cultures exposed to OGD; and (iii) selective inhibitors of the two pathways largely reduced the protective activity of A_1 or mGlu3 receptor agonists in astrocytes challenged with OGD.

Akt or ERK1/2 can phosphorylate several pro-apoptotic proteins leading to suppression of death signals. One of these is ASK1, which triggers an apoptogenic kinase cascade causing the phosphorylation/activation of JNK and p38 MAPK (Sumbayev and Yasinska, 2005). CCPA or LY379268 induced an early and sustained phosphorylation/inhibition of ASK1 at serine 83, a site that is phosphorylated by PI3K/Akt (Kim et al., 2001a). As expected, both drugs partially reduced the activation of the stress-related JNK and p38 MAPK pathways in cultures challenged with OGD. This might be one of the PI3K/Akt-dependent mechanisms whereby activation of A_1 and mGlu3 receptors protect astrocytes against apoptosis. In addition, CCPA and LY379268 changed the balance between the pro-apoptotic protein, Bad, and the anti-apoptotic protein, Bcl-X_L. Bad is the only member of the Bcl-2 family whose expression is up-regulated significantly during the early stages of an ischemic insult in astrocytes (Chen et al., 2005). Bad associates with Bcl-X_L preventing Bcl-X_L from exerting anti-apoptotic effects in ischemic astorcytes (Chen et al., 2005). CCPA or LY379268 increased the expression of Bcl-X_L and reduced the OGD-induced increase in Bad levels by phosphorylating Bad at sites targeted by PI3K/Akt and ERK1/2. Phosphorylated Bad is inactivated and no longer able to counteract Bcl-X_L antiapoptotic activity.

Interestingly, activation of the ERK1/2 MAPK pathway by CCPA or LY379268 was greater in cultures exposed to OGD than in control cultures and this is noteworthy because the ERK1/2

MAPK is considered as one of the major protective pathways in ischemic astrocytes (Chen et al., 2005). Why OGD amplifies the activation of the ERK1/2 MAPK pathway mediated by A_1 or mGlu3 receptors is unknown. Part of this activation was sensitive to LY294002, suggesting that activation of PI3K is upstream to ERK1/2 MAPK. Other mechanisms are likely to be involved. Activation of the PI3K and ERK1/2 pathways may be triggered by the βγ subunits of the Gi protein, as reported for other G protein-coupled receptors (Marinissen and Gutkind, 2001). Inhibition of cAMP formation (i.e. the canonical transduction pathway coupled to A_1 and mGlu3 receptors) may also be involved because cAMP limits membrane localisation of phosphoinositide-dependent kinase-1 (Kim et al., 2001b), which is a direct effector kinase of Akt.

In conclusion, our data suggest that activation of A_1 and mGlu3 receptors produces a strong pro-survival effect in astrocytes degenerating in response to "ischemic-like" conditions. At least in culture, these receptors effectively respond to pharmacological activation in spite of the large amounts or adenosine and glutamate released during OGD. A_1 or mGlu3 receptor agonists cater the potential to exert a variety of beneficial effects during ischemia. These drugs can protect astrocytes and neurons at the same time, and can also stimulate astrocytes to produce neurotrophic/neuroprotective factors (Bruno et al., 1998; Ciccarelli et al., 1999; D'Onofrio et al., 2001). LY379268 is a member of a growing list of brain-permeant and highly potent mGlu2/3 receptor agonists that are under pre-clinical development for the treatment of anxiety, drugs addiction, and other CNS disorders (Kim et al., 2005; Schiefer et al., 2004). These drugs might be highly effective in limiting astrocyte death in neurodegenerative disorders. A systemic use of A_1 receptor agonists has long been precluded owing to the occurrence of peripheral side effects. However, innovative delivery systems are now available that may allow a systemic use of A_1 receptor agonists in neurodegenerative disorders (Dalpiaz et al., 2005).

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. *Evaluation of OGD-induced apoptosis in rat cultured astrocytes.*

Astrocytes were cultured in serum-free medium for 24 hours, and then exposed exposed to OGD for 3 hours. Apoptosis was assessed by DAPI nuclear staining (A), the MTS assay (B), and caspase 3 activity (C). Values are means ± SEM of three independent experiments in duplicate. ***p*<0.01vs. controls (unpaired Student's *t* test). RFLU = relative fluorescence units. D) Immunoblot analysis of pro-caspase 3 in control astrocytes and in astrocytes exposed to OGD. At the end of OGD, cells were maintained in DMEM without serum for further 9 h. Densitometric values are means ± SEM from three independent experiments. ****p*<0.001 vs. controls (unpaired Student's *t* test).

Figure 2. *Molecular pathways activated by OGD in cultured astrocytes*

Cultures were grown in serum-free medium for 24 hours, and then exposed to OGD for different times. Western blot analysis of phosporylated (p-) ERK1/2 (A), p-Akt (B), p-ASK1-ser83 (B), p-JNK (C), p-p38 (**D**), p-Bad-ser112 and -ser136 (F), total Bad, and Bcl-X_L (E) is shown. Densitometric values are means ± SEM, from 3 independent experiments. **p*<0.05, ***p*<0.01, ****p*<0.001 (One-Way ANOVA + Dunnett's test) vs. control astrocytes.

Figure 3. *Protective effect of CCPA and LY379268 against astrocyte apoptosis induced by OGD.*

In (A), different concentrations of CCPA (2.5-75 nM) or LY379268 (0.25-7.5 μM) were applied to the cultures 1 hour prior to OGD exposure. In (B and C) cells were pre-treated with DPCPX or LY341495 30 min prior to the addition of CCPA or LY379268, respectively. Pre-treatment with pertussis toxin *(*PTX) was carried out for 16 hours. In (D), concentrations of CCPA or LY379268 corresponding to their IC_{25} and IC_{50} values were applied alone or in combination 1 hour prior to OGD. All drugs were maintained during the 3 hours of OGD exposure. Apoptosis was assessed 24 hours after the beginning of the experiment by DAPI staining. Results are expressed as percentage

of apoptotic cells and are means ± S.E.M. of 8 values from 4 independent experiments. ***p<0.001 (One-Way ANOVA + Dunnett's test) vs. control astrocytes.

Figure 4. *Pharmacological activation of A_1 or mGlu3 receptors stimulates the PI3K pathway andinduces ASK1 phosphorylation in cultured astrocytes.*

Astrocytes were starved for 24 hours, and then exposed to increasing concentrations of CCPA or LY379268 for 30 minutes (A**)** or to 30 nM CCPA or 5 µM LY379268 for different times (B). In (C)**,** astrocytes were treated with either the PI3K inhibitor, LY294002, or the receptor antagonists, DPCPX or LY341495, 30 min prior to the addition of CCPA or LY379268, whereas pre-treatment with pertussis toxin *(*PTX) was carried out for 16 hours. Levels of phosphorylated Akt/PKB (pAkt) and ASK1 at serine 83 (pASK1-ser83) were determined by Western blot analysis (60 µg of proteins were loaded per lane). Densitometric values are means ± SEM, from 3 independent experiments.. *p<0.05, **p<0.01, ***p<0.001 (One-Way ANOVA *plus* Dunnett's test) vs. control astrocytes.

Figure 5. *Activation of A_1 or mGlu3 receptors stimulates the ERK1/2 MAPK pathway in cultured astrocytes.*

Astrocytes were starved for 24 hours, and then exposed to increasing concentrations of CCPA or LY379268 for 30 minutes (A) or to 30 nM CCPA or 5 µM LY379268 for different times (B). In (C**),** astrocytes were treated with either the MEK inhibitor, U0126, or the receptor antagonists, DPCPX or LY341495, 30 min prior to the addition of CCPA or LY379268, whereas pre-treatment with pertussis toxin *(*PTX) was carried out for 16 hours. Levels of phosphorylated ERK1/2 (pERK1/2) and total ERK1/2 were determined by Western blot analysis (10 µg of proteins were loaded per lane). Densitometric values are means ± SEM, from 3 independent experiments. *p<0.05, **p<0.01, ***p<0.001 (One-Way ANOVA *plus* Dunnett's test) vs. control astrocytes.

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Figure 6. *Pharmacological activation of A_1 or mGlu3 receptors promotes Bad phosphorylation and increases the cytosolic content of the anti-apoptotic protein Bcl-X_L.*

Astrocytes were starved for 24 hours, and then treated with 30 nM CCPA or 5 μM LY379268 for 1 (A, B) or 4 (C) hours. At the end of the treatment, cells were switched into fresh serum-free medium for additional 2 hours. In some experiments, astrocytes were treated with either LY294002 or U0126) 30 min prior to the addition of CCPA or LY379268.. Levels of phosphorylated Bad and levels of Bcl-X_L were examined by Western blotting (50 μg of proteins were loaded per lane). Values are means ± SEM from 3 independent experiments. **p<0.01, ***p<0.001(One-Way ANOVA + Dunnett's test) vs. control astrocytes.

Figure 7. *Pharmacological activation of A_1 or mGlu3 receptors stimulates the PI3K and ERK1/2 MAPK pathways, and reduces the stimulation of the p38MAPK and the JNK pathways in cultured astrocytes exposed to OGD.*

Astrocytes were starved for 24 hours, and then pre-treated with 30 nM CCPA or 5 μM LY379268 s 1 h prior to exposure to OGD, in the absence or presence of LY294002 (30 μM) or U0126 (10 μM) (both added 30 min prior to receptor agonists). Levels of p-ERK1/2 (A), p-Akt (B), p-JNK (C) and p-p38 (D) were evaluated by Western blot analysis (20 or 50 μg of proteins were loaded per lane in panel A or in panels B-D, respectively). Values are means ± SEM from 3 independent experiments. ***p<0.01 vs. basal values (unpaired Student's *t* test); °p<0.05, °°p<0.01, °°°p<0.001 (One-Way ANOVA plus Dunnett's test) vs. OGD (control) values.

Figure 8. *Pharmacological activation of A_1 or mGlu3 receptors reduces Bad and increases Bcl-X_L levels in cultured astrocytes exposed to OGD.*

Astrocytes were starved for 24 hours, and then treated with 30 nM CCPA or 5 μM LY379268 1 hour prior to OGD exposure. When present, LY294002 (30 μM) or U0126 (10 μM) were added 30 min prior to receptor agonists. Levels of Bad (A), Bcl-X_L (B), and phosphorylated Bad at serine

112 or 136 (C, D) were evaluated by Western blotting. Values are means ± SEM from 3 independent experiments. ***p<0.01 vs basal values (unpaired Student's t test); °p<0.05, °°p<0.01, °°°p<0.001 (One-way ANOVA + Dunnett's test) vs OGD (control) values..

Figure 9. *Pharmacological inhibition of the PI3K/Akt and ERK1/2 MAPK pathways with LY294002 and UO126 attenuates the protective activity of CCPA and LY379268 against apoptosis in cultured astrocytes exposed to OGD.*.

CCPA (30 nM) and LY379268 (5 μM) were applied to the cultures 1 hours prior to OGD. When present, LY294002 (30 μM) or U0126 (10 μM) Were added 30 min prior to receptor agonists. Apoptosis was assessed 24 hours later by either DAPI staining (A) or measurements of caspase 3 activity (B). RFLU = relative fluorescence units (RFLU). Values are mean ± SEM from four independent experiments. ***p<0.001 vs. basal values (no treatment) (unpaired Student's t test); °°p<0.01 vs. OGD control values (One-way ANOVA plus Dunnett's test).

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Figure 1

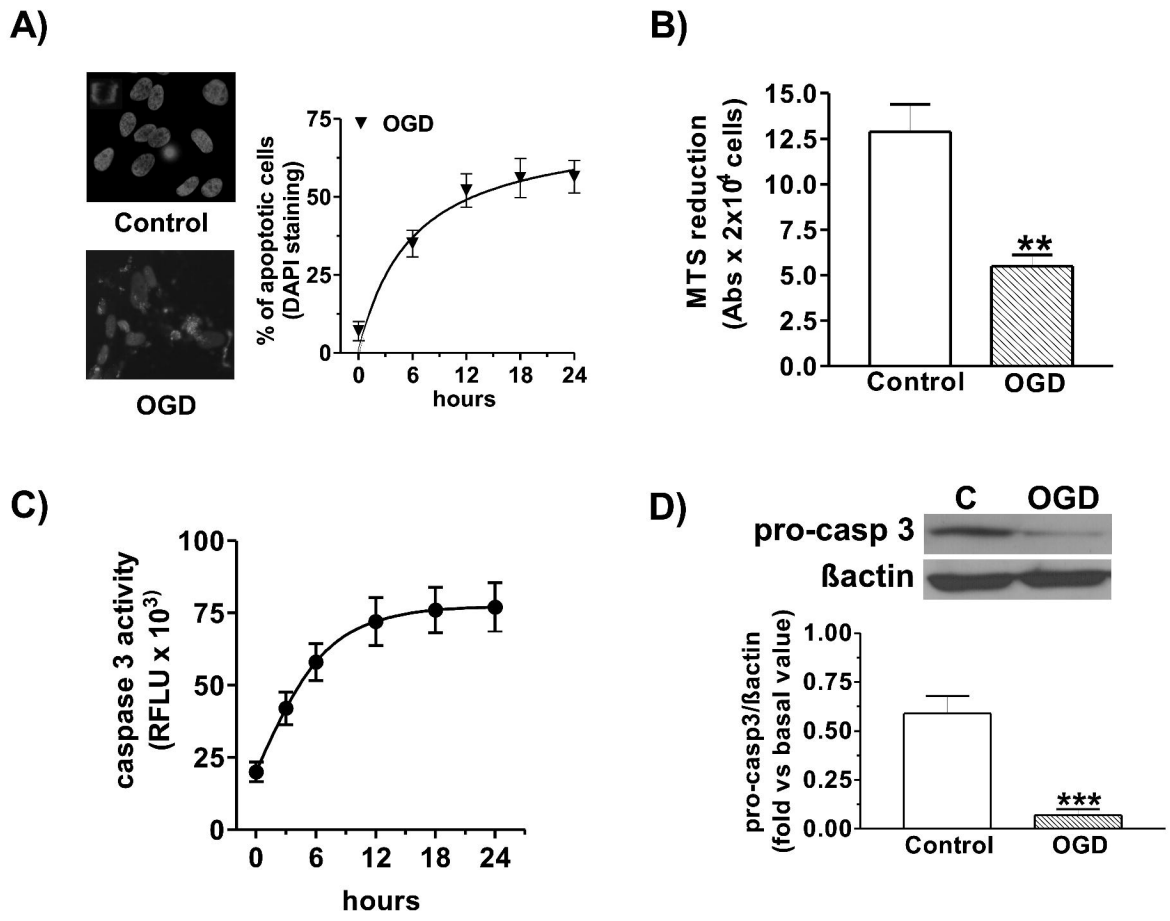

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Figure 2

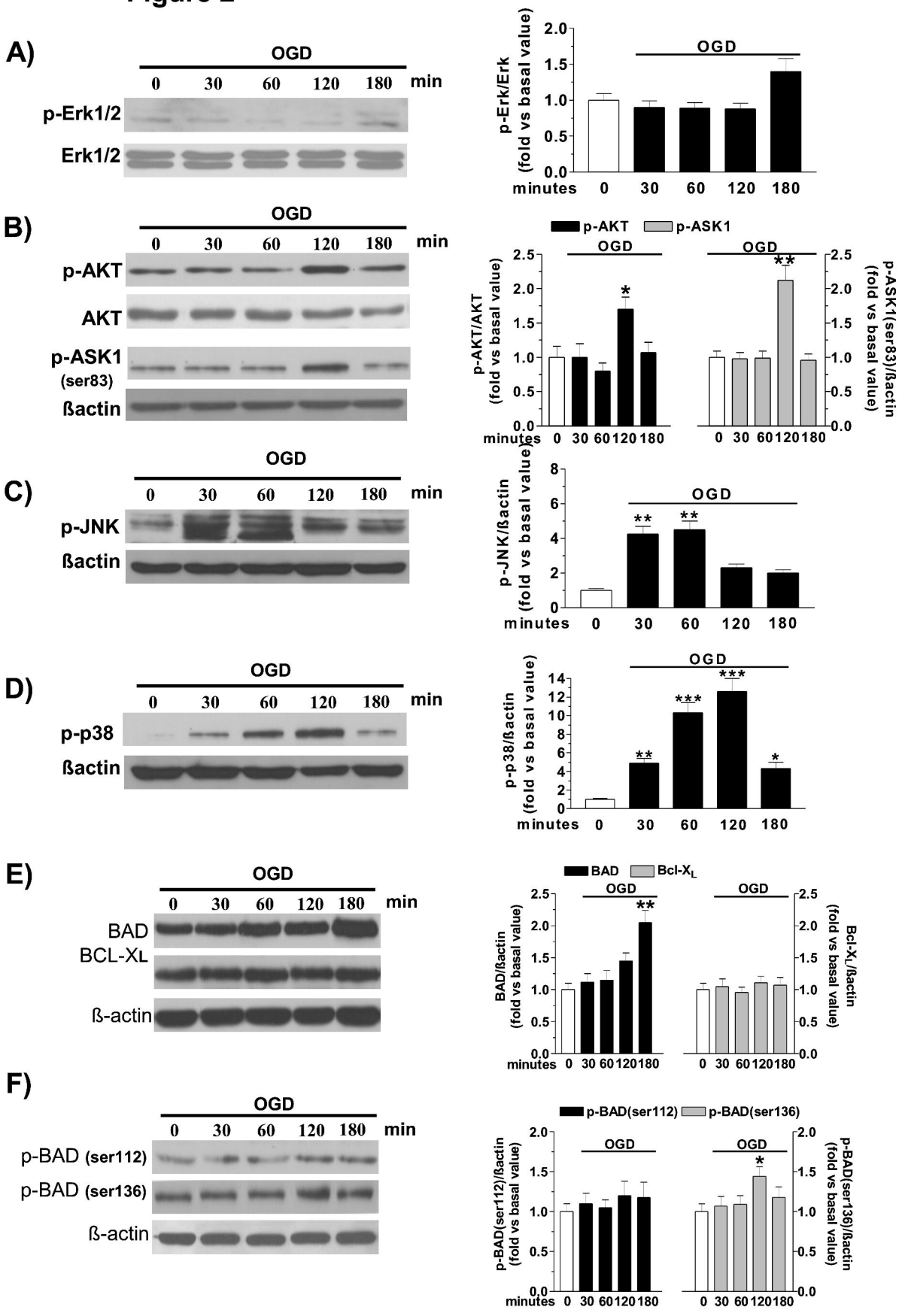

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Figure 3

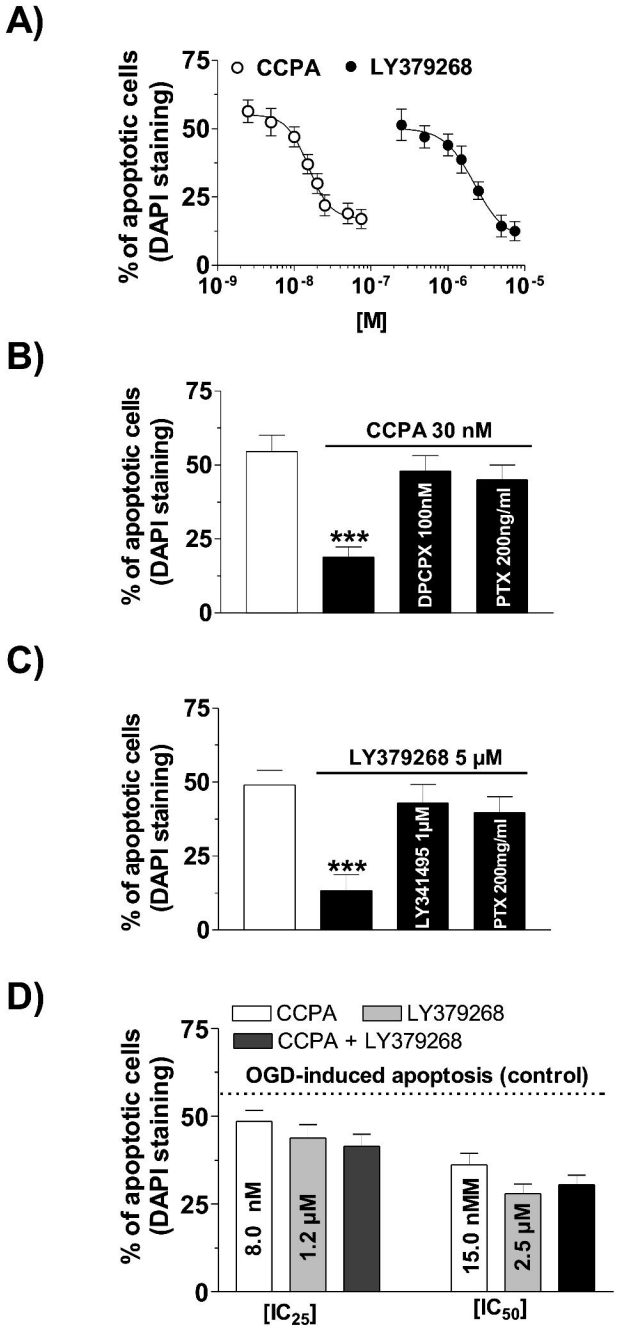

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Figure 4

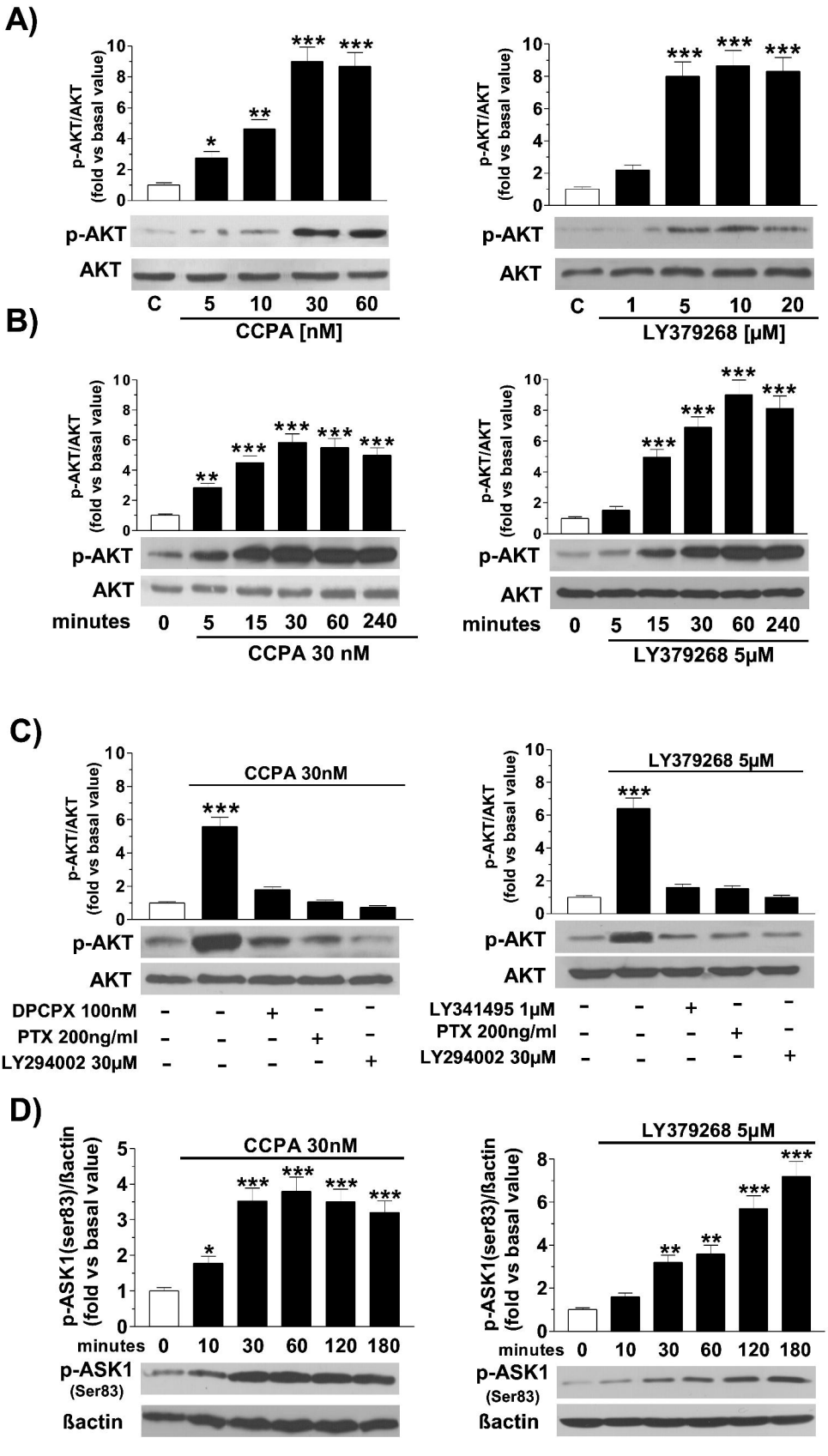

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Figure 5

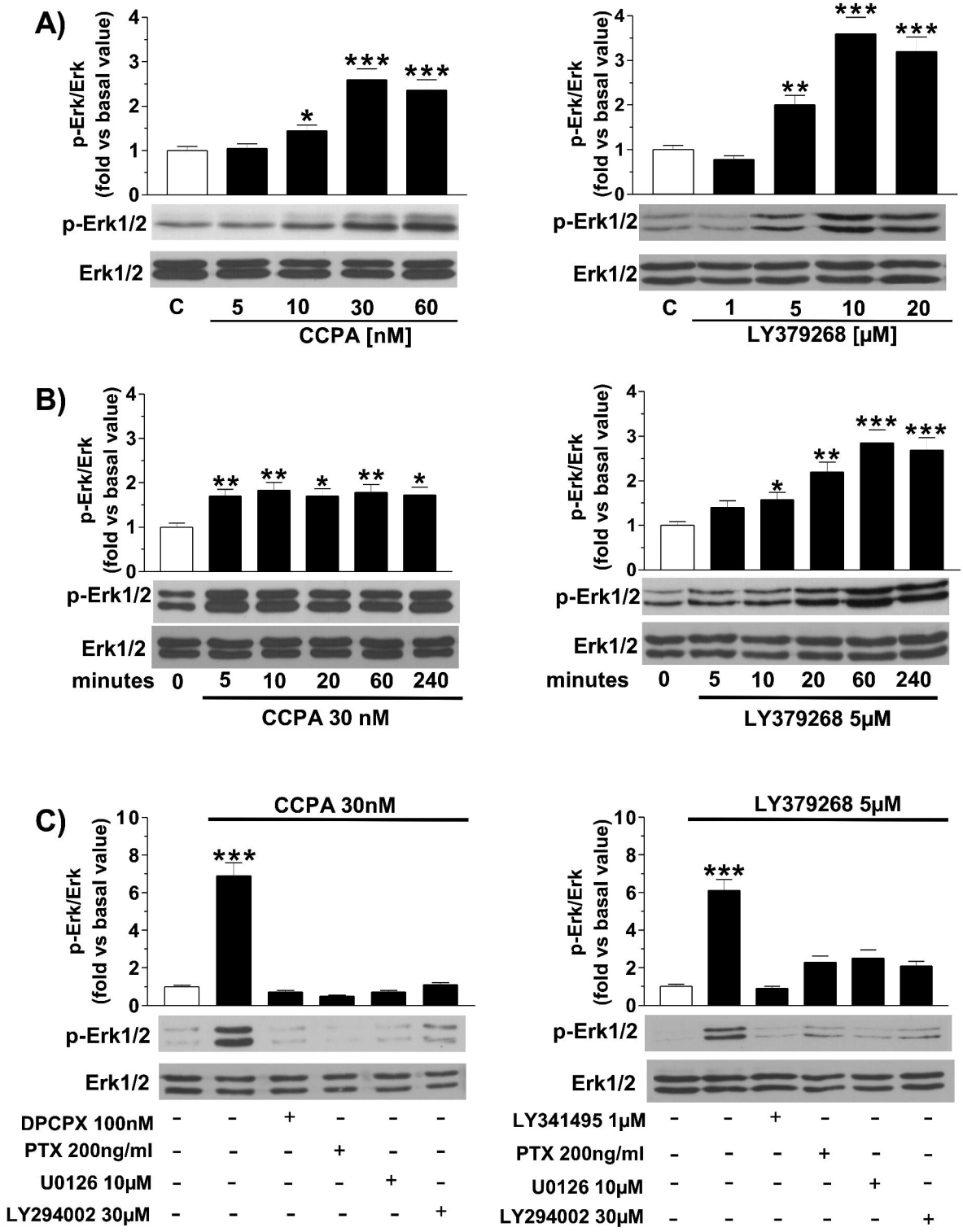

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Figure 6

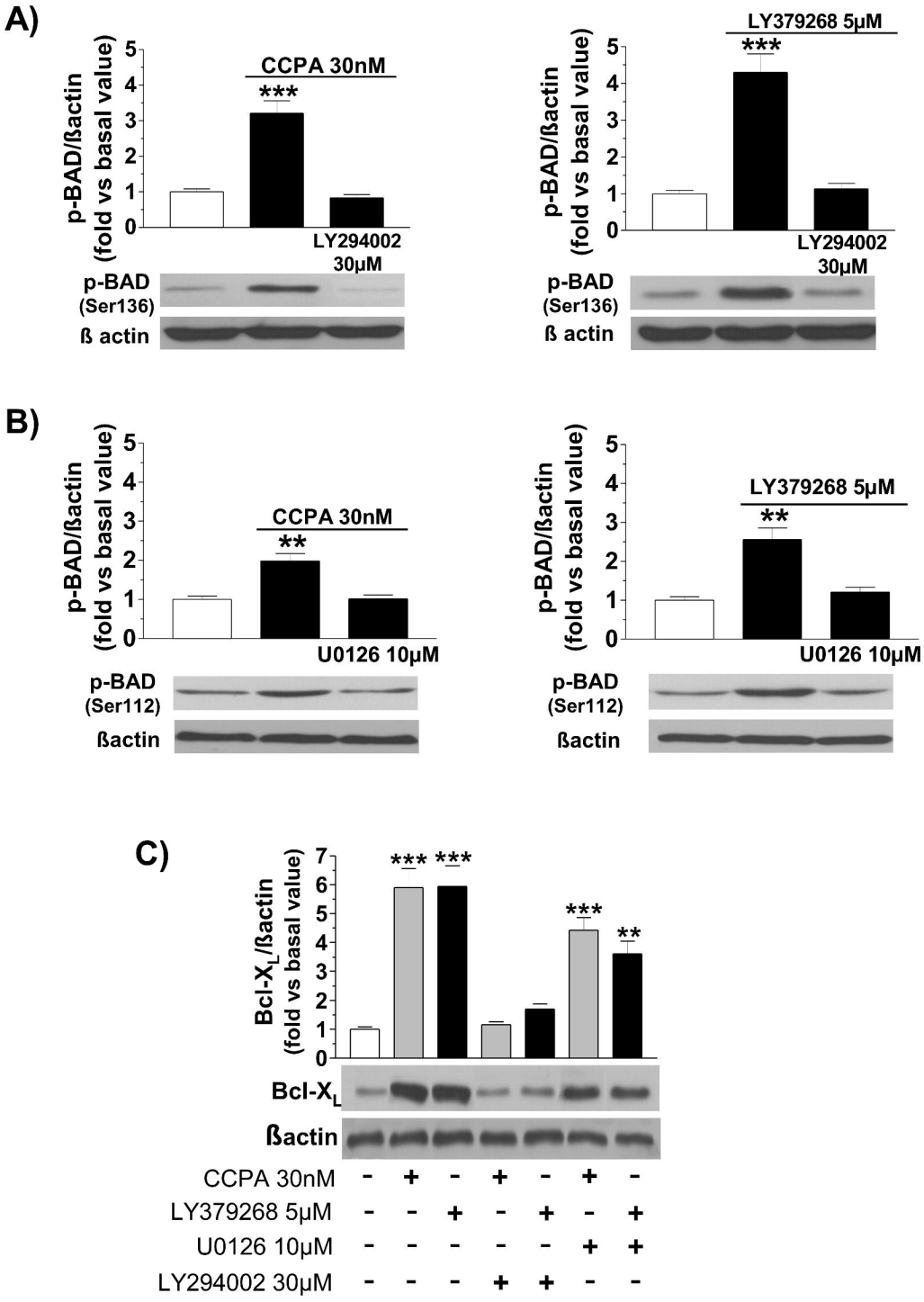

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Figure 7

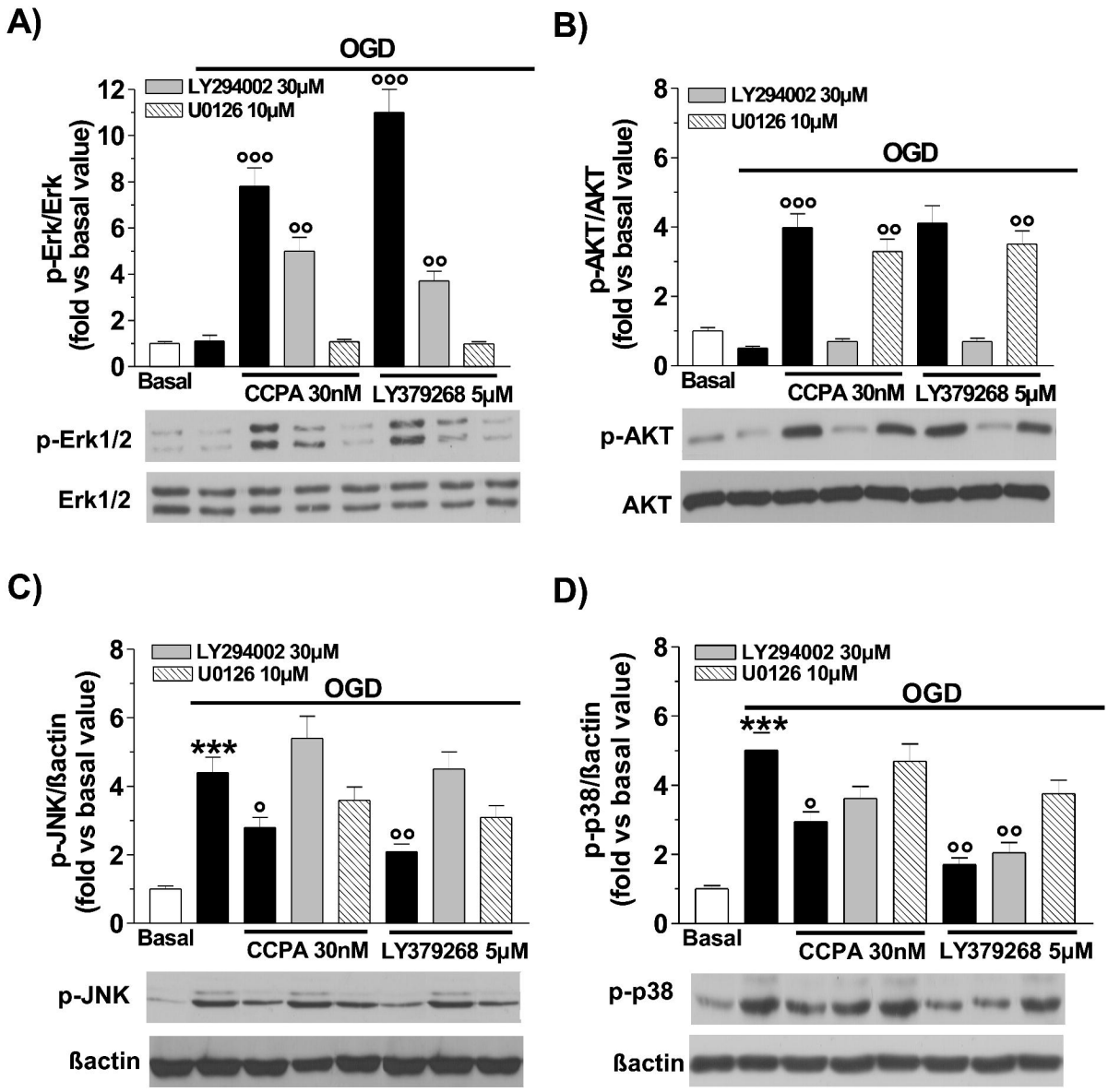

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Figure 8

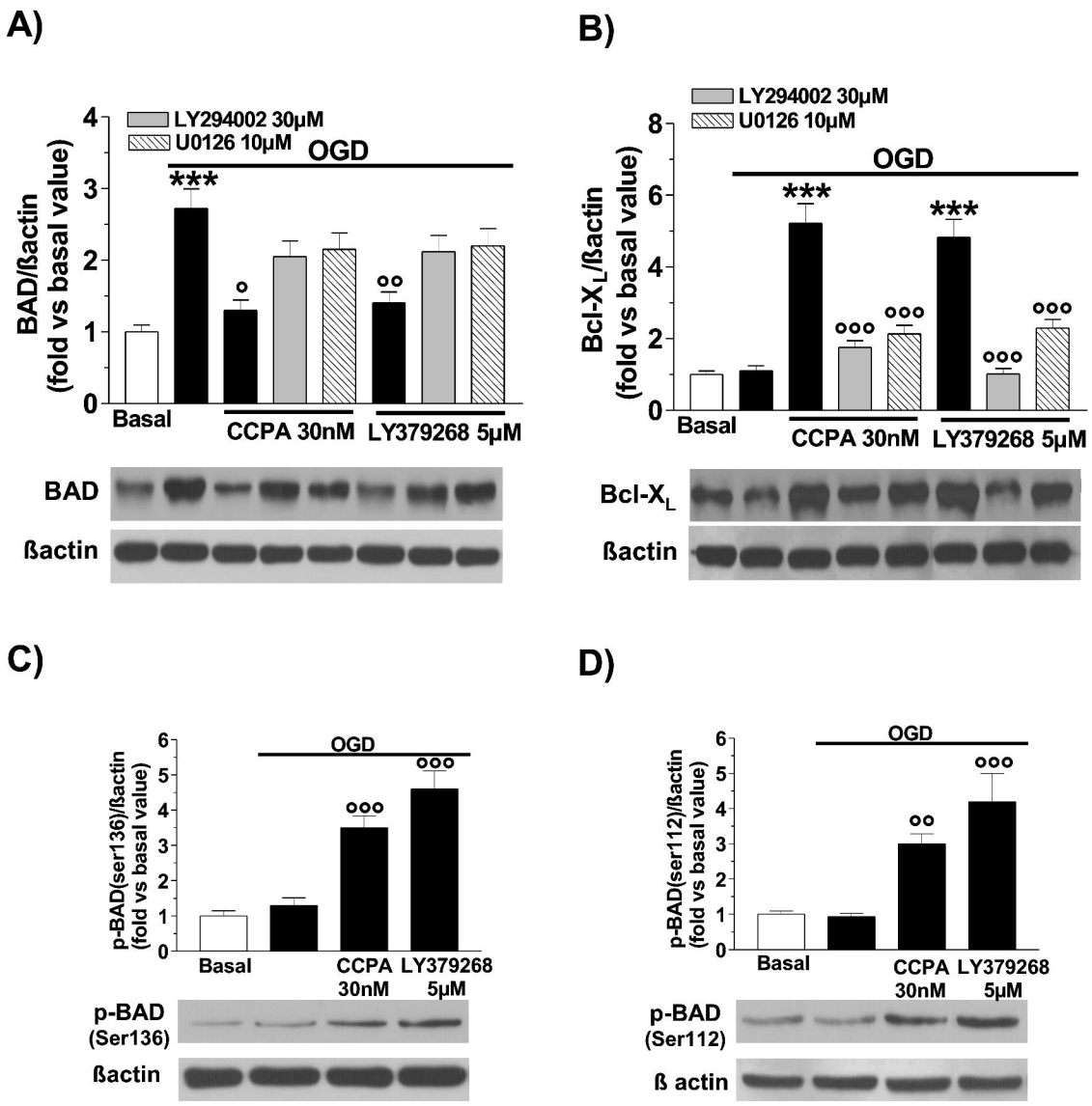

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Figure 9