Astrocyte Resilience to Oxidative Stress Induced by Insulin-like Growth Factor I (IGF-I) Involves Preserved AKT (Protein Kinase B) Activity*

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Disruption of insulin-like growth factor I (IGF-I) signaling is a key step in the development of cancer or neurodegeneration. For example, interference of the prosurvival IGF-I/AKT/FOXO3 pathway by redox activation of the stress kinases p38 and JNK is instrumental in neuronal death by oxidative stress. However, in astrocytes, IGF-I retains its protective action against oxidative stress (1). The molecular mechanisms underlying this cell-specific effect to unveil new strategies to treat neurodegenerative diseases may be relevant. The present work provides evidence that IGF-I stimulates AKT activity in astrocytes but not in neurons. This may be explained by phosphorylation of the phosphatase PTEN at the plasma membrane in response to IGF-I, which allows translocation of phosphatidylinositol 3-kinase (PI3K) to the plasma membrane where it catalyzes the formation of the lipid second messenger phosphatidylinositol 3,4,5-

This article has been withdrawn by the authors. The pAKT and AKT immunoblots in Fig. 1 C and the DAPI and DCF2 staining for control cells treated with either vehicle or IGF-I in Fig. 5A were published previously in Figs. 3E and 4B, respectively, from Genis, L., Dávila, D., Fernandez, S., Pozo-Rodríguez, A., Martinez-Murillo, R., and Torres-Alemán, I. (2014) Astrocytes require insulin-like growth factor I to protect neurons against oxidative injury. F1000Res 3, 28 (10.12688/f1000research.3-28.v2). Fig. 2A was published previously in Fig. 3C in Dávila, D. and Torres-Alemán, I. (2008) Neuronal death by oxidative stress involves activation of FOXO3 through a two-arm pathway that activates stress kinases and attenuates insulin-like growth factor I signaling. Mol. Biol. Cell 19, 2014–2025.

IGF-I² is a powerful reactive oxygen species scavenger (ROS) that can act as a natural antioxidant through a pathological process, such as oxidative stress, by a mechanism that interferes with the activity of p38 kinase (3). Moreover, IGF-I induces survival effects in neurons (1). One of the main downstream targets of IGF-I is the Ser/Thr kinase AKT (2), which mediates cell survival and proliferation (3). Upon its activation, the IGF-I receptor recruits and phosphorylates IRS docking proteins (4), which allows translocation of phosphatidylinositol 3-kinase 3-kinase (PI3K) to the plasma membrane where it catalyzes the formation of the lipid second messenger phosphatidylinositol 3,4,5-

The abbreviations used are: IGF-I, insulin-like growth factor I; mTORC2, mTOR complex 2; PI(3,4,5)P³, phosphatidylinositol 3,4,5-trisphosphate; PTEN, phosphatidylinositol-3,-4,-5-phosphate 3-phosphatase; ROS, reactive oxygen species; SOD, superoxide dismutase; IRS, insulin receptor substrate; carboxy-HDCFDA, 6-carboxy-2,7'-dichlorodihydrofluorescein diacetate; GTP, GTP-binding protein; MG, MGF, AKT, mitogen-activated protein kinase; MFOXO3, FOXO3 triple mutant T32A/S253A/S315A; JIP-1, c-Jun N-terminal kinase-interacting protein 1; PTEN4, mutant of PTEN (alanine substitutions of Ser-380, Thr-382, Thr-383, and Ser-385); DN-FOXO3, dominant negative FOXO3; GFP, glial fibrillary acidic protein; TK, thymidine kinase; Ras, RAS related protein.

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2 The abbreviations used are: IGF-I, insulin-like growth factor I; mTORC2, mTOR complex 2; PI(3,4,5)P³, phosphatidylinositol 3,4,5-trisphosphate; PTEN, phosphatidylinositol-3,-4,-5-phosphate 3-phosphatase; ROS, reactive oxygen species; SOD, superoxide dismutase; IRS, insulin receptor substrate; carboxy-HDCFDA, 6-carboxy-2,7'-dichlorodihydrofluorescein diacetate; GTP, GTP-binding protein; MG, MGF, AKT, mitogen-activated protein kinase; MFOXO3, FOXO3 triple mutant T32A/S253A/S315A; JIP-1, c-Jun N-terminal kinase-interacting protein 1; PTEN4, mutant of PTEN (alanine substitutions of Ser-380, Thr-382, Thr-383, and Ser-385); DN-FOXO3, dominant negative FOXO3; GFP, glial fibrillary acidic protein; TK, thymidine kinase; Ras, RAS related protein.

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modulation by the kinase p38 and 2) phosphorylation of PTEN by IGF-I, which leads to its cytosolic translocation. Armoring of AKT activation by IGF-I in astrocytes contributes to normalize ROS levels and to prevent cell death during oxidative stress. Of note, the neuroprotective role of astrocytes is also enhanced by these adaptations. These results point out the importance of AKT activation for astrocyte survival during oxidative stress and reinforce the idea that modulation of astrocytes by IGF-I forms part of the brain responses to oxidative damage.

**Experimental Procedures**

**Animals and Reagents**—Postnatal day 3 and 7 Wistar rats were used (Harlan, Spain). All efforts were made to minimize suffering and reduce the number of animals. Animals were kept under light/dark conditions following European Union guidelines (directive 86/609/EEC) and handled according to institutionally approved procedures. Antibodies to phospho-AKT (Ser-473), phospho-p38MAPK (Thr-180/Tyr-182), p38MAPK, c-Jun N-terminal kinases (JNKs), FOXO3, phospho (Thr-32) FOXO3, PTEN, and phospho-PTEN (Ser-380/Thr-382/383) were from Cell Signaling Technology (Danvers, MA). IGF-I receptor (C-20), AKT1/2 (H-136), R-RAS, and phospho-JNK (Thr-183/Tyr-185) (G7) antibodies were obtained from Santa Cruz Biotechnology. Antibody to CuZn-SOD was from AbCam (Cambridge, UK). Antibodies to β-actin and GFAP together with DAPI, propidium iodide, and hydrogen peroxide (H2O2) were purchased from Sigma. IGF-I was obtained from Prospec-Tany Technogene (University Medical Centre, Utrecht, The Netherlands).

**Plasmids**—pECE-FOXO3 was kindly provided by M. E. Greenberg (Harvard Medical School, Boston, MA). p6xDBE-luc (reporter luciferase containing six copies of the DAF16 family protein-binding element) and pRL-TK (TK-Renilla luciferase) were a kind gift from B. M. Burginger (University Medical Centre, Utrecht, The Netherlands). pCDNA3-AKT-CA (constitutively active AKT) was kindly provided by S. Pons (Biomedicine Institute, Consejo Superior de Investigaciones Científicas, Barcelona, Spain). Dominant negative FOXO3 (DN-FOXO3) was generated as described (22). pCDNA3-JIP-1 (c-Jun N-terminal kinase-interacting protein 1) was kindly provided by M. Dickens (University of Leicester, Leicester, UK). pCEV-MEKK was obtained through the generosity of M. J. Marinissen (Instituto de Investigaciones Biomedicas, Consejo Superior de Investigaciones Científicas, Madrid, Spain). The pSG5L-PA-Ptena construct contains alanine substitutions of Ser-380, Thr-382, Thr-383, and Ser-385 and was a kind gift from W. R. Sellers (Harvard Medical School, Boston, MA).

**Astrocyte Culture and Transfections**—Astrocyte cultures were prepared from postnatal day 3 rats as described (23). Cells were grown on Dulbecco’s modified Eagle’s medium/F-12 (DMEM/F-12) supplemented with 10% fetal calf serum. After 12 days, astrocytes were seeded at 2.5 × 10^5 or 1.25 × 10^5 cells/well in 6- and 12-well culture plates, respectively. The day of the experiment cells were starved for 3 h and treated with IGF-I (10^−7 M) and/or H2O2 at doses of 50–150 μM. Inhibitory drugs were given 45 min before treatments. We used H2O2 as an oxidant stimulus because it is an endogenously produced ROS that serves as a precursor to hydroxyl radicals and possesses redox signaling capacities (24). For transfection, astrocytes were seeded at 2.5 × 10^5 or 1.25 × 10^5 cells/well in 6- and 12-well culture plates, respectively, and after 16 h, constructs were mixed with FuGENE HD (Roche Applied Science) in a 1:3 ratio and added following the manufacturer’s instructions. Alternatively, astrocytes were electroporated (2 × 10^5 astrocytes with 2 μg of DNA or shRNA) before seeding using an astrocyte Nucleofector kit (Lonza, Switzerland). After electroporation, cells were plated to obtain a final cell density on the day of the experiment similar to that obtained with the transfection method. All experiments were performed after 48–72 h. The transfection efficiency was 20–30% for FuGENE HD and 60–80% for electroporation as assessed with GFP vector. At least three independent experiments were performed in duplicate wells.

**Cerebellar Granule Neuron Culture and Transfections**—Cerebellar granule neurons were produced from P7 rats as follows: in brief, cells were plated at a density of 4 × 10^6 cells/ml on poly-1-lysine (1 μg/ml) coated 12-well plates or 0.45 × 10^6 cells/ml on 12-well plates in 5% CO2 in Neurobasal with 10% FBS supplemented with 2 mM glutamine, 10 mM HEPES, and 10 mM sodium pyruvate. Cells were treated with IGF-I (10^−7 M), H2O2 (50–150 μM), and/or inhibitory drugs as above.

**Co-cultures of Neurons and Astrocytes**—Co-cultures were produced as described previously (16). In brief, wild type astrocytes (1.25 × 10^5/well) were seeded on 12-well plates with coverslips using DMEM/F-12 plus 10% FBS and 16 h later transfected with appropriate constructs. After 48–72 h, cerebellar granule neurons were isolated and plated onto astrocytes. We used forebrain astrocytes and cerebellar granule neurons because in our experience the forebrain and cerebellum yielded very high numbers of astrocytes and neurons, respectively (thus minimizing animal use). Nevertheless, in previous studies, we carried out co-cultures with neurons and astrocytes from the same region (forebrain), obtaining identical results (16). Culture medium was changed to DMEM/F-12 plus B27, 4 mM glutamine, and 25 mM KCl (the latter only in the case of neurons). Two days later, co-cultures were treated with 100 nM IGF-I ± 50–150 μM H2O2. After 24 h, cells were fixed and immunostained.
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Cell Assays—Viability of astrocytes or neurons was assessed in different ways to assure reproducibility of results using 12-well plates. In the first assay, cells were stained after respective treatments with propidium iodide (2 µg/ml). Propidium iodide-positive cells were counted in a Leica CTR 6000 fluorescence microscope (Wetzlar, Germany). In the second assay, cell cultures were transfected with a GFP-pCMV vector and the different constructs under evaluation in a 1:5 ratio. GFP+ cells were scored before treatment to determine baseline survival (time 0) and at different times thereafter. In these two assays, cells were counted in 12 different and random fields per well at 40× magnification. In the third assay, the amount of lactate dehydrogenase released from damaged cells into the culture medium was used to quantify cell death. Lactate dehydrogenase levels were measured at various times with a commercial kit (Roche Diagnostics). In co-cultures, cellular viability was assessed in immunostained coverslips by counting the total number of viable β-III-tubulin+ (neuronal marker) and GFAP+ (astrocyte marker) cells, respectively, in 12 different and random fields per coverslip at 40× magnification. Cellular viability was determined by plasma membrane integrity and absence of nuclear alterations detected with DAPI. The percentage of viable neurons and astrocytes in each treatment was expressed relative to vehicle independent experiments. All assays were done in triplicate dishes in at least three independent experiments.

Immunoassays—For immunostaining, cell cultures were washed with PBS and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer for 15 min. Cells were incubated in 1% Nonidet P-40, 150 mM NaCl, 1% triton, 20 mM Tris, pH 7.4, 10% glycerol, 1 mM CaCl2, 1 mM MgCl2, 400 µM sodium vanadate, 0.2 mM PMSF, 1 µg/ml leupeptin, 0.5 µg/ml aprotinin, 0.1% phosphatase inhibitor mixtures I and II (Sigma-Aldrich). To normalize for protein load, membranes were reblogged (ReBlot, Chemicon) and incubated with an appropriate control antibody (see “Results”). Levels of the protein under study were expressed relative to protein load. Different exposures of each blot were collected to ensure linearity and to match control levels for quantification. Densitometric analysis was performed using Quantity One program (Bio-Rad). A representative blot is shown from a total of at least three independent experiments.

Luciferase Assays—Cells were transfected with a reporter construct bearing six canonical FOXO binding sites (6xDBE-luciferase) and co-transfected with different constructs as indicated in each experiment. Transfections were performed in triplicate dishes. Luciferase counts were normalized using TK-RENilla luciferase. At given times, cells were lysed in passive lysis buffer, and luciferase activity was analyzed using a luminometer and the Dual-Luciferase assay kit according to the manufacturer’s instructions (Promega). Background luminescence was subtracted. Luciferase activity was expressed as -fold change of control levels.

Subcellular Fractionation—Membrane and cytosolic fractions were obtained from wild type or transfected astrocytes seeded on 6-well plates (1.25 × 105/well) as described (26). The quality of the fractionations was determined by assaying for the presence of the cytosolic protein Cu,Zn-SOD and the membrane protein R-RAS.

ROS Measurement—ROS generation was assessed by two different methods. Astrocytes cultured on coverslips were treated
with the fluorogenic marker carboxy-H$_2$DCFDA (Molecular Probes) for 30 min at 37 °C with protection from the light. After incubation, cells were gently washed three times with warm DMEM and mounted. Pictures were taken at 40× magnification using a Leica fluorescence microscope (Germany). A representative picture is shown. The percentage of astrocytes positive for the carboxy-H$_2$DCFDA marker was quantified using ImageJ software. Using this ROS marker it is not possible to distinguish endogenous ROS from exogenously applied H$_2$O$_2$. Nevertheless, we compared this method with the oxidation of luminol (that detects superoxide anions (O$_2^-$)) that distinguishes H$_2$O$_2$ from other ROS. To this end, we used a specific kit (Sigma). Half a million astrocytes were added to luminometer tubes containing the reagents for luminol oxidation and different concentrations of H$_2$O$_2$ (0, 50, and 100 μM) in a final volume of 200 μl. Ten minutes later the chemiluminescence signal was determined. Results obtained with carboxy-H$_2$DCFDA and with the kit based on luminol oxidation were similar (data not shown). All assays were done in triplicate dishes in at least three independent experiments.

PTEN Lipid Phosphatase Activity—Phosphate released from substrates was measured with a Malachite Green Phosphatase Assay (Echelon). After treatments, astrocyte cultures were washed with ice-cold PBS and lysed with PIK buffer (see above). 250 μg of total protein was immunoprecipitated with a monoclonal anti-mouse PTEN antibody. Lipid phosphatase PTEN activity was measured in 50 μl of PTEN activity buffer (100 mM Tris-HCl, pH 8.0, 10 mM DTT) containing water-soluble d(+)-sn-1,2-di-O-octanoylglycerol,3-O-phospho-linked phosphatidylinositol phosphate. Samples were incubated for 30 min at 22 °C with gentle shaking before measuring absorbance at 620 nm. Inorganic phosphate release was quantified by comparison with a standard curve of KH$_2$PO$_4$ in distilled H$_2$O.

Statistical Analysis—Data are expressed as mean ± S.E. Differences between groups were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test.
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variance followed by a Newman-Keuls or t test. A p value < 0.05 was considered significant.

Results

Activation of AKT by IGF-I in Astrocytes Is Not Affected by Oxidative Stress—Previous evidence showed that oxidative stress elicited by H$_2$O$_2$ interfered with IGF-I signaling and with its cytoprotective effect on neurons (9). Specifically, H$_2$O$_2$ (50–100 µM) decreased activation of the prosurvival kinase AKT induced by 100 nM IGF-I in a dose-dependent manner (Fig. 1, A and B). However, in astrocytes, the cytoprotective effect of IGF-I was preserved during oxidative stress in sharp contrast to neurons (16). Different doses of H$_2$O$_2$ (50–150 µM), able to induce oxidative stress in astrocytes (16), not only did not prevent the activation of AKT detected 15 min after IGF-I (100 nM)
addition but also significantly enhanced it (Fig. 1C). Thus, we next elucidated the molecular mechanisms underlying differential responses of neurons and astrocytes.  

**AKT Activation by IGF-I in Astrocytes Is Not Affected by p38 Redox Activation**—In neurons, interference by H$_2$O$_2$ of IGF-I-induced activation of AKT was mediated by the stress kinase p38 (9). Thus, redox activation of p38 prevented the phosphorylation of the docking protein IRS-1 (Tyrr-612) induced by IGF-I, leading to the down-regulation of the PI3K/AKT pathway (9). Pretreatment with the p38 inhibitor SB239063 (7.5 μM) prevented its activation by H$_2$O$_2$ in neurons, restoring IRS-1 phosphorylation and AKT activation by IGF-I (24). We tested whether this molecular mechanism could interfere with IGF-I signaling in astrocytes. We first observed that H$_2$O$_2$ did not prevent IRS-1 phosphorylation after IGF-I treatment in astrocytes (Fig. 2B), which is in clear contrast to that observed in neurons (9) (Fig. 2C). Nevertheless, H$_2$O$_2$ induced p38 activity in astrocytes just as in neurons (Fig. 2D). Furthermore, we also observed in astrocytes exposed to H$_2$O$_2$ (50–150 μM) that pretreatment with SB239063 prevented p38 activation without up-regulating phosphorylation of IRS-1 or AKT by IGF-I (Fig. 2D). On the contrary, SB239063 significantly down-regulated phosphorylation of IRS-1 in astrocytes exposed to the highest dose of H$_2$O$_2$ (150 μM) (Fig. 2D). These results indicate that activation of p38 by H$_2$O$_2$ does not prevent AKT activation by IGF-I in astrocytes.  

**IGF-I Induces Phosphorylation of PTEN to Facilitate AKT Activation in Astrocytes**—By quantification of AKT (Ser-473) protein levels, we observed that activation of AKT was significantly reduced in astrocytes than in neurons, both under normal and oxidative stress conditions. In astrocytes, IGF-I induced (5 min after its addition) phosphorylation of the phosphatase PTEN at three specific residues (Ser-380, Thr-382, and Thr-383) of its C-terminal tail (Fig. 3A). This increase in PTEN phosphorylation was not affected by co-treatment with H$_2$O$_2$ (50–150 μM), a condition we did not observe in neurons treated with IGF-I where we observed the opposite effect, a decrease of PTEN phosphorylation (Fig. 3, A and B). These residues form part of a serine/threonine cluster located at the PTEN tail whose phosphorylation can decrease its phosphatase activity (27, 28) or induce its cytosolic translocation, preventing PIP$_3$ dephosphorylation at the membrane (28, 29). However, whereas H$_2$O$_2$ (100 μM) reduced the phosphatase activity of PTEN, IGF-I did not produce any significant effect (Fig. 3C). Nevertheless, we detected that PTEN phosphorylation mainly occurred at the membrane of astrocytes treated with IGF-I alone or in combination with H$_2$O$_2$ (Fig. 3D). A time course analysis showed that PTEN translocates to the cytosol after its phosphorylation 15 min after IGF-I treatment (Fig. 3E). This translocation was enhanced by co-administration of H$_2$O$_2$ (Fig. 3E). Phosphorylation of these residues is essential for the translocation of PTEN from the membrane to the cytosol after addition of IGF-I + H$_2$O$_2$ because levels of mutant PTENA4 (where Ser-380, Thr-382 and -383, and Ser-385, also a member of the serine/threonine cluster, were mutated to alanine) were not affected by treatment with IGF-I and H$_2$O$_2$ (Fig. 3E). Confirming the specificity of these events in astrocytes, we did not observe PTEN phosphorylation at the membrane of neurons treated with IGF-I and H$_2$O$_2$ (50 μM) (data not shown). Finally, we analyzed whether PTEN phosphorylation was involved in the activation of FOXO3 in astrocyte cultures treated for 5 min with IGF-I (100 nM) and H$_2$O$_2$ (100 μM). While FOXO3 translocation was enhanced in normal and under oxidant conditions, these results indicate that PTEN phosphorylation promote AKT activity in astrocytes.
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Confirming our previous evidence (16), IGF-I-mediated inactivation of FOXO3 protected astrocytes from H₂O₂ treatment (Fig. 4D).

FOXO3 activation by oxidative stress also depends on the activation of JNKs (9, 32). This pathway was also present in astrocytes because transfection with a JNK-interfering protein, JIP-1, reduced FOXO3 activation by H₂O₂ (Fig. 4E). Previously, we had described in neurons how the blockade of IGF-I signaling by oxidative stress was necessary for JNKs to activate FOXO3, unveiling a sequential activation of FOXO3 that depended first on down-regulation of its phosphorylation by AKT and second on its activation by JNKs (9). Bearing in mind these results, we examined in astrocytes whether AKT activation by IGF-I could prevent JNK activation of FOXO3 during oxidative stress. Initially, we observed lower levels of phospho-JNK1 and -JNK2 (Thr-183/Tyr-185) in H₂O₂ (100 µM)-treated...
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Akt Activation by IGF-I Reduces ROS Levels in Astrocytes—

Activation of Akt has previously been involved in up-regulation of ROS detoxification mechanisms (35, 36). We analyzed whether Akt activation by IGF-I or, alternatively, Akt-CA reduced ROS levels in astrocytes. Two different methods were used. Total ROS levels (endogenous ROS and those generated from exogenously applied \text{H}_2\text{O}_2) were assessed with the fluorescent marker cytochrome-C (Cyo-C). Akt-CA significantly countered the ROS fluorescence signal induced by \text{H}_2\text{O}_2 (Fig. 5A and B). We also observed that Akt inhibition by pretreatment with \text{LY294002} neutralized the effects of IGF-I on ROS levels (Fig. 5, A and B). The other method, based on the oxidation reaction, assesses levels of \text{O}_2^- generated by endogenous ROS. Astrocytes transfected with Akt-CA showed significantly lower levels of \text{O}_2^- than control cells (n = 3). These results suggest that Akt activation could be crucial to prevent FOXO3 activation and cell death in astrocytes during oxidative stress. FOXO3 inactivation would depend on two Akt-mediated steps: phosphorylation of FOXO3 and inactivation of JNKs.

Akt Activation by IGF-I Reduces ROS Levels in Astrocytes—

Akt Activation by IGF-I reduces FOXO3 activity in normal conditions (Fig. 5E). In contrast, Akt-CA expression mimicked the effect of IGF-I, up-regulating Cu,Zn-SOD levels both in normal and oxidant conditions (Fig. 5E). These results illustrate the antioxidative role of Akt in astrocytes.

Akt Activation by IGF-I Is Involved in Neuroprotection by Astrocytes—

In view of the cytoprotective and antioxidative effects mediated by Akt activation in astrocytes during oxidative stress, we analyzed whether this activation could also have an impact on neuroprotection. Neurons were cultured alone or with astrocytes in the presence of \text{H}_2\text{O}_2 (50 \mu\text{M}) and IGF-I (100 \mu\text{M}). As expected (9, 16), IGF-I did not prevent cell death by \text{H}_2\text{O}_2 when neurons were cultured alone (Fig. 6A). However, when co-cultured with astrocytes, IGF-I significantly increased neuronal survival (Fig. 6A). This confirmed a neuroprotective effect mediated by IGF-I signaling in astrocytes. To test whether this effect depended on Akt activation, we transfected astrocytes with a PTEN4 mutant or a control vector to prevent this activation as demonstrated previously (Fig. 3G). We observed that PTEN4 expression significantly reduced survival of neurons in co-cultures treated with IGF-I and \text{H}_2\text{O}_2 compared with co-cultures where astrocytes were transfected with a control vector (Fig. 6B). To confirm the importance for the neuronal survival of IGF-I-protected astrocytes with Akt expression mimicked by Akt-CA or their control vectors, we used a co-culture system. IGF-I and Akt-CA or their control vectors were co-administered with astrocytes in the presence of \text{H}_2\text{O}_2 (50 \text{ –} 100 \mu\text{M}). Akt-CA treatment significantly increased survival of neurons (Fig. 6C). This suggested that Akt activation is important for astrocyte-mediated neuroprotection (Fig. 6D). These results support the involvement of Akt activation in astrocyte-mediated neuroprotection in vivo.

FIGURE 4. Akt activation by IGF-I prevents FOXO3-mediated cell death in astrocytes during oxidative stress. A, astrocyte death 12 h after \text{H}_2\text{O}_2 (100 \mu\text{M}) addition was determined by measuring lactate dehydrogenase (LDH) released to the medium. Transfection of astrocytes with DN-FOXO3 protected them (**, p < 0.001 versus \text{H}_2\text{O}_2-treated cells transfected with control construct; \text{n} = 3). B, IGF-I (100 \text{ nm}) increased phospho-FOXO3 (pFOXO3) (Thr-32 and FOXO3 levels in the cytosolic fraction 4 h after its addition (*, p < 0.05 versus control cells; \text{n} = 3). Co-administration of \text{H}_2\text{O}_2 (100 \mu\text{M}) enhanced this effect (**, p < 0.01 versus control cells; *, p < 0.05 versus IGF-I treated cells; \text{n} = 3). C, B-Actin served as a loading control. D, astrocytes were co-transfected with a luciferase reporter vector containing six copies of the DAF16 family protein-binding element and either the WT FOXO3 or MFOXO3 (insensitive to IGF-I) construct. IGF-I (100 \mu\text{M}) significantly reduced FOXO3 activity in non-\text{H}_2\text{O}_2-treated astrocytes transfected with WT FOXO3 (**, p < 0.01 versus control cells; \text{n} = 4). Alternative expression of MFOXO3 abrogated IGF-I effects (*, p < 0.05 versus WT FOXO3-transfected cells; \text{n} = 4). E, \text{H}_2\text{O}_2 (100 \text{ nm}) treatment increased FOXO3 transcriptional activity; however, it did not prevent IGF-I-mediated FOXO3 inactivation (**, p < 0.001 versus \text{H}_2\text{O}_2-treated cells; \text{n} = 4). Alternative expression of MFOXO3 abrogated IGF-I effects on \text{H}_2\text{O}_2-treated astrocytes (**, p < 0.001 versus WT FOXO3-transfected cells; \text{n} = 4). F, \text{H}_2\text{O}_2 (50 – 100 \mu\text{M}) co-administration significantly prevented \text{H}_2\text{O}_2-mediated lactate dehydrogenase release (**, p < 0.001 versus \text{H}_2\text{O}_2-treated cells; \text{n} = 3). Transfection of astrocytes with MFOXO3 (insensitive to IGF-I) significantly prevented the cytoprotective effect of IGF-I in oxidative stress conditions (**, p < 0.001 versus \text{H}_2\text{O}_2-treated cells and WT FOXO3-transfected cells; \text{n} = 3). E, JIP-1-transfected astrocytes showed lower FOXO3 activity than control astrocytes in the presence of \text{H}_2\text{O}_2 (100 \mu\text{M}) (**, p < 0.01 versus control cells transfected with control construct; \text{n} = 4). F, \text{H}_2\text{O}_2 (50 – 100 \mu\text{M}) co-administration up-regulated phospho-JNK1 (pJNK1) and -2 (pJNK2) (Thr-183 and Tyr-185) levels in IGF-I (100 \text{ nm})-treated astrocytes. Pretreatment with the PI3K/Akt inhibitor LY294002 (25 \mu\text{M}) prevented Akt phosphorylation (Ser-473) (pAkt) and significantly enhanced JNK phosphorylation (**, p < 0.01 and *, p < 0.05 versus \text{H}_2\text{O}_2-treated cells without LY294002; \text{n} = 3). G, astrocytes transfected with MEKK-CA showed a significantly higher FOXO3 activity than control astrocytes (*, p < 0.05 versus cells transfected with control construct; \text{n} = 4). Co-transfection with an Akt-CA construct prevented this effect (**, p < 0.001 versus Akt-CA cells only transfected with MEKK-CA; \text{n} = 4). Error bars represent S.E. a.u., absorbance units.
factor FOXO3 and the expression of ROS-detoxifying enzymes such as Cu,Zn-SOD. Finally, we also observed that by preserving AKT activation the neuroprotective role of astrocytes is maintained during oxidative stress insults (these results are summarized in Fig. 7).

Our group previously described a p38-mediated interference of the IGF-I/PI3K/AKT pathway induced by oxidative stress in neurons (9). Numerous lines of evidence in models of diabetes, cardiovascular dysfunction, and obesity confirm the role of p38 as a possible mediator of IGFs/insulin resistance induced by oxidative stress (37–42) and the potential benefits of its inhibition (43, 44). p38 redox activation prevents IRS-1/2 phosphorylation by IGF-I or insulin, blocking in this way PI3K and AKT activation (45). Here, we confirmed that H2O2 treatment stim-
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Our results showed that in astrocytes, but not in neurons, IGF-I induced PTEN phosphorylation in a specific serine/threonine cluster of amino acids (Ser-380, Thr-382, and Thr-383) at its C-terminal tail. This confirms previous work describing that this phosphorylation can interfere with the presence of PTEN at the plasma membrane, which in turn results in increased AKT activation (28, 29, 50), and that oxidative stress induced by H₂O₂ enhances this effect. PTEN phosphorylation is induced by the prosurvival kinase casein kinase 2 (51–53), which could be a novel target of IGF-I-mediated signaling in astrocytes.
Translocation of PTEN from the membrane to the cytosol took place within 15 min after IGF-I treatment, whereas our previous results had shown a transient reduction of PTEN mRNA and protein levels 6 h after IGF-I addition (54), indicating that IGF-I could use both short and long term mechanisms to regulate PTEN activity. Collectively, these results suggest that PTEN inhibition may be a key mechanism to preserve IGF-I-mediated AKT activation in astrocytes during oxidative stress conditions.

We have also revealed that preservation of AKT activation by IGF-I provides important advantages for astrocyte survival during oxidative stress. First, AKT inactivates FOXO3, a transcription factor that is key to trigger cell death after oxidative stress both in neurons and astrocytes (9, 16). Hence, AKT activation prevented FOXO3 activation by JNKs, the signaling pathway that stimulates its proapoptotic role in neurons during oxidative stress (9, 32). These results highlight the importance of AKT activation by IGF-I in astrocytes to tilt the balance between inhibitory and excitatory signals of FOXO3, preventing its proapoptotic effects during oxidative stress. Second, AKT activity reduces ROS levels during oxidative stress. Previous observations already indicated antioxidative actions of IGF-I in astrocytes (16). We describe here that one of them, the up-regulation of the antioxidative enzyme Cu,Zn-SOD, depends specifically on AKT activation. Additional antioxidative mechanisms related to AKT activity could also participate in IGF-I cytoprotection, as for example up-regulation of the transcription factor Nrf2 (36).

Astrocytes are coupled to neurons to provide ROS detoxification support during oxidative stress insults (21). Several works also suggest that the release by astrocytes of soluble and insoluble factors could contribute to their neuroprotective role (55, 56). Supporting this idea, our group has recently described that IGF-I cooperates with stem cell factor secreted by astrocytes to protect neurons against oxidative stress (16). The present results suggest that preservation of AKT activity in astrocytes can be key for IGF-I neuroprotection. Expression of CA-AKT in astrocytes partially mimicked IGF-I neuroprotection in co-cultures with neurons exposed to H2O2, whereas expression of a PTEN mutant insensitive to IGF-I inhibition prevented AKT activation in astrocytes and reduced IGF-I neuroprotection. AKT-mediated neuroprotection probably includes its effect on ROS detoxification (57) and up-regulation of secreted factors such as stem cell factor, whose promoter can be regulated by transcription factors targeted by AKT (58, 59).

Furthermore, PTEN inhibition in astrocytes could be neuroprotective in pathologies associated with oxidative stress. Supporting this idea, PTEN inhibition has shown cytoprotective effects in animal models of oxidative stress-associated pathologies such as diabetes, obesity, and cardiac ischemia (60–63). However, evidence about the neuroprotective role of PTEN inhibition in brain ischemia is contradictory (64, 65). PTEN
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displayed multiple functions in the brain, including tumor suppression, axonal outgrowth, astroglisisi, and cognitive function regulation (66–69), and its total inhibition could affect all of them in a nonspecific manner. Therefore, further research to develop more precise modulators of PTEN and appropriate timings of administration may help develop its possible neuroprotective role.

Overall, the results presented in this study reinforce the notion that IGF-I could display antioxidative actions in specific cellular contexts and types. We demonstrate that in astrocytes these actions depend, at least in part, on the preservation of AKT activation. This is achieved through molecular adaptations targeting p38, PTEN, and FOXO3 that result in increased resilience of astrocytes to IGF-I resistance induced by oxidative stress. Our results also suggest that activation of AKT in astrocytes by growth factors such as IGF-I, which is produced by astrocytes during stress situations (16, 70), may be part of an endogenous brain defense mechanism against oxidative stress injury.

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