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Modulation by insulin-like growth factor I of the phosphatase PTEN in astrocytes

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Abstract

Characterization of intracellular pathways underlying the pleiotropic actions of insulin-like growth factor-I (IGF-I) on brain cells is incomplete. We analyzed IGF-I signalling on astrocytes through the canonical phosphatidylinositol 3-kinase (PI3K)/Akt pathway and focused on possible changes in PTEN, a phosphatase that modulates IGF-I signalling by inhibiting Akt activation and, in turn is positively regulated by PI3K. After exposure of astrocytes to IGF-I, PTEN mRNA and protein levels were reduced and its phosphatase activity diminished. Inhibition of PTEN involved activation of a PI3K/protein kinase C (PKC) pathway that decreased in a proteasome-dependent step the levels of the transcription factor Egr-1, a key regulator of PTEN levels in astrocytes, causing decreased binding of Egr-1 to the PTEN promoter. Enhanced mitogenesis in PTEN siRNA-transduced astrocytes after IGF-I suggested that reduced PTEN may be a permissive factor for the mitogenic activity of IGF-I. Subsequent recovery of reduced PTEN required also activation by IGF-I of PI3K to recruit in this case protein kinase A (PKA) which stimulated Egr-1 levels and, consequently PTEN synthesis. Because basal levels of PTEN in astrocytes are also governed by PI3K, IGF-I appears to modulate PTEN in astrocytes by redirecting its homeostasic control through PI3K in a timed fashion.

Keywords: IGF-I; Astrocyte; PI_3K; PTEN; PKA; Egr-1; PKC

1. Introduction

Insulin-like growth factor I (IGF-I) is a wide-spectrum neuroactive factor expressed in the central nervous system and many other tissues [1,2]. In the brain, IGF-I promotes neuroprotection by modulating all types of brain cell functions in a distinct fashion [3]. Intriguingly, IGF-I appears to recruit the same intracellular cascades in its target cells not only in the brain but elsewhere; these include the canonical phosphatidylinositol 3-kinase (PI3K)/AKT and Ras/Mitogen activated protein kinase (MAPK) pathways [4,5] known to be involved in the growth and differentiating effects of IGF-I. However, the actions of IGF-I on its different target cells are quite specific. For instance, in astrocytes IGF-I exerts proliferative effects [6], while in neurons it is a potent pro-survival signal [7] and in oligodendrocytes participates in their differentiation [8]. Therefore, a better understanding of the molecular pathways underlying cell-specific actions of IGF-I on brain cells is required.

Phosphatase and tensin homolog deleted from chromosome 10 (PTEN) is a dual lipid/protein phosphatase [9–11] that dephosphorylates phosphatidylinositol 3,4,5-triphosphate (PIP_3) and in this way inhibits the PI3K/AKT kinase pathway [12–14]. Modulation of the expression of this phosphatase is essential in regulating cell viability [15–17], while in the brain PTEN appears to play a role in neuronal differentiation and in proliferation of astrocytes [18]. Recently, a positive modulation of PTEN by PI3K was reported [19]. Because IGF-I induces activation of the IRS/PI3K/Akt in target cells, including astrocytes [6], modulation of PTEN in astrocytes by IGF-I appears likely. We explored this possibility because while searching for cell-specific actions of IGF-I on brain cells we found that PTEN mRNA levels were modulated in forebrain astrocytes but not in cerebellar neurons. We now show a biphasic regulation of PTEN by IGF-I in astrocytes through PI3K. This timed modulation involves protein kinase C (PKC), as a negative regulator, and protein kinase A

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(PKA), as a positive modulator, converging at the upstream regulatory transcription factor Early growth response-1 (Egr-1).

2. Materials and methods

2.1. Antibodies and reagents

Culture media, serum and supplements were from Invitrogen (USA). Human recombinant IGF-I was from GroPep (Australia). Wortmannin, LY294002, H89, Bisindolylmaleimide IX (Ro813220), PD98059, Cycloheximide and Ro-32-0432 were from Calbiochem (USA). Forskolin, MG132, the phorbol ester 12-*O*-tetradecanoyl phorbol-13-acetate (TPA), and picropodophyllin (P) were purchased from Sigma (USA). In all cases, drugs were added at the lowest concentration that produces the maximal effect. Antibodies against N-end PTEN (sc-9145), AKT (sc-8318), Egr-1 (sc-110), insulin-like growth factor-1 receptor beta subunit (IGF-IR-β) (C-20, sc-713), and PKA Iα reg (C-14, sc-18800) were from Santa Cruz (USA). Monoclonal anti-β-actin (clone AC-74, Sigma) was used to normalize protein load in Western blots. Phosphospecific antibodies against Phospho-PTEN (Ser380/Thr382/383) and Phospho-AKT (Ser 473) were from Cell Signalling (USA). Phosphorylated IGF-I receptor from AbCam was also used. Secondary HRP-anti-mouse, HRP-anti-rabbit and HRP-goat antibodies were from Bio-Rad (USA). Anti-GADPH antibody was purchased from Affinity Bio Reagents (ABR) (USA).

2.2. Plasmids and reporter constructs

The pGL3-P10 plasmid (kindly provided by E.D. Adamson, the Burnham Institute) was used to obtain the 1,978-bp PTEN promoter and cloned into a SEAP reporter vector (Clontech, pPten-SEAP). pCMV-IGF-IR (K1003R) a derivative of pBS-IGF-IR (K1003R, from D. Le Roith, Mt. Sinai) in which the IGF-IR (K1003R) is a kinase dead [20]. IGF-I receptor beta-subunit was cloned into a peGFPNI vector (Clontech). pCDNA3.1 (Invitrogen), pCDNA3-AKT wild type and pCDNA3-AKT kinase dead were also used. The AKT constructs were provided by S. Pons (Instituto de Biomedicina). A protein kinase A-α I regulatory subunit kinase dead was kindly provided by Stanley MacKnight (University of Washington) and cloned into the pCDNA3.1 expression vector.

2.3. Cell cultures

Astrocyte and neuronal cultures were prepared from postnatal day 3 (P3) rat telencephalon and P7 rat cerebellum, respectively, as described [21,22]. Astroglial cultures were grown on Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. After 10–15 days astrocytes were seeded in a 12-well plate. Cerebellar granule neurons were grown (2×10^6 cells/well) on polystyrene dishes with Neurobasal +B27 (Gibco, USA), glutamine, and 25 mM KCl. Under these conditions, 95% of the cells are neurons (β3-tubulin-positive). Cultures were placed in a humidified incubator at 37 °C and 5% CO_2 atmosphere. The day of the experiment, the medium was replaced by Neurobasal plus 25 mM KCl in neuronal cultures and in astrocytes cultures with serum-free DMEM. After 3 h IGF-I (at the indicated concentrations) was added while inhibitors or other drugs were given 30 min before IGF-I treatment. The C6 rat glioma and the U373MG and T98G human glioblastomas were cultured in DMEM.

2.4. Trasient transfections and gene reporter assays

Astrocytes were seeded at 50,000 cells/well in a 12-well culture plate 24 h before transfection to reach ~70% confluence by the time of transfection. Transient transfections were performed with 500 ng of each construct with Fugene 6 (Roche Applied Science) following the manufacturer's instructions. After 16–24 h, the medium was replaced with serum-free medium and 3 h later treated with 100 nM IGF-I. 25 μl of medium were used to measure PTEN promoter activity with the SEAP reporter system (BD Biosciences) using CSPD substrate (Tropix, USA) and quantified in a luminometer (Berthold Detection Systems, Germany). All samples were done in triplicate and independent experiments repeated at least four times. Transfections were normalized by co-transfection with a green fluorescent protein (peGFPN1) expression vector.

2.5. siRNA interference

Three different annealed siRNAs were pre-designed for rat Egr-1 and PTEN genes (RefSeq Number NM_012551 and NM_031606, respectively) by Ambion. The siRNA sequences used for Egr-1 were 5′GGACTTAAAGGCTCTTAATAtt3′, 5′CCTTTTCTCCTAGGACAATtt3′ and 5′GGACAAGAAAGCAGACAAAtt3′ and oligonucleotides to silence PTEN expression were 5′CCCTTTTGTGAAGATCTTGtt3′, 5′GGTTTTTGGATTCAAAGCAtt3′and 5′GGAACAATATTGATGATGTtt3′. Annealed siRNAs were dissolved in 250 μl of RNAse free water at 20 mM. siPORTNeoFx transfection reagent (Ambion) was used 3:1(v/v) in OPTI-MEM I reduced serum medium (Gibco). After 15 min at room temperature the RNA/transfection agent complexes were IGF-I in panel C dispensed into the empty well of a 12-well culture plate. Then, astrocytes were transferred to the culture plate containing the RNA/transfection agent at a cell density of 9×10^4 cells/well. After gently mixing, cells were incubated for different times as indicated. A positive control with a validated siRNA targeting rat glyceraldehyde-3-phosphate dehydrogenase (GADPH) and a negative control siRNA scrambled sequence that bears no homology to human, rat or mouse genomes were used in parallel (Ambion).

2.6. Western blots

Western blotting was performed as described previously [22]. Cultures were washed once with ice-cold PBS and lysed with PIK buffer (150 mM NaCl, 20 mM Tris HCl pH: 7.4, 1% Nonidet P-40, 1 μg/ml aprotinin, 1 μg/ml leupeptin and 1 mg/ml phenylmethylsulfonyl fluoride). To normalize for protein load, membranes were reblotted (Re-Blot plus mild solution, Chemicon, USA) and incubated with an anti-β-actin antibody. Levels of the protein under study were expressed relative to protein load in each lane as determined by β-actin content. Different exposures of each blot were collected to ensure linearity and to match control levels for quantification. Densitometric analysis was performed using Molecular Image Program (BioRad). A representative blot is shown from a total of at least three independent experiments.

2.7. RT-PCR

Total RNA from astrocyte cultures was isolated with TRIzol Reagent (Invitrogen) and used as template to synthesize cDNA using Mo-MLV reverse transcriptase (Promega, USA). Relative quantitative RT-PCR was performed using the QuantumRNA Kit (Ambion, USA) according to the manufacturer's conditions. PCR was performed with the following primers for PTEN cDNA: 5′ GACAGCCATCATCAAAGAGA 3′ and 5′ATCCAGGTGATTCTTTAACAG 3′, and normalized with the Universal 18S Internal Standard. Samples were loaded into a 1% agarose gel and bands were quantified by densitometric analysis using Analysis Image Program (BioRad).

2.8. Chromatin Immunoprecipitation (ChiP) assay

A Chromatin Immunoprecipitation Kit (Upstate) was used following the manufacturer's conditions. Astrocyte cultures (1×10^6 cells) were crosslinked after the different treatments with 1% formaldehyde (10 min at 37 °C). The cells were lysed, nuclear fractions collected and resuspended in SDS lysis buffer. Chromatin was sonicated and the suspension was precleared with Salmon Sperm DNA/Protein agarose-50% slurry. Chromatin from 1×10^6 cells was then incubated with 3 μg of anti-Egr-1 polyclonal antibody (Santa Cruz) or control without antibody and rotated overnight at 4 °C. The immune complexes were precipitated, washed, eluted and cross-links reversed. After treatment with proteinase K, phenol extraction and ethanol precipitation, immunoprecipitated chromatin was resuspended in 25 μl of H_2O. 5 μl of immunoprecipitate or 50 ng of total input DNA was used for 25 cycles of PCR amplification with the following primers specific to rat PTEN promoter: ChiP1 5′GACTCTTTGTGCACTGCG3′ and ChiP2 5′CAGCGGCTCAAC TCTCAA3′. PCR reactions were loaded on a 2% agarose gel and visualized by ethidium bromide staining.

2.9. PTEN lipid phosphatase activity

Phosphate released from substrates was measured with a Malachite Green Phosphatase Assay (Echelon, USA). After treatments, astrocyte cultures were

washed with ice-cold PBS and lysed with PIK buffer (see above). 250 μg of total protein were 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*-octanoylglyceryl, 3-*O*-phospho-linked-phosphatidylinositol phosphate (PI(3,4,5)P3). 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 to a standard curve of KH_2PO_4 in distilled H_2O.

2.10. PKC and PKA activity

The PepTag assay (Promega) utilizes a brightly coloured fluorescent peptide substrate that is highly specific to PKC or PKA kinase, respectively. Phosphorylation by the kinase changes the net charge of the substrate from +1 to −1, thereby allowing the phosphorylated and nonphosphorylated versions of the substrate to be separated on an agarose (0.8%) gel. The phosphorylated species migrates towards the positive electrode, while the nonphosphorylated substrate migrates towards the negative electrode. After treatment, astrocyte cultures (1×10^6) were washed once with ice-cold PBS and lysed .Total lysates (10–25 μg in 10 μl) were incubated with the respective kinase reaction mixture (25 μl) following the manufacturer's protocol. After adding 80% glycerol (1 μl), the samples were loaded onto an agarose gel (0.8% in 50 mM Tris–HCl, pH 8.0) and run at 100 V for 15–30 min. The bands were visualized under UV light. Immediately after photographing the gel, the negatively charged phosphorylated bands were excised and heated at 95 °C until melted. The volume was adjusted to 250 μl with water. A hot agarose solution (175 μl) was added to a separate tube containing 75 μl of gel solubilization solution, 100 μl of glacial acetic acid, and 150 μl of distilled water. Using absorbance reading at 570 nm, the number of units of kinase activity in each slice of agarose was measured following the manufacturer's instructions.

2.11. Cell proliferation assay and cell counting

Cell proliferation was measured with a DHL Cell viability kit (Anaspec). This fluorescence method detects cytoplasmic lactate dehydrogenase (LDH) as an indicator of cell numbers. Astrocyte cultures were seeded in 48-well culture plates (1×10^3cells/well). After 3 h of serum starvation cells were treated with 100 nM IGF-I, and 40 μl of resazurin (that reacts with endogenous LDH to produce the reaction) was added per well and incubated for 30 min at 37°C in the dark. Fluorescence was measured at an excitation/emission wavelength of ~540/590. In cell counting experiments, cells (9×10^4 cells) were plated onto 12-well plates and 24 h after siRNA transfection (see above) serum starved and stimulated with IGF-I or vehicle for the indicated times. Cells were gently washed with PBS, trypsinized, harvested, and resuspended. Cell number was determined by counting with the aid

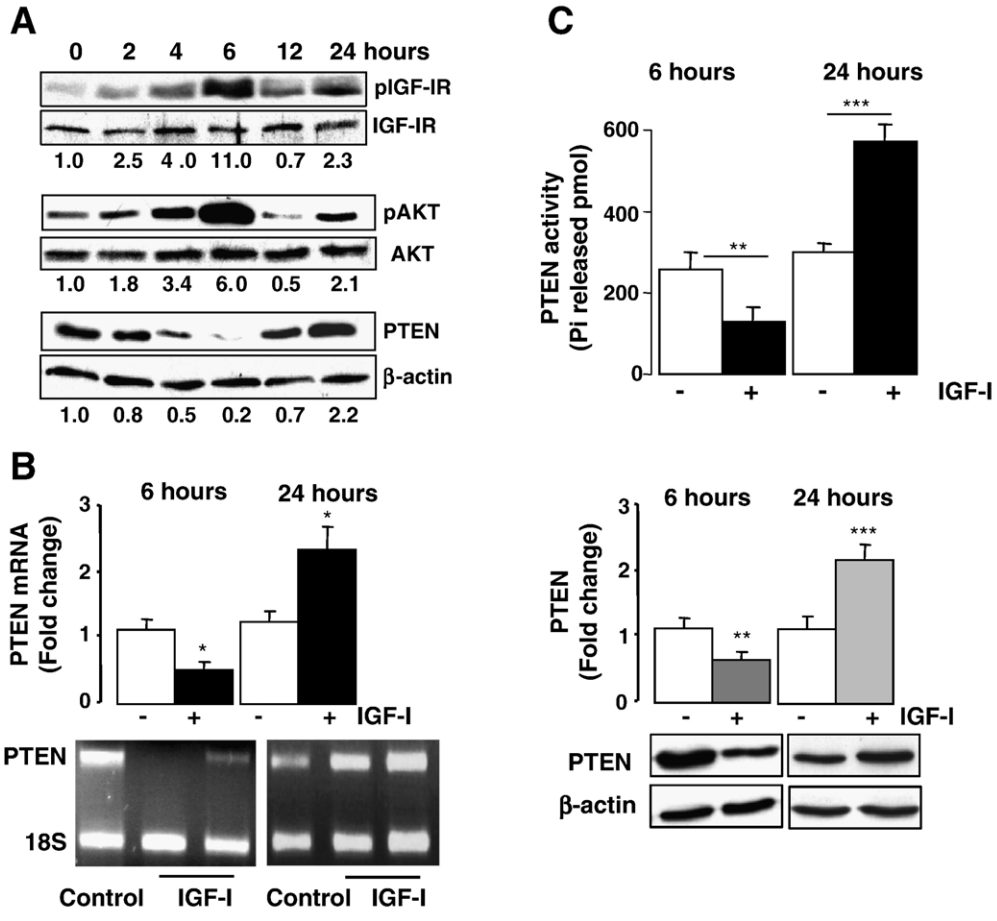

Fig. 1. IGF-I modulates PTEN in astrocytes. A, Stimulation of astrocytes with 100 nM IGF-I resulted in increased levels of phosphorylation of the IGF-I receptor and Akt while levels of PTEN were modulated in a biphasic manner. Cells were exposed from 0 to 24 h. Numbers below blots: fold changes of control levels of the ratios between phosphorylated and total IGF-I receptor and Akt, respectively, or total protein load (β-actin levels) for PTEN levels. Representative blots are shown. B, Changes in PTEN mRNA levels (left) after IGF-I paralleled those in protein levels (right): an early decrease (6 h) followed by an increase at later times (24 h). Left: representative gels stained with ethidium bromide and quantified (histograms). PTEN cDNA (480 bp) and the internal standard 18S (315 bp) are indicated ($n=3$). Right: representative western blots of PTEN and quantification histograms using β-actin for normalization ($n=7$). C, In parallel to the changes seen in PTEN levels, its lipid phosphatase activity was decreased 6 h after IGF-I and increased 24 h later ($n=3$). *$p<0.05$, **$p<0.01$, and ***$p<0.001$ vs respective controls.

of a hemacytometer under an inverted light microscope. All experiments were done in triplicate at least 3 times.

2.12. Statistics

A Student's *t*-test was used to calculate differences between groups; differences were considered significant at $p<0.05$.

3. Results

3.1. IGF-I modulates PTEN in astrocytes

In pilot studies analyzing cell-specific IGF-I signalling on brain cells, we observed a marked increase in the expression of PTEN mRNA 24 h after the exposure of forebrain astrocytes to IGF-I (Supplementary Fig. 1). We confirmed this initial observation by determining that the transcriptional activity of the PTEN promoter (p-PTEN) was increased in response to IGF-I. A dose-dependent stimulatory effect of IGF-I on PTEN promoter activity was observed up to 1 nM, while the stimulation reached a plateau at 24 h (not shown). Since 100 nM IGF-I produced the highest effect, we used this dose for subsequent studies to obtain robust responses to IGF-I. Other neurotrophic factors such as NGF (50 and 100 ng/ml) did not modify PTEN in astrocytes while BDNF (50 and 100 ng/ml) produced a prolonged modest, but significant decrease in the levels of this phosphatase at the lower dose tested (not shown).

Detailed characterization of the action of IGF-I on PTEN was then conducted. Exposure of forebrain astrocytes to IGF-I increased phosphoIGF-I receptor/phosphoAKT levels and elicited a biphasic change in PTEN levels; and initial decrease followed by a rebound (Fig. 1A). PTEN mRNA levels were affected in parallel;

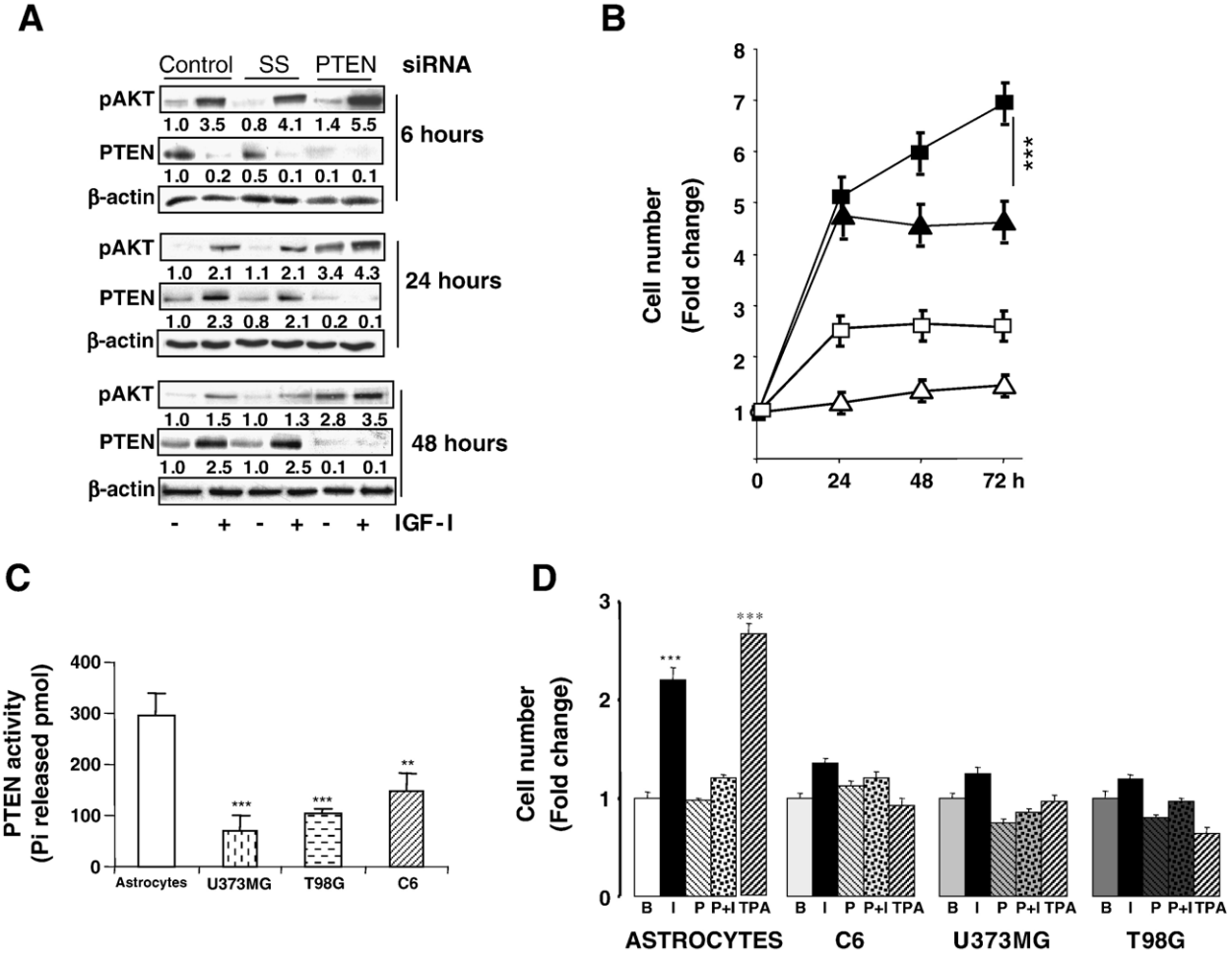

Fig. 2. Reduced PTEN levels and IGF-I mitogenesis on astrocytes. A, Reduction of PTEN protein levels by siRNA markedly diminished PTEN levels in the cultures while pAKT levels after IGF-I were higher. Non-transfected astrocytes (control), or astrocytes transfected with a scramble sequence siRNA (SS) showed no major changes in protein levels. Western blots were done after 6, 24 and 48 h of IGF-I exposure ($n=4$, representative blots are presented). Fold changes in protein levels (after normalization with β-actin) are shown. B, Inhibition of PTEN expression after 24 h of siRNA transfection resulted in significantly enhanced cell growth after IGF-I. Note that cell proliferation was markedly augmented even without IGF-I. Cell growth was expressed as percent of the number of cells at the start of the experiment (time 0). Control cultures received SSsiRNA. ($n=5$). ***$p<0.001$ IGF-I+siRNA PTEN vs Control+siRNA PTEN. Although not indicated, cell growth in siRNA PTEN-transfected cultures was significantly greater than control cultures receiving SSsiRNA alone. (■) PTEN siRNA+IGF-I, (□) SSsiRNA+IGF-I, (▲) PTEN siRNA control, (△) SS siRNA control. C, PTEN phosphatase activity was significantly lower in the 3 glioma cells lines tested. **$p<0.01$ and ***$p<0.001$ vs normal astrocytes. ($n=3$). D, While normal astrocytes proliferated in response to IGF-I (I) or the PKC agonist TPA (TPA), glioma cells did not. The IGF-I receptor antagonist picropodophyllin (P) blocked IGF-I activity in normal astrocytes and did not affect basal proliferation in the cell lines ($n=4$). ***$p<0.001$ vs basal levels (B).

reduced after 6 h and increased after 24 h (Fig. 1B). PTEN phosphatase activity was similarly affected; decreased after IGF-I exposure and enhanced at later times (Fig. 1C). However, levels of phosphorylated PTEN did not parallel these changes, as only at late times they were reduced (not shown). The modulatory actions of IGF-I on astrocyte PTEN were mediated through the IGF-I receptor (IGF-IR) since they were abrogated by transfection of a dominant negative (DN) IGF-IR mutant (Suppl Fig. 2A,B). On the contrary, overexpression of the wild type (WT) IGF-IR was sufficient to replicate IGF-I actions on PTEN (Suppl Fig. 2A,B). This is likely related to the fact that astrocytes in culture produce IGF-I [23]. Confirming previous gene-screens, the modulatory effect of IGF-I on PTEN was not observed in neurons. Neurons obtained from the cerebellum, forebrain or the hypothalamus were exposed to IGF-I but no change in PTEN levels was found (not shown). On the contrary, PTEN was modulated by IGF-I in astrocytes obtained from other brain regions such as the hippocampus or the cerebellum (not shown). The temporal pattern of changes in PTEN levels depended on sustained IGF-I stimulation. Exposure of the cells during 2 or 4 h to IGF-I was not enough to decrease PTEN levels at 6 h (Suppl Fig 2C). Similarly, PTEN levels at 24 h increased after 24 h of continuous exposure to IGF-I, but not after shorter exposure times of 6 or 12 h (Suppl Fig. 2C).

To determine the functional significance of the inhibition of PTEN by IGF-I, we tested the mitogenic activity of IGF-I in astrocytes transfected with PTEN siRNA to reduce its levels (Fig. 2A). As already reported [18], low levels of PTEN alone were sufficient to stimulate astrocyte proliferation markedly, but astrocyte proliferation was further increased by IGF-I (Fig. 2B). As expected, under low PTEN levels pAKT levels remained elevated after IGF-I, without returning to baseline values (Fig. 2A). However, in 2 human and 1 rat (C6) glioma cell lines with greatly reduced PTEN activity (Fig. 2C), IGF-I was not mitogenic (Fig. 2D). This lack of effect of exogenously added IGF-I in glioma proliferation was not due to autocrine production of this growth factor as glioma proliferation was not affected by picropodophyllin, an antagonist of the IGF-IR (Fig. 2D).

3.2. Pathways involved in PTEN inhibition by IGF-I

We searched for pathways involved in the modulatory effects of IGF-I on PTEN. Addition of the PI3K inhibitor LY294002 to astrocytes resulted in markedly reduced levels of PTEN (Fig. 3A), indicating that PI3K activity is required to maintain normal levels of this phosphatase in astrocytes. IGF-I was unable to rescue the inhibitory effect of PI3K inhibition, suggesting that PI3K is in the pathway that IGF-I modulates to regulate PTEN levels. Another PI3K inhibitor, wortmannin gave similar results (not shown). Downstream signalling of PI3K through AKT was not involved in the effect of IGF-I on PTEN, as dominant negative AKT did not

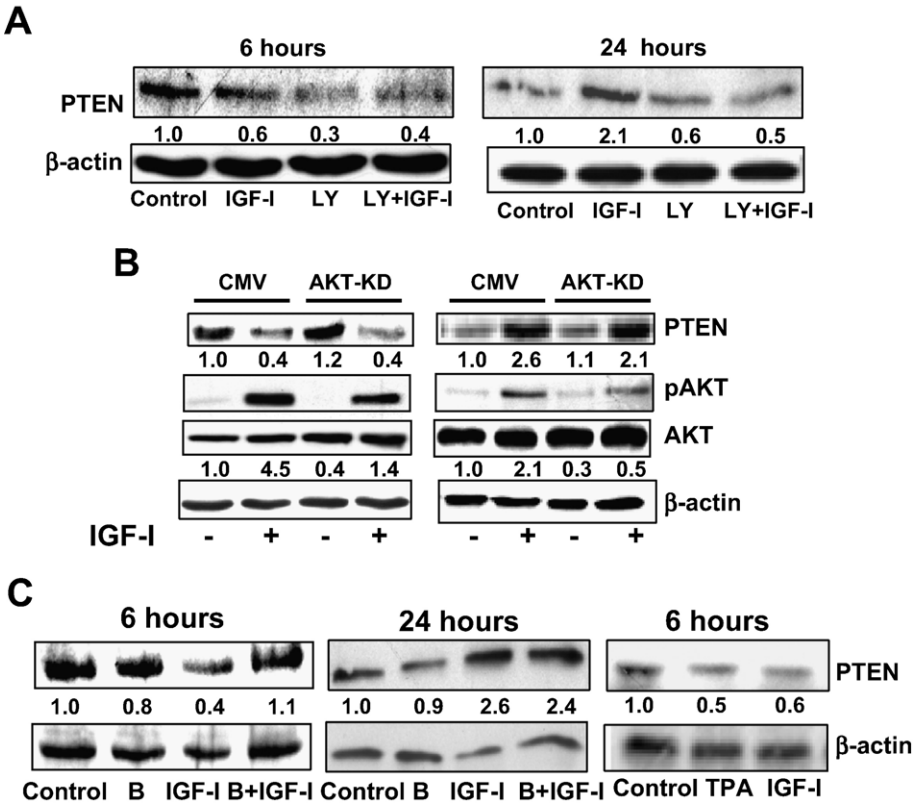

Fig. 3. Pathways involved in inhibition of PTEN by IGF-I. A, Blockade of PI3K activity with LY294002 (10 μM) inhibited basal levels of PTEN as well as interfered with the modulation at 6 and 24 h produced by IGF-I. B, While in DN-AKT-, but not in control-transfected (CMV) astrocytes, IGF-I was not able to stimulate AKT activity (pAKT/AKT ratio shown below the AKT blot), the modulatory effects of IGF-I on PTEN remained unaltered. C, Addition of the PKC inhibitor bisindolylmaleimide (B, 1 μM) to the cultures abrogated early (6 h) inhibition of PTEN by IGF-I without affecting late increases (24 h). The PKC stimulator TPA (100 nM) mimicked early effects of IGF-I on PTEN (right upper panel).Representative blots are shown in all panels. Levels are indicated as fold changes of controls as in previous figures.

affect the PTEN response to IGF-I (Fig. 3B). Activation of MAPK after IGF-I was not involved either because its blockade did not affected the inhibitory action of IGF-I on PTEN (Suppl Fig. 2D). Thus, we searched for alternative pathways downstream of PI3K. We found that the PKC inhibitor bisindolylmaleimide IX (B, 1 μM) specifically abrogated the reduction of PTEN levels after IGF-I (Fig. 3C). To confirm the involvement of this kinase we treated cells with the PKC-activating phorbol ester 12-*O*-tetradecanoyl phorbol-13-acetate (TPA, 100 nM). TPA mimicked the reduction of PTEN levels by IGF-I (Fig. 3C) as well as its proliferative effects in normal, but not in transformed astrocytes (Fig. 2D). Interestingly, increased PTEN levels found at later times after IGF-I were not affected by PKC interference, suggesting that this increase did not reflect a mere post-inhibitory rebound. Measurement of PKC activity confirmed that IGF-I activated this kinase at 6 but not at 24 h (Fig. 4A). IGF-I required PI3K to activate PKC because the PI3K inhibitor LY294002 blocked it (Fig. 4B). To determine which PKC isoform could be implicated in the modulatory actions of IGF-I, we exposed astrocytes to the concentration dependent inhibitor of PKCα, β and ε, Ro-32-0432 (ID_{50} of 10 μM, 30 μM, and 110 μM, respectively). At 10 μM, Ro-32-0432 already reverted the effects of IGF-I (Fig. 4C), pointing to an involvement of PKCα in the actions of IGF-I.

We then looked for mechanisms downstream of PKC activation leading to reduced PTEN levels. In astrocytes treated with the proteasome inhibitor MG132, PTEN protein levels were not reduced after IGF-I (Fig. 5A). This suggested that proteasome activity was involved in the inhibitory effects of IGF-I on PTEN. However, reduced PTEN mRNA after IGF-I (Fig. 1B) suggested that direct proteasome activity might not be involved. Rather, reduced PTEN mRNA could be due to decreased PTEN transcription. We therefore examined whether the transcription factor Early growth response-1 (Egr-1), involved in the response to IGFs and previously shown to positively modulate PTEN transcription [24,25] was upstream of the inhibitory action of IGF-I on PTEN. Indeed, we observed that IGF-I decreased the levels of Egr-1 (Fig. 5B). Furthermore, MG-132 addition to IGF-I-treated astrocytes abrogated this effect (Fig. 5C). Collectively, these data suggest that IGF-I induced degradation of Egr-1 through the proteasome. Reduced Egr-1 levels diminished the activity of this transcription factor and consequently, reduced PTEN expression. To further confirm this possibility, we determined whether decreased Egr-1 after IGF-I also depended on PKC activity. We observed that not only the pan-specific PKC inhibitor bisindolylmaleimide abrogated the effect of IGF-I (Fig. 5D), but also the isoform specific Ro-32-0432 eliminated the effect of IGF-I (Fig. 5E) at the same doses seen with PTEN (Fig. 4C). We also tested whether Egr-1 binding to the p-PTEN was modulated by IGF-I. As determined with a chromatin immunoprecipitation (ChIP) assay, reduced levels of Egr-1 protein after IGF-I (Fig. 5B)

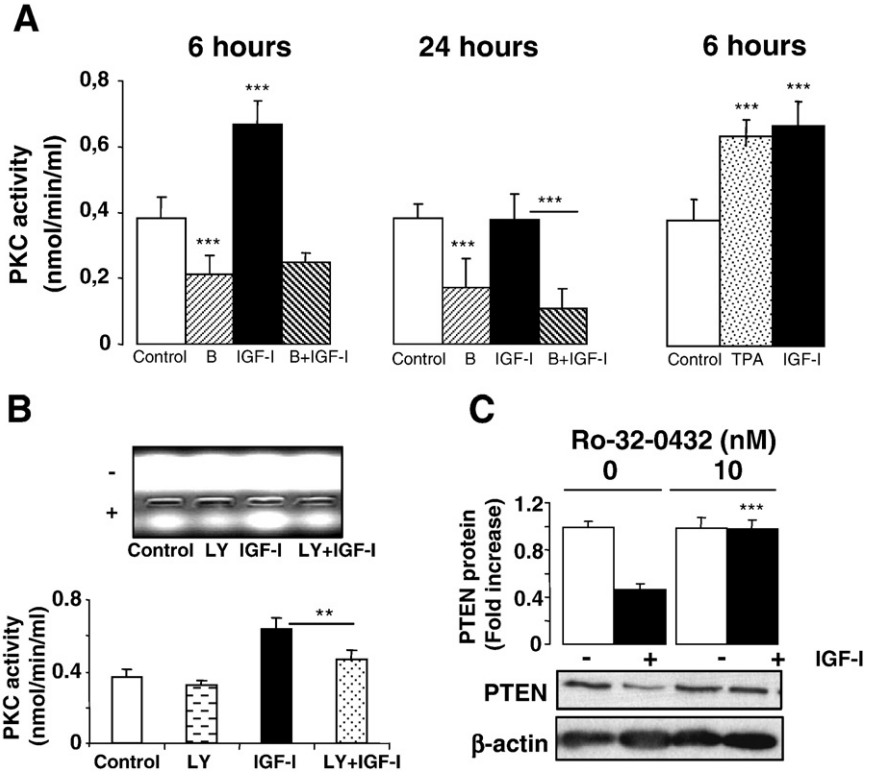

Fig. 4. IGF-I reduces PTEN through PKC. A, PKC activity is increased in astrocytes early after IGF-I addition (6 h) but not after 24 h. The PKC inhibitor Bisindolylmaleimide IX (B) reduced basal and IGF-I-stimulated PKC activity whereas the PKC agonist TPA mimicked the action of IGF-I at 6 h ($n=4$). B, PI3K is involved in stimulation of PKC by IGF-I as the PI3K inhibitor LY294002 (LY) abrogated the action of IGF-I. Representative agarose gels showing the bands of phosphorylated (+)/non-phosphorylated (−) PKC substrate. Quantitation of the excised bands is shown in histograms. C, Exposure to Ro-32-0432, a selective inhibitor of PKCα (10 nM), βI (30 nM) and ε (110 nM) isoforms reverted the PTEN protein decrease after 6 h of IGF-I already at 10 nM. Representative blots and quantitation histograms are shown ($n=3$). ***$p<0.001$, **$p<0.01$ vs respective control groups.

paralleled reduced interaction of Egr-1 with the p-PTEN (Fig. 5F). Finally, we inhibited Egr-1 through siRNA interference in astrocytes expressing a p-PTEN reporter vector and found that reduced Egr-1 resulted in reduced activity of p-PTEN in either the absence or the presence of IGF-I (Fig. 5G,H; $p<0.001$ vs SS-transfected astrocytes), indicating that Egr-1 is required to maintain the activity of p-PTEN.

3.3. Pathways involved in PTEN recovery after IGF-I

We next searched pathways underlying recovery of PTEN levels and activity after IGF-I-induced inhibition (Fig. 1), and found that the PKA inhibitor H89 (1 μM), abrogated late increases in PTEN after IGF-I without affecting the early inhibitory phase (Fig. 6A). That PKA is involved in this late phase is reinforced by the observation that the PKA agonist forskolin (2 μg/ml) reproduced the late action of IGF-I on PTEN (Fig. 6A). Furthermore, PKA kinase activity is activated by IGF-I after 24 h but not at 6 h (Fig. 6B). Accordingly, astrocytes transfected with a dominant negative PKA-α1 subunit showed a decreased expression of PTEN after 24 h of IGF-I (Fig. 6C). We also determined that activation of PKA through IGF-I required PI3K as LY294002 inhibited it (Fig. 6D). Altogether this data indicated that after IGF-I, an active PKA is necessary for the recovery of PTEN.

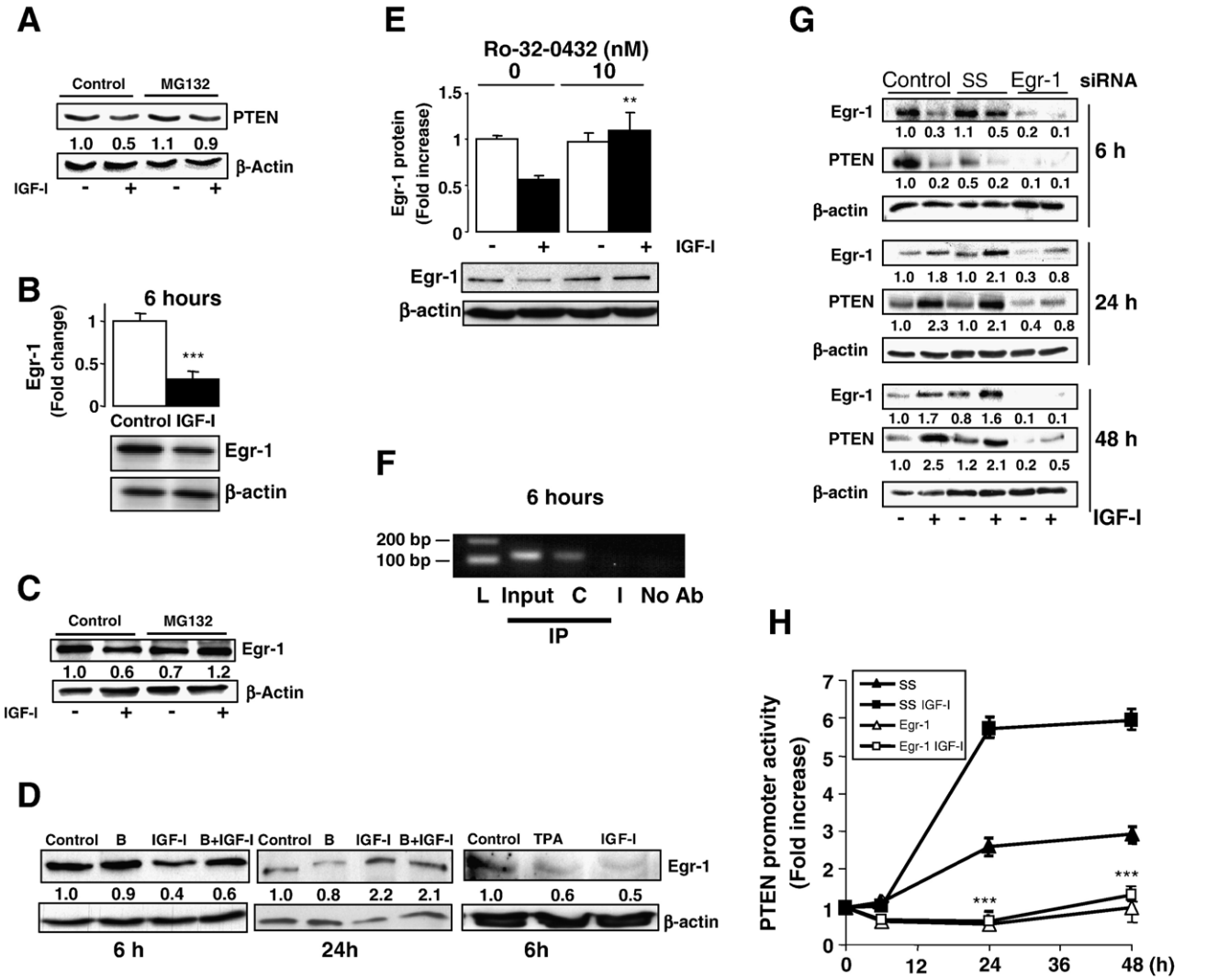

Fig. 5. IGF-I inhibits PTEN by decreasing Egr-1 levels in astrocytes. A, Incubation with the proteasome inhibitor MG132 (5 μM) abolished the reduction of PTEN. B, Levels of Egr-1 are reduced by IGF-I at 6 h. Densitometric analysis of western blots is shown in histograms. C, MG132 also blocked the reduction of Egr-1 by IGF-I (compare bands in lanes 2 and 4). D, Addition of the PKC inhibitor bisindolylmaleimide (B, 1 μM) to the cultures abrogated early (6 h) inhibition of Egr-1 by IGF-I without affecting late increases (24 h). The PKC stimulator TPA (100 nM) mimicked early effects of IGF-I (blots in the right). E, The isoform specific inhibitor Ro-32-0432 reverted the decrease of Egr-1 after 6 h of IGF-I already at 10 nM. F, IGF-I reduces the binding of Egr-1 to the PTEN promoter at 6 h, as shown by chromatin immunoprecipitation assays (ChiP). A non-immune control (No Ab) and a non-specific antibody (Input) were used as controls. (L, ladder; C, control and I, IGF-I). Detection of the captured PTEN GC-rich 113-bp promoter fragment was performed by PCR. G, Reduced Egr-1 levels with siRNA reduces PTEN levels in astrocytes and disrupts the biphasic regulation of PTEN by IGF-I. Astrocytes were cotransfected with Egr-1 siRNA (30 nM) or a siRNA scramble sequence (SS) as a control. H, Accordingly, the activity of the PTEN promoter decreased regardless of the presence or absence of IGF-I when Egr-1 levels were decreased by siRNA. Astrocytes were cotransfected with the respective siRNAs and a PTEN promoter reporter vector. Egr-1 and PTEN levels are shown as fold change after normalization with β-actin levels. Representative experiments are shown. n = at least 3 independent experiments.

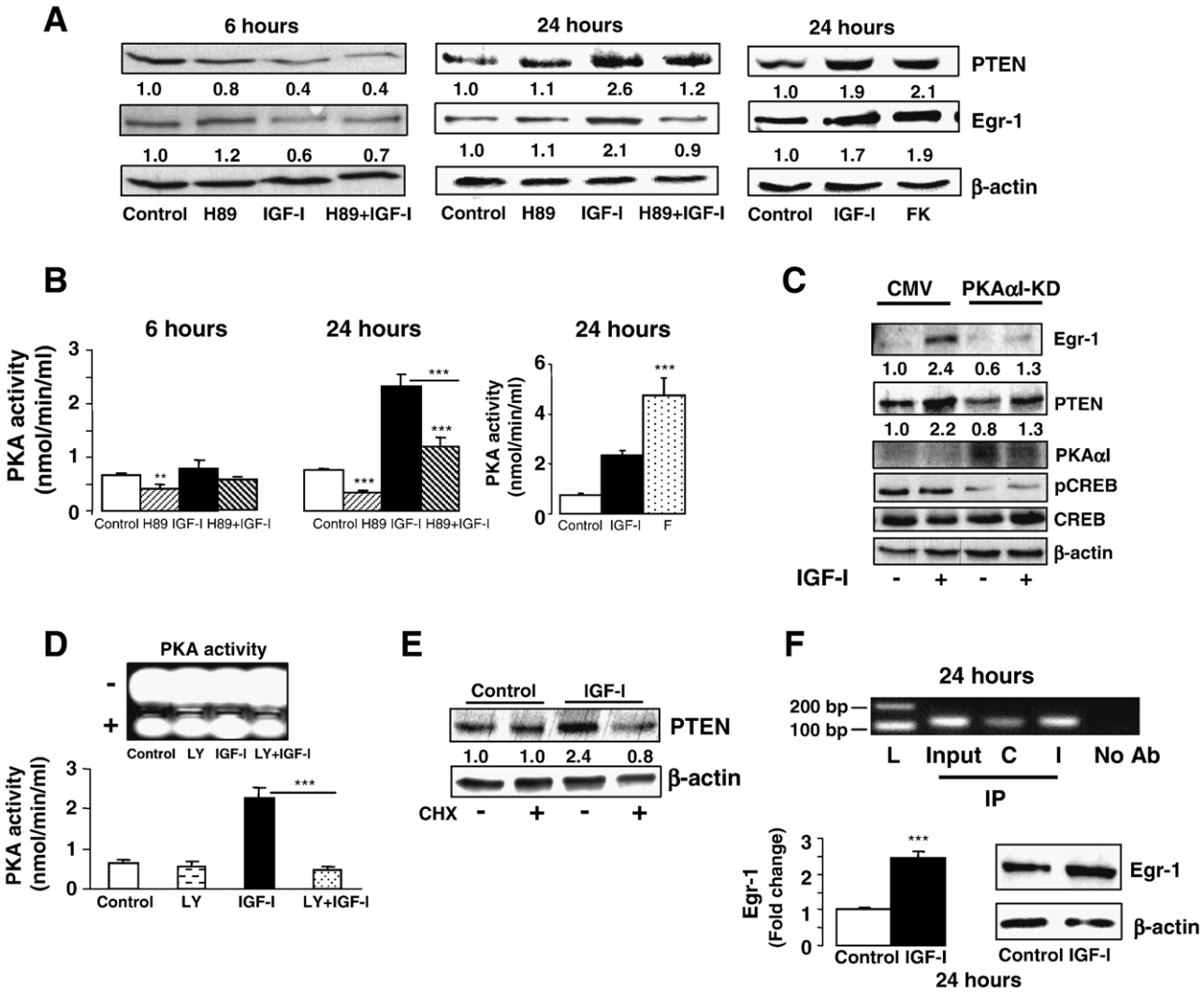

Fig. 6. Recovery of PTEN after IGF-I depends on PKA and Egr-1. A, Inhibition of PKA with H89 (1 μM) blocked late but not early effects of IGF-I on Egr-1 and PTEN (24 and 6 h, respectively). Exposure to forskolin (2 μg/ml, F), a PKA agonist, resulted in stimulation of Egr-1 and PTEN at 24 h, mimicking the action of IGF-I. (n=4). B, PKA activity is increased in astrocytes after 24 h but not after 6 h of IGF-I. Inhibition with H89 or stimulation with forskolin resulted in expected changes in PKA activity. C, In the presence of a dominant negative PKA (PKAαI–KD) IGF-I only weakly stimulates Egr-1 and PTEN levels at 24 h. CMV: mock-transfected controls. Note that levels of PKAαI were increased in transfected cultures while levels of pCREB, that is downstream of PKA activation were reduced. Representative blots are shown (n=3). D, PI3K is involved in stimulation of PKA by IGF-I as LY294002 (LY) abrogated the action of IGF-I. Representative gels showing the bands of phosphorylated (+)/non-phosphorylated (−) PKA substrate and quantitation histograms are shown. E, Inhibition of protein synthesis with cycloheximide (CHX, 10 μg/ml) abrogated stimulation of PTEN by IGF-I at 24 h. F, IGF-I stimulates binding of Egr-1 to the PTEN promoter at 24 h. Note the larger band corresponding to the PTEN promoter in IGF-I (I)-treated astrocytes. (L, ladder; C, control). A parallel increase in levels of Egr-1 was observed after 24 h of IGF-I. Densitometric analysis of western blots is shown in histograms (n=3). ***p<0.001, **p<0.01 vs respective controls. Representative blots are shown.

The increase in PTEN protein levels depended on "de novo" protein synthesis as cycloheximide (CHX) abrogated it (Fig. 6E). In addition, because PTEN expression depends on Egr-1 levels (Fig. 5F), we confirmed that Egr-1 levels were increased after 24 h of IGF-I, and in a PKA-dependent fashion (Fig. 6A,C). Furthermore, with the ChiP assay we observed that in parallel to increased levels of Egr-1, binding of this transcription factor to the p-PTEN was increased 24 h after addition of IGF-I to the astrocyte cultures (Fig. 6F).

4. Discussion

IGF-I signalling is associated with a great variety of cellular functions (reviewed in [26]. However, our understanding of the molecular pathways underlying IGF-I signalling on target cells is far from complete. The present results point to a novel PI3K-dependent pathway in astrocytes that comprises timed activation of PKC and PKA by IGF-I which in turn results in biphasic modulation of the transcription factor Egr-1 and its downstream target the phosphatase PTEN. Timed regulation of PTEN was not found in neurons, which provides a clear example of cell-specific signalling by IGF-I on brain cells. The fact that IGF-I (and its mimetic TPA) was not mitogenic in various glioma cell lines sharing in common low PTEN levels, while in normal astrocytes the mitogenic response was enhanced under low PTEN conditions, suggests that the reduction of PTEN through PI3K/PKC is a permissive, but not a a key step in the mitogenic actions of IGF-I. Conversely, the late increase in PTEN through

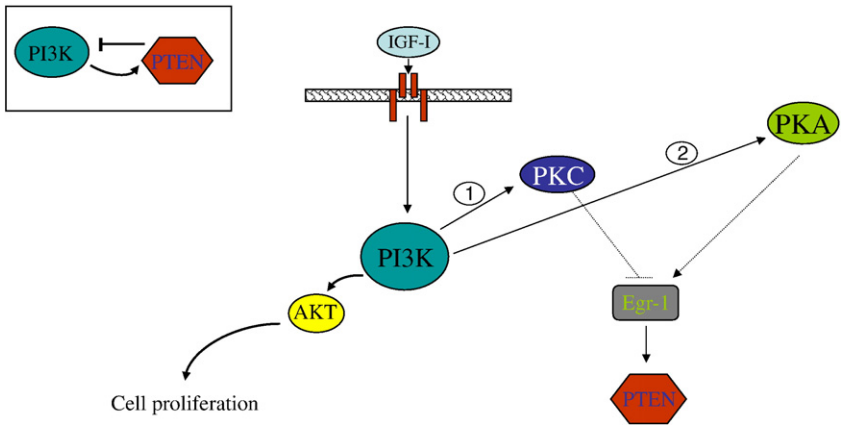

Fig. 7. Homeostatic regulation of PTEN by PI3K in astrocytes is modulated by IGF-I. In the steady state, there is a reciprocal regulation of PTEN and PI3K (inset). Upon activation of the IGF-I receptor, the PI3K/Akt pathway is activated to enhance cell proliferation. However, as shown now by our results, IGF-I activation of PI3K in astrocytes also leads to timed activation of PKC and PKA. 1) initial activation of PKC and reduction of Egr-1 levels (probably by directing its proteasome degradation through an as yet unknown process) that are crucial to maintain PTEN levels in the cell, and consequently PTEN levels decrease; and 2) delayed activation of PKA that results in enhanced levels of Egr-1 (probably through enhanced transcriptional activity induced by PKA-stimulated CREB) that eventually restores PTEN levels. Dotted pathways indicate the need for further analysis because additional unknown steps are likely involved. While the mechanism involved in timed activation of PKC and PKA by PI3K remains to be established it is probably related to relative levels of PI3K activity.

PI3K/PKA appears as an homeostatic recovery of PTEN function in astrocytes with no relation to the mitogenic effects of IGF-I. Indeed, overexpression of PTEN in wild type astrocytes did not modify the mitogenic activity of IGF-I (not shown). This is in line with the proposal that PTEN is a constitutively active phosphatase subject to negative regulation [27]. At any rate, and as recently outlined [28], it is difficult to assign a specific physiological significance for PTEN regulation and further work in this regard is warranted.

However, these observations allow to start delineating the existence of a complex network between IGF-I and PTEN through PI3K (Fig. 7), and suggest that the regulation of PTEN activity not only includes reversible postraslational modifications such as phosphorylation or oxidation but may also include the regulation of its cellular levels. The latter seems to involve the growth factor-sensitive transcription factor Egr-1, which has been shown previously to be involved in downstream signalling by IGFs [25,29,30] and to be upstream of PTEN [25]. This is confirmed by the present findings showing that the levels of Egr-1 are a critical factor in regulating PTEN synthesis in astrocytes. Reduced levels of Egr-1 after IGF-I were accompanied by decreased binding of Egr-1 to p-PTEN and lower levels of PTEN mRNA. Inhibition of Egr-1 by IGF-I was mediated by PI3K-dependent activation of PKC-previously shown to have either inhibitory or stimulatory effects on this transcription factor [31,32]. Activation of PKC by IGF-I led to enhanced degradation of Egr-1 by the proteasome pathway as the inhibitory drug MG132 abrogated the decrease in Egr-1/PTEN after IGF-I. However, the link between PKC activation and Egr-1 degradation requires further analysis. Conversely, recovery of PTEN after IGF-I includes activation in a PI3K-dependent fashion of PKA, which in turn results in increased Egr-1, greater Egr-1 binding to the PTEN promoter and enhanced PTEN mRNA and protein levels. Indeed, the activity of the p-PTEN is regulated by Egr-1 levels. That recovery of PTEN levels is through de novo protein synthesis is reinforced by the observation that cycloheximide blocks it. Significantly, the Egr-1 gene promoter has cAMP-sensitive consensus sites [33], and our preliminary data show that activation of the downstream transcription factor CREB may be involved in PKA regulation of Egr-1. Therefore, increased transcriptional activity of the Egr-1 gene in response to PKA may drive recovery of PTEN levels.

While the existence of additional intermediate steps along this intricate pathway needs to be determined, in particular the cellular mechanism supporting delayed activation by IGF-I of PKA through PI3K, these findings provide a physiological context for the recently described regulatory action of PI3K on PTEN [19]. Our observations also provide a deeper insight into the role of PI3K on PTEN regulation showing that it can be either negative (through PKC) or positive (through PKA).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamcr.2007.10.020.

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