IGF-I inhibition of apoptosis is associated with decreased expression of prostate apoptosis response-4

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Abstract

The neuronal damage caused by ischemic brain injury is associated with increased apoptosis. IGF-I exposure promotes neuronal defense and survival against ischemic insult by inhibiting apoptotic processes. We investigated the role of prostate apoptosis response-4 (Par-4), a proapoptotic gene the expression of which is increased after ischemic injury, in IGF-I-mediated inhibition of apoptosis using PC12 cells exposed to oxygen-glucose deprivation (OGD). The OGD insult resulted in significant increases in apoptotic cell death and Par-4 expression, which were prevented by the treatment of cells with an antisense oligonucleotide of Par-4. IGF-I treatment prior to OGD insult significantly reduced the number of apoptotic cells and the OGD-induced increase in Par-4 expression. OGD-induced nuclear translocation of

Par-4 was also attenuated by IGF-I treatment. In addition, we demonstrated that the anti-apoptotic effect of IGF-I was blocked by chemical inhibition of a mitogen activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), or protein kinase A (PKA), but not by a protein kinase C inhibitor. Finally, pretreatment of cells with a MAPK or PI3K inhibitor attenuated IGF-I-induced inhibition of Par-4 expression, suggesting that the MAPK and PI3K pathways contribute to IGF-I-induced Par-4 suppression. In contrast, a PKA inhibitor failed to alter the inhibitory effect of IGF-I on Par-4. These findings indicate that in PC12 cells exposed to OGD insult, IGF-I protects cells from apoptosis, at least in part through the inhibition of Par-4 expression.

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Introduction

Insulin-like growth factor (IGF)-I, a 70-amino acid polypeptide trophic factor, plays an important role in the regulation of metabolism, cellular function, growth, and differentiation (Feldman *et al.* 1997). The biological actions of IGF-I are mediated via the IGF-I receptor, a tyrosine kinase receptor (LeRoith *et al.* 1995). IGF-I has been known to successfully reduce the damage after ischemic lesions in experimental animal models (Guan *et al.* 1993, Tagami *et al.* 1997, Fernandez *et al.* 1999, Brywe *et al.* 2005). *In vitro*, IGF-I has been shown to act as a survival factor and inhibit apoptosis in a number of cell types (Rodriguez-Tarduchy *et al.* 1992, Chun *et al.* 1994, Parrizas *et al.* 1997, Russell *et al.* 1998, Delaney *et al.* 1999, Takadera *et al.* 1999).

The neuronal damage caused by ischemic brain injury is due to a reduction in the oxygen and glucose supply, i.e., oxygen-glucose deprivation (OGD). The OGD insult, followed by reoxygenation, is thought to be an *in vitro* model of ischemic injury mimicking the pathological conditions of stroke. Although the precise mechanistic pathway of ischemic neuronal cell death is still not clearly understood, one of the mechanisms involved is apoptosis (Mattson *et al.* 2000). Prostate apoptosis response-4 (Par-4) is

a proapoptotic gene initially identified in prostate tumor cells undergoing apoptosis (Sells *et al.* 1994). The expression of endogenous Par-4 is increased after transient focal ischemia in mice. Moreover, antisense oligonucleotide of Par-4 protects cultured cells against apoptosis and reduces damage after ischemic brain injury (Culmsee *et al.* 2001). These findings indicate that the increase in Par-4 levels plays a pivotal role in ischemic neuronal cell death. Considering that IGF-I acts as an anti-apoptotic factor, we hypothesized that IGF-I may inhibit apoptosis through the suppression of Par-4 expression in ischemic injury. However, to the best of our knowledge, the role of Par-4 in IGF-I-mediated inhibition of apoptosis has never been investigated. Therefore, to test this hypothesis, we examined the effect of IGF-I on the regulation of Par-4 expression using PC12 cells exposed to OGD insult.

Materials and Methods

Materials

Human recombinant IGF-I was obtained from Sigma Chemical Co. RPMI 1640 medium was from Gibco/Invitrogen. Primary antibodies to Par-4 and β -actin were

obtained from Abcam Inc. (Cambridge, UK). PD98059, wortmannin, LY294002, H89, and GF109203X were from Tocris (Ellisville, MO, USA). Par-4 antisense oligonucleotide (5'-ATA GCC GCC GGT CGC CAT GTT-3') and nonsense oligonucleotide (5'-CCG TGT CTG ATC TTC GTG CGT-3'; Duan *et al.* 1999) were purchased from Bioneer Co. (Daejeon, South Korea). All tissue culture reagents were obtained from Gibco/Invitrogen, and all other reagents were obtained from Sigma unless otherwise indicated.

Cell cultures

PC12 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin, in a humidified atmosphere of 5% CO₂. The cells were plated on collagen (50 µg/ml)-coated 100 mm dishes or 24-well plates. To determine whether IGF-I protects PC12 cells from OGD insult, cells were pretreated with IGF-I (10⁻⁸ M) or vehicle (saline) for 24 h, and then cells were exposed to OGD or maintained under normoxic conditions. Experiments were performed by adding the following pharmacological inhibitors to the culture media, PD98059 (50 µM), wortmannin (200 nM), LY294002 (2 µM), GF109203X (5 µM), or H89 (5 µM).

OGD

PC12 cells were exposed to OGD as previously described, with some modifications (Tabakman *et al.* 2004, Chung *et al.* 2007). On the day of the experiment, the regular high glucose (4.5 mg/ml) RPMI 1640 medium was replaced with OGD medium (glucose-free RPMI supplemented with 1% FBS). Cultures were then placed in a humidified 37 °C incubator within the Hypoxic Workstation (Daiki Sciences Co. Ltd by Ruskinn Technology, Bridgend, Mid Glamorgan, UK) containing a gas mixture of 0.1% O₂, 5% CO₂, and 94.9% N₂ for 3 h to initiate the OGD insult. OGD was terminated by replacing the OGD medium with normal culture medium containing 4.5 mg/ml glucose, and cultures were incubated for an additional 18 h under normoxic conditions.

Cell death and apoptosis

Cell viability was measured by the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described (Chen *et al.* 1996). Histone-complexed DNA fragments were quantified by cell death detection ELISA (Roche) according to the manufacturer's protocol.

Immunocytochemistry and confocal microscopy

For the evaluation of intracellular localization of Par-4, cells were fixed and probed with a Par-4 antibody and a secondary

antibody conjugated with the fluorescent dye. Nucleus was visualized by staining with DAPI. Fluorescence was captured using a 40× objective lens on a Carl Zeiss LSM 510 Meta (Oberkochen, Germany) confocal microscope (485 nm excitation and 535 nm emission).

Western blot analysis

Cells were lysed in a buffer containing 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 140 mM NaCl, 1% (w/v) Nonidet P-40, 1 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, 50 mM NaF, and 10 µg/ml aprotinin. Cell lysates were separated by 12% SDS-PAGE and electrotransferred to a polyvinylidene difluoride membrane (Bio-Rad). The membranes were soaked in blocking buffer (1× Tris-buffered saline, 1% BSA, and 1% nonfat dry milk) for 1 h and incubated overnight at 4 °C with the primary antibody. Blots were developed using a peroxidase-conjugated anti-rabbit IgG and a chemiluminescent detection system (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). The bands were visualized using a ChemicDoc XRS system (Bio-Rad) and quantified using Quantity One imaging software (Bio-Rad). The Par-4 band intensity was adjusted by the β-actin band intensity.

RT-PCR

Total RNA from PC12 cells was extracted using the Qiagen RNeasy Mini Kit (Qiagen Inc.) according to the manufacturer's instructions and was reverse transcribed using the Superscript II reverse transcriptase (Life Technologies Inc.) at 42 °C with random hexamer priming. An RNA control tube containing all RT reagents except the reverse transcriptase was included as a negative control to monitor genomic DNA contamination. The mRNA levels of Par-4 were measured by RT-PCR using β-actin as the internal standard. The sequences of primers for Par-4 and β-actin were as follows: Par-4, sense 5'-ACA ACA GTG ACA CGC TGG AG-3' and antisense 5'-CAG CCA ATA GGA AGG TCT GC-3' and β-actin, sense 5'-ATG GGT CAG AAG GAC TCC TAC G-3' and antisense 5'-AGT GGT ACG ACC AGA GGC ATA C-3'.

Statistical analysis

Data are presented as the mean ± S.E.M. (*n* = 4 per treatment). Each experiment was repeated at least twice. Statistical analysis between groups was performed using one-way ANOVA and the Holm-Sidak method for multiple comparisons using SigmaStat for Windows Version 3.10 (Systat Software Inc. Point Richmond, CA, USA). *P* < 0.05 was considered as statistically significant.

Results

Par-4 antisense oligonucleotide attenuates OGD-induced PC12 cell death and apoptosis

As shown in Fig. 1A, exposure of cells to OGD insult resulted in a significant increase in Par-4 protein levels, which were subsequently significantly decreased by the treatment with Par-4 antisense oligonucleotide. In order to determine whether the increase in Par-4 levels by the OGD insult plays a role in apoptotic PC12 cell death, we assessed cell survival in cultures treated with Par-4 antisense oligonucleotide. After a 3-h exposure of cells to OGD insult followed by 18-h reoxygenation, ~50% of the cells were not viable as determined by the MTT assay (Fig. 1B). However, treatment of cells with Par-4 antisense oligonucleotide significantly inhibited OGD-induced cell death. The apoptotic DNA fraction was significantly increased from 7.5% (normoxia) to 57.9% by OGD insult, whereas Par-4 antisense oligonucleotide treatment significantly decreased the percentage of apoptotic DNA to 30.9% (Fig. 1C) in comparison with cells treated with a control oligonucleotide with a scrambled sequence (54.6%).

IGF-I protects PC12 cells against OGD insult and inhibits the OGD-induced activation of Par-4 mRNA and protein expression

To investigate whether IGF-I may inhibit OGD-induced cell death in PC12 cells, we assessed the effect of IGF-I on the cell death induced by OGD insult, which causes apoptotic cell death of PC12 cells *in vitro* (Tabakman *et al.* 2004). Treatment of cells with 10^{-8} M IGF-I for 24 h significantly inhibited OGD-induced cell death (Fig. 2A). OGD-induced increase in the percentage of apoptotic DNA was also decreased by the pretreatment of cells with IGF-I (Fig. 2B). Next, we investigated the effect of IGF-I on OGD-induced activation of Par-4 expression and found that IGF-I significantly inhibited the up-regulation of the Par-4 mRNA and protein levels caused by OGD insult (Fig. 2C and D).

We also examined the effect of IGF-I pretreatment on Par-4 immunoreactivity in cells exposed to OGD insult. As shown in Fig. 3, PC12 cells exhibited a predominant localization of endogenous Par-4 in the cytoplasm with relatively low levels of expression in the nucleus. However, Par-4 exhibited a homogenous distribution pattern when cells were exposed to OGD insult, suggesting that Par-4 has translocated to the nucleus. In contrast, nuclear translocation of Par-4 was attenuated after the cells were pretreated with IGF-I.

MAPK, phosphatidylinositol 3-kinase (PI3K), and protein kinase A (PKA) pathways mediate IGF-I's anti-apoptotic effects against OGD insult

To further clarify the mechanisms of IGF-I-induced anti-apoptotic effect in PC12 cells exposed to OGD insult, we

Figure 1 Par-4 antisense oligonucleotide treatment inhibits OGD-induced induction of Par-4 and protects PC12 cells from OGD-induced apoptotic cell death. (A) Western blot analysis of Par-4 protein levels in a culture maintained under normoxic conditions (lane 1); a culture exposed to OGD insult for 3 h (lane 2); a culture pretreated with 25 μ M Par-4 antisense (lane 3) or nonsense oligonucleotide (lane 4) for 2 h; and then exposed to OGD for 3 h. Similar results were obtained in a separate experiment. (B) Cell viability measured by the MTT assay. (C) DNA fragmentation, a marker of apoptosis, measured by ELISA. Values are the mean $\pm$ S.E.M. ($n=4$). Each experiment was repeated twice. * $P<0.05$ versus vehicle-treated cells exposed to OGD; † $P<0.05$ versus Par-4 antisense oligonucleotide-treated cells.

Figure 2 IGF-I protects PC12 cells from OGD-induced apoptotic cell death and inhibits OGD-induced induction of Par-4. PC12 cells were pretreated with vehicle or IGF-I (10^{-8} M) for 24 h, and then cells were exposed to OGD for 3 h followed by 24-h reoxygenation or maintained under normal conditions (normoxia). (A) Cell viability measured by the MTT assay. (B) DNA fragmentation, a marker of apoptosis, measured by ELISA. (C) Par-4 mRNA levels measured by the RT-PCR assay. (D) Par-4 protein levels were assessed by western blot analysis. Values are the mean $\pm$ s.e.m. ($n=4$). Each experiment was repeated twice. * $P < 0.05$ versus vehicle-treated cells exposed to OGD; † $P < 0.05$ versus IGF-I-treated cells.

tested whether pretreatment of the cells with the MAPK inhibitor PD98059 (50 μ M), the PI3K inhibitors LY294002 (2 μ M) and wortmannin (200 nM), the protein kinase C (PKC) inhibitor GF109203X (5 μ M), or the PKA inhibitor H89 (5 μ M) impaired the anti-apoptotic activity of the IGF-I against OGD insult. As shown in Fig. 4, all of these inhibitors, except GF109203X, significantly blocked the anti-apoptotic effects of IGF-I. These data suggest that IGF-I inhibits OGD-induced apoptosis via the activation of the MAPK, PI3K, and PKA pathways.

PI3K and MAPK pathways mediate IGF-I's inhibitory effects on Par-4 expression

We further determined which pathways were involved in the IGF-I-induced inhibition of Par-4 expression in cells exposed to OGD insult. Pretreatment of cells with PD98059 (50 μ M), LY294002 (2 μ M), or wortmannin (200 μ M) significantly attenuated IGF-I-induced inhibition of Par-4 protein levels in OGD insult-subjected PC12 cells (Fig. 5A–C), suggesting that the MAPK and PI3K pathways contribute to IGF-I-induced

Figure 3 OGD-induced nuclear translocation of Par-4 is attenuated by IGF-I. PC12 cells were pretreated with vehicle or IGF-I (10^{-8} M) for 24 h, and then cells were exposed to OGD for 3 h followed by 24-h reoxygenation or maintained under normal conditions (normoxia). Cells were fixed and probed with Par-4 antibody to detect the expression of Par-4. Nuclei were visualized by staining with DAPI. Intracellular localization of Par-4 was visualized by confocal microscopy. Par-4 images are shown in the upper panels, and an overlay of the Par-4 and DAPI images are shown in the lower insets (scale bar=20 μ m).

Par-4 suppression. In contrast, H89 pretreatment failed to alter the inhibitory effect of IGF-I on Par-4 expression (Fig. 5D).

Discussion

In this study, we have demonstrated that IGF-I treatment protects PC12 cells from the apoptotic stimuli of OGD insult, consistent with the previous report (Yoshida *et al.* 2004). It is shown that IGF-I inhibited OGD-induced apoptosis through the suppression of Par-4 expression. The protective effects of IGF-I were dependent on the activities of the MAPK, PI3K, and PKA signaling pathways. It is also demonstrated that IGF-I-mediated inhibition of Par-4 expression was mediated via the activation of MAPK and PI3K signaling pathways.

In the present study, it is shown for the first time that IGF-I treatment prevents OGD-induced increase in Par-4 expression. Rapid up-regulation of Par-4 has been observed in animal and cell culture models of ischemic brain injury, and suppression of Par-4 production and function by an antisense oligonucleotide of Par-4 prevents neuronal cell death in experimental stroke models (Chan *et al.* 1999, Duan *et al.* 1999, Culmsee *et al.* 2001). Consistent with these findings, we observed that Par-4 mRNA and protein levels were remarkably increased in PC12 cells exposed to OGD insult. We also found that treatment of cells with Par-4 antisense oligonucleotide attenuated Par-4 induction and apoptotic cell death caused by OGD insult. These results substantiate the critical role of Par-4 in ischemic brain injury. It is considered that the elevation of Par-4 levels is an early event positioned upstream of mitochondrial dysfunction and caspase activation

(Duan *et al.* 1999) in the apoptotic cascade in neuronal cells. The ability of Par-4 to induce apoptosis involves multiple mechanisms. Par-4 activates the Fas death receptor signaling pathway and inhibits the activation of nuclear factor- κ B (NF- κ B) and activator protein-1 (AP-1) (Chakraborty *et al.* 2001), while it down-regulates Bcl-2 expression in PC12 cells (Camandola & Mattson 2000, Guo *et al.* 2001). Over-expression of Par-4 in PC12 cells increases intracellular calcium levels, which may be involved in the induction of apoptosis (Guo *et al.* 2001). It has been reported that nuclear translocation of Par-4 is essential for the induction of apoptosis and inhibition of the NF- κ B transcriptional activity by Par-4 (El-Guendy *et al.* 2003). The immunohistochemical findings of this study showed that Par-4 was localized primarily in the cytoplasm of PC12 cells, as is observed in normal tissues (Boghaert *et al.* 1997). We also found that OGD exposure resulted in a translocation of Par-4, which translocation was attenuated by pretreatment of cells with IGF-I. Therefore, we assume that the protective effect of IGF-I is mediated, at least in part, through a suppression of Par-4 expression and translocation into the nucleus during OGD.

IGF-I exerts potent neurotrophic, neurogenic, and neuroprotective/anti-apoptotic effects (Russo *et al.* 2005). The ability of IGF-I to promote neuronal survival is associated with its ability to prevent the apoptosis induced by various insults. Several evidences suggest that endogenous IGF-I plays a significant role in recovery from insults such as hypoxia-ischemia (Komoly *et al.* 1992, Beilharz *et al.* 1998, Clawson *et al.* 1999, Hinks & Franklin 1999). In this study, we showed that IGF-I protected PC12 cells from OGD-induced cell death by inhibiting apoptosis. This finding mirrors a report of Yoshida *et al.* (2004), in which IGF-I protected primary

Figure 4 PI3K, MAPK, and PKA pathways mediate the anti-apoptotic effect of IGF-I. PC12 cells were preincubated with 200 nM wortmannin for 30 min, 2 μ M LY294002 for 30 min, 50 μ M PD98059 for 1 h, 5 μ M GF109203X for 30 min, or 5 μ M H89 for 30 min and then treated with IGF-I (10^{-8} M) for 24 h, and then cells were exposed to OGD for 3 h followed by 24-h reoxygenation or maintained under normal conditions (normoxia). (A) Cell viability measured by the MTT assay. (B) DNA fragmentation, a marker of apoptosis, measured by ELISA. Values are the mean $\pm$ S.E.M. ($n=4$). Each experiment was repeated twice. * $P<0.05$ versus vehicle-treated cells exposed to OGD; † $P<0.05$ versus IGF-I-treated cells.

cortical neurons from the cell death induced by OGD insult. IGF-I binding to its receptor leads to the activation of the PI3K-Akt pathway and the ras-raf-MEK-ERK pathway, the two main IGF-I receptor signaling cascades (Kooijman 2006). In agreement with previous papers demonstrating that IGF-I inhibits apoptosis through the activation of these two signaling pathways (Campana *et al.* 1999, Mehrhof *et al.* 2001, Kang *et al.* 2003, Maldonado *et al.* 2005), we have shown that the protective effect of IGF-I appears to be

mediated through the activation of the PI3K and MAPK pathways because the PI3K inhibitors LY294002 and wortmannin, and the MAPK inhibitor PD98059 blocked the IGF-I's protective effect against OGD insult. The activation of cAMP response element-binding protein (CREB) is another pathway that has been shown to mediate the protective effect of IGF-I in some cell types (Pugazhenthir *et al.* 1999, Mehrhof *et al.* 2001, Leininger *et al.* 2004, Maldonado *et al.* 2005). In fact, the activation of PKA by forskolin (D'Mello *et al.* 1993) or the cAMP analog dibutyryl-cAMP (Hartikka *et al.* 1992) protects neuronal cells from apoptosis. Therefore, we examined whether H89, a PKA inhibitor, attenuated the effect of IGF-I on inhibiting apoptotic cell death in the present study. The anti-apoptotic ability of IGF-I was significantly blocked when cells were pretreated with H89, suggesting that activation of the PKA pathway contributes to the protective effect of IGF-I. However, the role of PKA pathway in the prevention of cell death by IGF-I seems to be cell type-specific. In pancreatic β -cells, IGF-I increases CREB phosphorylation, which is inhibited by H89 treatment, whereas the protection mediated by IGF-I is not affected by PKA inhibition (Liu *et al.* 2002). In contrast, our results show that PKC does not mediate the anti-apoptotic action of IGF-I in PC12 cells, in agreement with the observations in cardiac myocytes (Foncea *et al.* 2000). Taken together, our analyses using inhibitors of the PI3K, MAPK, PKC, and PKA pathways demonstrate that the protective effect of IGF-I appears to be mediated in part through the activation of PI3K, MAPK, and PKA pathways, with essentially no contribution of the PKC pathway.

Finally, we investigated which pathways were involved in the inhibitory effect of IGF-I on Par-4 expression. We demonstrated that IGF-I-induced suppression of Par-4 was attenuated when cells were pretreated with inhibitors of the PI3K and MAPK pathways. Akt1, a key cell survival protein, which is activated by PI3K and functionally involved in various anti-apoptotic processes, binds to Par-4 and results in both Par-4 phosphorylation and inactivation of its proapoptotic potential (Goswami *et al.* 2005). The ERK-MAPK pathway is thought to be involved in cell growth and survival, and activation of the ERK1/2 pathway protects astrocytes from ischemic injury (Jiang *et al.* 2002). It has been reported that the inhibitory effect of a Par-4 antisense oligonucleotide on glutamate-induced apoptosis is eliminated by PD98059 (Lu *et al.* 2005). Taken together, the data suggest that the IGF-I inhibitory effect on Par-4 expression is mediated through the activation of the PI3K and MAPK pathways. However, the precise mechanisms how IGF-I signaling interferes with Par-4 expression remain to be determined.

In summary, IGF-I protects against apoptosis in PC12 cells exposed to OGD insult through an inhibition of the expression and nuclear translocation of Par-4. The protective effect of IGF-I in these cells is mediated by the PI3K, MAPK, and PKA pathways, but not the PKC pathway. We also demonstrated that the PI3K and MAPK pathways are involved in the suppressive effect of IGF-I on Par-4 expression.

Figure 5 PI3K and MAPK pathways are involved in IGF-I-induced inhibition of Par-4 induced by OGD. PC12 cells were preincubated with (A) 200 nM wortmannin for 30 min, (B) 2 μ M LY294002 for 30 min, (C) 50 μ M PD98059 for 1 h, or (D) 5 μ M H89 for 30 min and then treated with IGF-I (10^{-8} M) for 24 h, and then cells were exposed to OGD for 3 h followed by 24-h reoxygenation. Par-4 protein levels were assessed by western blot analysis. Values are the mean $\pm$ S.E.M. ($n=4$). Each experiment was repeated twice. Group means that do not share a common letter (a and b) are significantly different ($P < 0.05$).

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