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Insulin-activated Akt rescues Aβ oxidative stress-induced cell death by orchestrating molecular trafficking

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Summary

Increasing evidence indicates that Alzheimer's disease, one of the most diffused aging pathologies, and diabetes may be related. Here, we demonstrate that insulin signalling protects LAN5 cells by amyloid-β42 (Aβ)-induced toxicity. Aβ affects both activation of insulin receptors and the levels of phospho-Akt, a critical signalling molecule in this pathway. In contrast, oxidative stress induced by Aβ can be antagonized by active Akt that, in turn, inhibits Foxo3a, a pro-apoptotic transcription factor activated by reactive oxygen species generation. Insulin cascade protects against mitochondrial damage caused by Aβ treatment, restoring the mitochondrial membrane potential. Moreover, we show that the recovery of the organelle integrity recruits active Akt translocation to the mitochondrion. Here, it plays a role both by maintaining unimpaired the permeability transition pore through increase in HK-II levels and by blocking apoptosis through phosphorylation of Bad, coming from cytoplasm after Aβ stimulus. Together, these results indicate that the Akt survival signal antagonizes the Aβ cell death process by balancing the presence and modifications of common molecules in specific cellular environments.

Key words: amyloid-beta; insulin; Akt; apoptosis; cell survival; mitochondrion.

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder that currently affects nearly 2% of the population in industrialized countries, and the risk of AD dramatically increases in individuals beyond the age of 65–70. Alzheimer's disease patients lose their memory and cognitive abilities, and even their personalities may change dramatically. These changes are because of the progressive dysfunction and death of nerve cells that are responsible for the storage and processing of information. Although drugs can temporarily improve memory, at the present time, there are no treatments available to stop or reverse the inexorable neurodegenerative process.

Even though it is not clear why memory is specifically targeted in AD, increasing evidence indicates that the functional and morphological deterioration of specific memory synapses is caused by potent neurotoxins that accumulate in the AD brain. Among the known neurotoxins, a relevant role is played by aggregates of the amyloid-β–peptide (Aβ), a peptide generated by proteolytic cleavage of the amyloid precursor protein (Klein *et al.*, 2004). Fibrillar forms of Aβ found in amyloid plaques were previously considered the major cause of neuronal damage in AD, but recently it has been discovered that the Aβ soluble oligomers also known as A-derived diffusible ligands are the more potent neurotoxins (Lambert *et al.*, 1998; Gong *et al.*, 2003). Unlike the insoluble fibrils, ADDLs are diffusible molecules that attach with specificity to particular synapses, acting as pathogenic ligands (Gong *et al.*, 2003). However, Aβ, in all its various aggregation forms, induces neurodegeneration using the programmed cell death (apoptosis) mechanism, in different experimental or transgenic models (Magrané *et al.*, 2005; Oakley *et al.*, 2006; Picone *et al.*, 2009).

It has been hypothesized that cellular mechanisms exist that could physiologically protect synapses against ADDL toxicity. Such active synaptic protection mechanisms could contribute to preserving cognitive function in normal individuals, and recent reports are consistent with the possibility that one such protective mechanism could be provided by CNS insulin signalling (De Felice *et al.*, 2009). Insulin resistance, that is the reduced ability of insulin to stimulate glucose utilization, is a syndrome associated with type II diabetes, hypertension and obesity (Biddinger & Kahn, 2006). It is caused by impairment of the peripheral insulin receptor (IR), a tyrosine kinase protein, playing a pivotal role in the regulation of peripheral glucose metabolism and energy homeostasis. Insulin receptors are also present in the brain and are involved in synaptic activities required for learning and memory (Zhao & Alkon, 2001). Recently, the intriguing suggestion that CNS insulin signalling can express a novel type of insulin-resistant diabetes linked to AD has been proposed (Baskin *et al.*, 1983a,b, 1988, 1993, 2001; Craft & Watson, 2004; de la Monte *et al.*, 2006).

Soluble Aβ oligomers induce pathological changes that include oxidative stress (Serrano & Klann, 2004; Schmitt, 2005a,b; Oddo *et al.*, 2006). Cell metabolism generates potentially harmful reactive oxygen species (ROS). At moderate levels, ROS act as second messenger for different cellular functions (Dröge, 2002). At the same time, a variety of mechanisms protect cells against ROS excess. However, chronic and/or abrupt increases in ROS levels above a physiological threshold may trigger cell death by interfering with normal cellular mechanisms.

Binding of insulin to IR activates phosphatidyl inositol 3 kinase (PI3K), which in turn, activates the Ser/Thr-kinase Akt (Dudek *et al.*, 1997). The PI3K-Akt signalling pathway is responsive to trophic factors, metabolic signals and environmental stress and regulates survival, growth, differentiation and other homeostatic functions. For instance, Aβ specifically inhibits the activation of Akt through a mechanism preventing its direct interaction (Lee *et al.*, 2009).

Akt promotes cell survival by inactivating certain proapoptotic mediators such as transcription factors of the forkhead (FOXO) family (Biggs *et al.*, 1999; Brunet *et al.*, 1999) or the Bcl2 antagonist Bad protein. FOXO transcription factors are key players in cell death/life pathways. In neurons, FOXO has been involved mostly in cell death processes such as those because of trophic deprivation (Gilley *et al.*, 2003) or ROS excess (Lehtinen *et al.*, 2006). Moreover, Akt is able to confer mitochondrial protection through phosphorylation of the apoptotic protein Bad (del Peso

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et al., 1997). Both oxidative stress and apoptosis are mechanisms involved in neurodegeneration, and both cause mitochondrial dysfunction indicating that the integrity of this organelle is relevant to cell death or survival pathways. Oxidative stress disrupts mitochondrial integrity through the opening of a large channel referred to as the permeability transition (PT) pore, thus producing mitochondrial depolarization, a key event to initiate the cell death cascade. The PT pore has been seen to be composed of the voltage-dependent anion channel, adenine nucleotide translocator, cyclophilin D and hexokinase-II (HK-II) proteins, although perhaps other molecular constituents are present. Opening of the PT pore favours cytochrome c release and apoptosis activation. HK-II has a role in mitochondrial protection, and interestingly, the ability of Akt to protect against cytochrome c release and apoptosis has been shown to be decreased after HK-II dissociation from mitochondria (Majewski *et al.*, 2004). Moreover, it has been demonstrated that PT pore opening and mitochondrial integrity are regulated by HK-II phosphorylation after Akt activation (Miyamoto *et al.*, 2008).

Recently, we have demonstrated that insulin is capable of reducing toxicity induced by Aβ oligomers by inhibition of the intrinsic apoptotic pathway (Di Carlo *et al.*, 2010). Here, in an effort to elucidate the link between AD and diabetes, we have addressed our studies to test the hypothesis that insulin signalling provides a physiological defence mechanism to counteract the death programme triggered by Aβ oligomers. In particular, we have investigated whether insulin is able to prevent or reverse damage caused by oxidative stress induced by Aβ, and we have identified which signal molecules are involved in the neuroprotection programme and in which cellular compartment they act.

Results

Insulin protects neuroblastoma cells against rAβ42 oligomer toxicity

To evaluate the dose and incubation time of insulin necessary to interfere with cell damage induced by rAβ42 oligomers, we have performed a viability assay. As a first step to ascertain whether the size of the rAβ42 aggregates is maintained during the incubation time of the toxicity

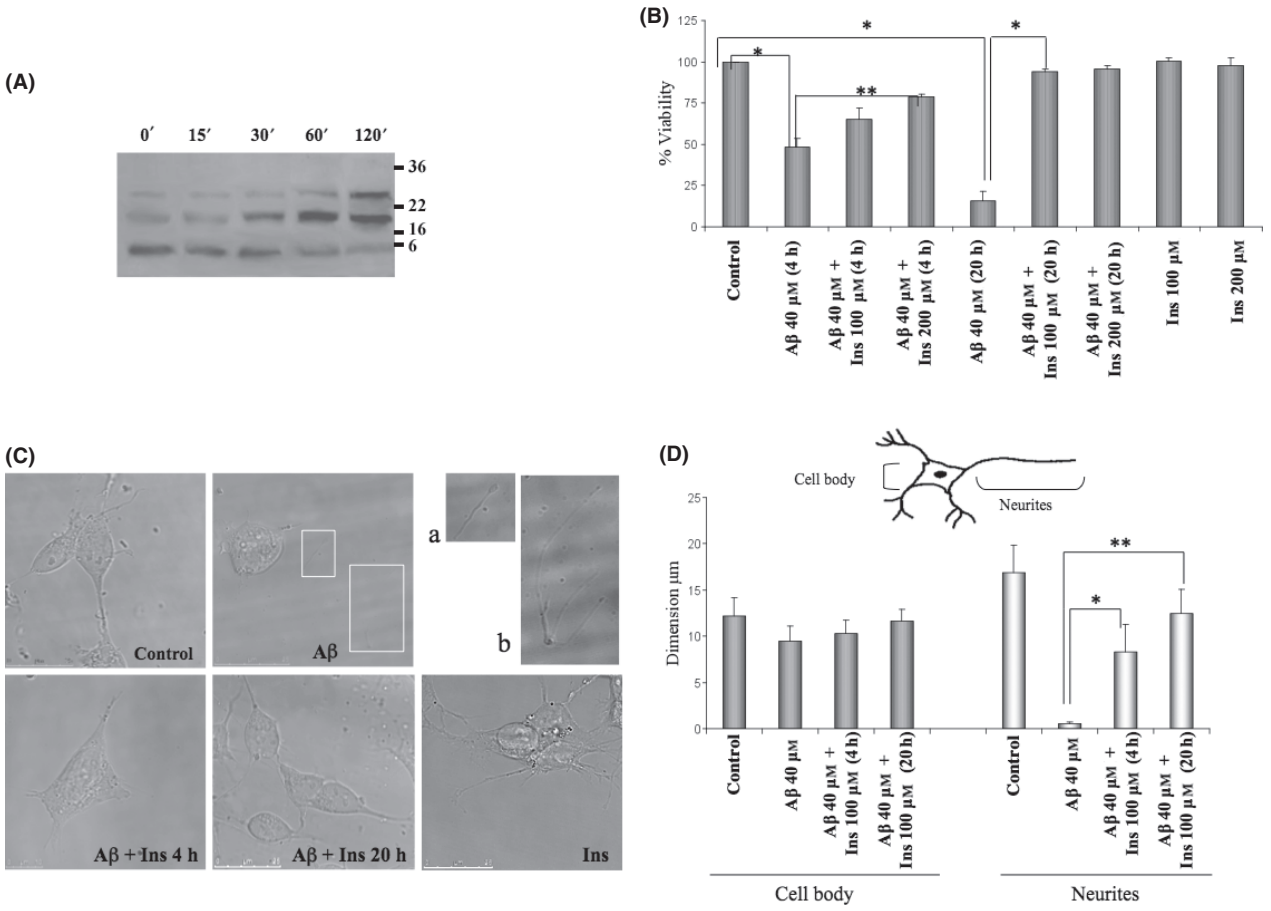

Fig. 1 rAβ42 oligomers-induced toxicity is rescued by insulin in a dose- and time-dependent manner. (A) Time evolution of rAβ42 oligomers during incubation at 37°C. Aliquots of oligomers prepared at pH 7.2 and diluted in RPMI at 37°C were taken at different incubation times and loaded on a nondenaturing PAGE. Molecular weight is indicated on the right. (B) LAN5 neuroblastoma cells were untreated (control) or treated with rAβ42 oligomers 40 μM (Aβ40μM) or with rAβ42 oligomers 40 μM and insulin 100 μM (Aβ40μM + Ins 100 μM) or with rAβ42 oligomers 40 μM and insulin 200 μM (Aβ40μM + Ins 200 μM) or with insulin 100 μM (Ins 100 μM) and 200 μM (Ins 200 μM) for 4 and 20 h of recovery, as described in M&M. *$P < 0.01$, **$P < 0.05$, vs. indicated groups. (C) Representative morphological images of LAN5 untreated cells (control) or treated with rAβ42 oligomers (Aβ) or with rAβ42 and insulin 100 μM for 4 and 20 h (Aβ + Ins 4 h, Aβ + Ins 20 h) and with insulin 100 μM alone (Ins). a and b show high-magnification images of broken neurites contained in the rectangles indicated in Aβ42. (D) Histogram of cell body and neurites dimensions: the minor diameter size was considered for the cell body. The value was expressed in micrometre. *$P < 0.03$, **$P < 0.05$, vs. indicated groups.

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experiments, an aliquot of rAβ42 taken at different incubation times was loaded on a nondenaturing PAGE. In agreement with our previous data obtained by light scattering measurements (Picone *et al.*, 2009), we detected the bands corresponding to monomers or small oligomers (pentamers as the larger species) that were stable up to 2 h of incubation. During the time course experiments, we have observed only a difference in the relative population of the monomeric and oligomeric species (Fig. 1A). For the viability assays, LAN5 neuroblastoma cells were preincubated with the same amount of oligomers and then treated with different insulin concentrations (100 and 200 μM) for 4 and 20 h. The percentage of cell viability was measured by an MTS assay. As shown in Fig. 1(B), cells treated with rAβ42 oligomers only showed a mortality of about 50% and 85%, compared with the control, if incubated for 4 or 20 h, respectively. When insulin was added at different concentrations, a recovery of cell viability was observed, and the best result was obtained with the longest treatment time, thus suggesting a protective role of insulin against Aβ toxicity. Moreover, to visualize the results obtained by the viability assay, the morphological effect on untreated and differently treated cells was observed by microscopic inspection (Fig. 1C). Neurons treated with rAβ42 oligomers showed different steps of degeneration and morphological changes resulting in a modification of the cellular body and a reduction in the neurites. Moreover, after a more careful inspection, we detected some broken neurites in the culture medium (a and b insets of Fig. 1B). In contrast, cells treated with rAβ42 oligomers and insulin appear to recover the regular cell morphology (Fig. 1C). By image analysis, we observed that no relevant differences were present in the cell body, whereas neurites recover their physiological length in proportion to the time of exposure to insulin, suggesting that a process of regeneration was occurred (Fig. 1C,D).

Aβ oligomers induce loss of activated IR in neuronal cells

As shown above, treatment of LAN5 cells with rAβ42 oligomers produced morphological changes reverted by insulin. On the basis of this result, we have investigated whether the protection provided by insulin could involve its receptor activation. Confocal immunofluorescence microscopy analysis has shown that subcellular distribution of active IR was strikingly different on neurons depending on their cell history (Fig. 2A). LAN5 cells treated with rAβ42 oligomers did not show active IR immunoreactivity, as expected. In contrast, cells treated with insulin showed a dotted staining along the cell body and, mainly, in the neurites. Analysis of the samples previously exposed to rAβ42 oligomers and then to insulin showed the presence of dotted staining in the cell body and in minor amount in the short neurites. By image analysis, immunoreactivity in cell body of rAβ42- and insulin-treated cells was a little less elevated as compared to the level of insulin-treated cells, whereas the main difference was detected in neurites of the two samples in agreement with their different length because of the treatment (Fig. 2B). Considering the whole cell, the data strongly

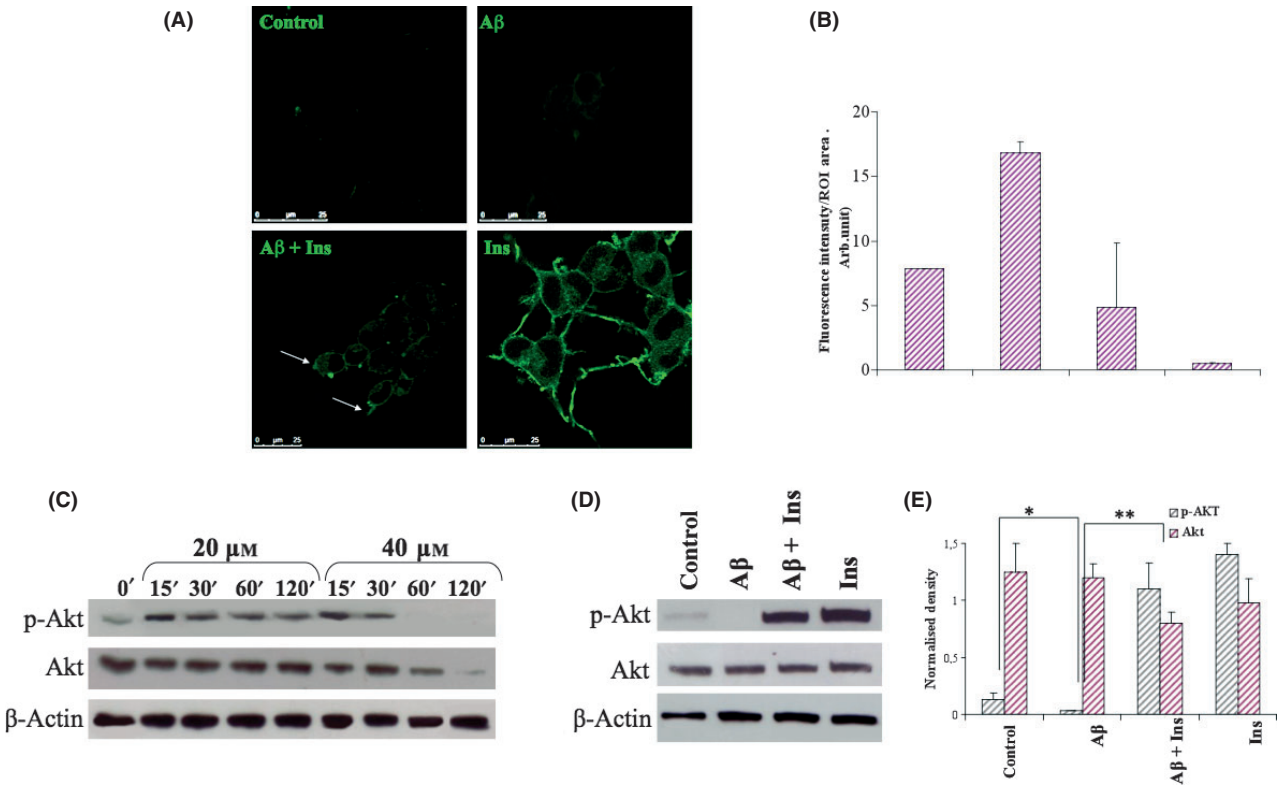

Fig. 2 rAβ42 oligomers reduce active insulin receptors (IRs). (A) LAN5 untreated cells as control (control) or treated with rAβ42 oligomers (Aβ) or with rAβ42 oligomers and insulin (Aβ + Ins) or with insulin alone (Ins) were immunolabelled with anti-phosphorylated IR. The arrows indicate the short neurites. (B) Histogram of immunofluorescence quantification levels in cell body and neurites of LAN5 treated with insulin alone (Ins cell body, Ins neurites) or rAβ42 and insulin (Aβ + Ins cell body, Aβ + Ins neurites). (C) Immunoblot of LAN5 proteins untreated or treated with rAβ42 oligomers 20 or 40 μM for 15 and 30 min or 1 and 2 h and incubated with anti-Akt, anti-phospho-Akt (p-Akt) and anti-β-actin E) Activation of Akt in LAN5 in the presence of rAβ42 alone (Aβ) or rAβ42 and insulin (Aβ + Ins) or insulin alone (Ins). (D–F) Quantification of immunoreactivity was performed using densitometric analysis; uniformity of gel loading was confirmed with β–actin utilized as standard. $*P < 0.02$, $**P < 0.01$, vs. indicated groups.

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suggest that IR response was lowered by Aβ oligomer pretreatment and that Aβ triggers insulin resistance. Moreover, it is known that activation of the IR induces PI3K/Akt cell signalling whose function has been associated with cell survival. To analyse how Aβ oligomers can affect Akt activation, LAN5 cells were exposed at two Aβ concentrations for different times (15, 30, 60 and 120 min). Akt was transiently activated at an early time (15 min) for both the rAβ42 oligomers concentrations. At intermediate and late times (30, 60 and 120 min), the kinase was gradually deactivated (Fig. 2C). To explore whether the insulin protection against Aβ toxicity was mediated through this signalling, we analysed Akt activation by a Western blot. As shown previously in the presence of rAβ42, Akt phosphorylation is inhibited. In contrast, high level of phosphorylated Akt is present in LAN5 treated with rAβ42 and then with insulin, suggesting that the recovery of viability after rAβ42 stimulus needs Akt activation (Fig. 2D,E).

Insulin inhibits Aβ-induced intracellular ROS generation via Akt activation

Several pieces of evidence suggest that Aβ is able to generate free radicals and oxidative damage, and the increase in ROS level elicits neuronal death (Reddy, 2006). Moreover, it has been demonstrated that insulin protects cells from apoptosis by the activation of IRs leading to a decrease in oxidative stress (Kang *et al.*, 2003). To investigate whether in LAN5 cells, insulin can affect intracellular ROS accumulation produced by Aβ addition, we performed a fluorimetric assay in which the fluorescence is directly proportional to the concentration of hydrogen peroxide inside the cell (Fig. 3). LAN5 cells were incubated with rAβ42 alone or with insulin or with H_2O_2 as a positive control. Moreover, to test the possibility that PI3K/Akt signalling was involved in ROS reduction, we utilized the wortmannin, an inhibitor of PI3K, at different concentrations (Fig. 3A,B). Fluorimetric analysis and microscopic observations have shown that the fluorescence intensity of Dichlorofluorescein (DCF) oxidized by intracellular ROS was increased if cells were incubated with rAβ42 or H_2O_2 alone. On the contrary, the presence of insulin caused a reduction in DCF fluorescence intensity, elicited by rAβ42 addition. Moreover, we did not find a decrease in DCF fluorescence intensity when the cells were incubated with insulin together with wortmannin. The results obtained indicate that the protective role played by insulin after rAβ42 damage is exerted by inhibiting ROS accumulation, and this mechanism involves the Akt pathway.

Active Akt protects cells by Aβ-induced oxidative stress via FOXO3a phosphorylation

Forkhead transcription factors of the FOXO class are negatively regulated by the insulin/PI3K/Akt signalling pathway (Burgering & Medema, 2003). Direct phosphorylation by PI3K/Akt inhibits transcriptional activation by FOXO factors, causing their export from the nucleus to the cytoplasm. Further, in response to oxidative stress, FOXO factors activate a programme of gene expression that regulates apoptosis. To obtain evidence as to whether, in our model system, the inhibition of FOXO factors can be involved in protecting cells against rAβ42-induced oxidative stress, we analysed the phosphorylation state of Foxo3a under our experimental conditions. A Western blot of proteins extracted from cells treated with rAβ42 alone or with insulin was incubated with antibodies against both the unphosphorylated and phosphorylated forms of Foxo3a. Moreover, the same Western blot was incubated with anti-unphosphorylated and phosphorylated Akt and with anti-PrxIII, a Foxo3a direct gene target (Fig. 4A–D). Under

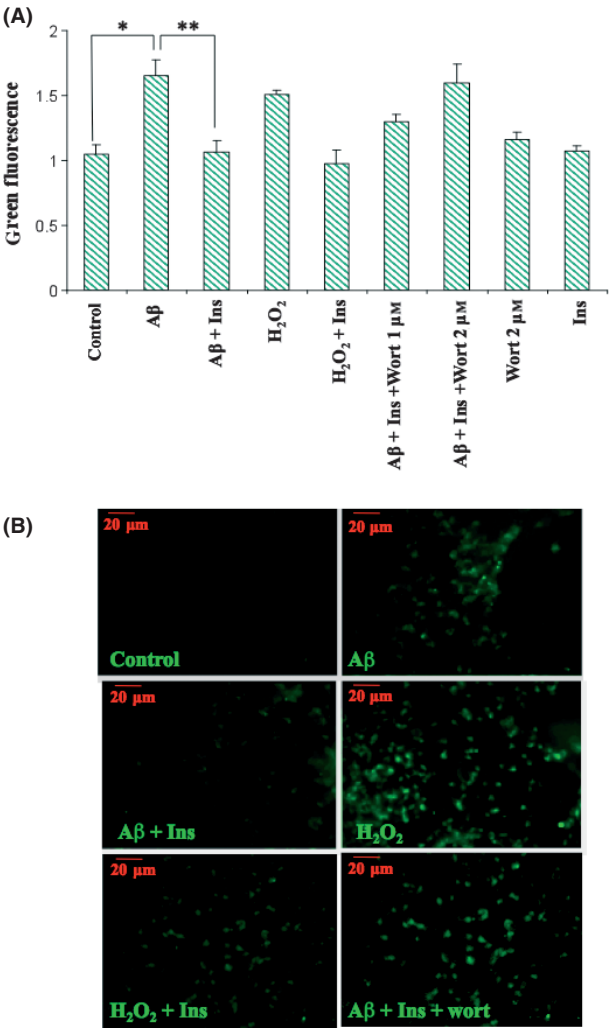

Fig. 3 Insulin, via active Akt, decreases reactive oxygen species (ROS) generated by rAβ42 oligomers in LAN5 cells. (A) Neuroblastoma cells were untreated (control) or treated with rAβ42 oligomers (Aβ) or with rAβ42 oligomers and insulin (Aβ + Ins) or with H_2O_2 (H_2O_2) or with H_2O_2 and insulin (H_2O_2 + Ins) or with rAβ42 oligomers, insulin and wortmannin (Aβ + Ins + wort) at 1 and 2 μM or with wortmannin 2 μM (wort 1 μM or wort 2 μM) or with insulin alone (Ins). After these treatments, DCFH-DA was added to the cells, and then, the samples were submitted to fluorimetric analysis. $^{*}P < 0.02$, $^{**}P < 0.05$, vs. indicated groups. (B) Representative fluorescent images of untreated LAN5 cells (control) or treated with rAβ42 oligomers (Aβ) or with rAβ42 oligomers and insulin (Aβ + Ins) or with H_2O_2 (H_2O_2) alone or with insulin (H_2O_2 + Ins) or with rAβ42 oligomers, insulin and wortmannin (H_2O_2 + Ins + wort) 2 μM.

stimulus by rAβ42 oligomers, Akt is not activated, and as expected, Foxo3a is mainly in the unphosphorylated form. According to literature, Foxo3a should be present in the nucleus where it activates gene expression as demonstrated by the presence of high levels of PrxIII protein. Administration of insulin after rAβ42 treatment, in contrast, activates Akt and Foxo3a, mainly present in its phosphorylated form, and is probably translocated to the cytoplasm, and consequently the level of expression of PrxIII is reduced. These results indicate that the inhibition of Foxo3a by Akt phosphorylation helps insulin to play a protective role against the oxidative stress induced by rAβ42 oligomers. Further, data suggest that this mechanism is feasible by moving Foxo3a to different subcellular compartments.

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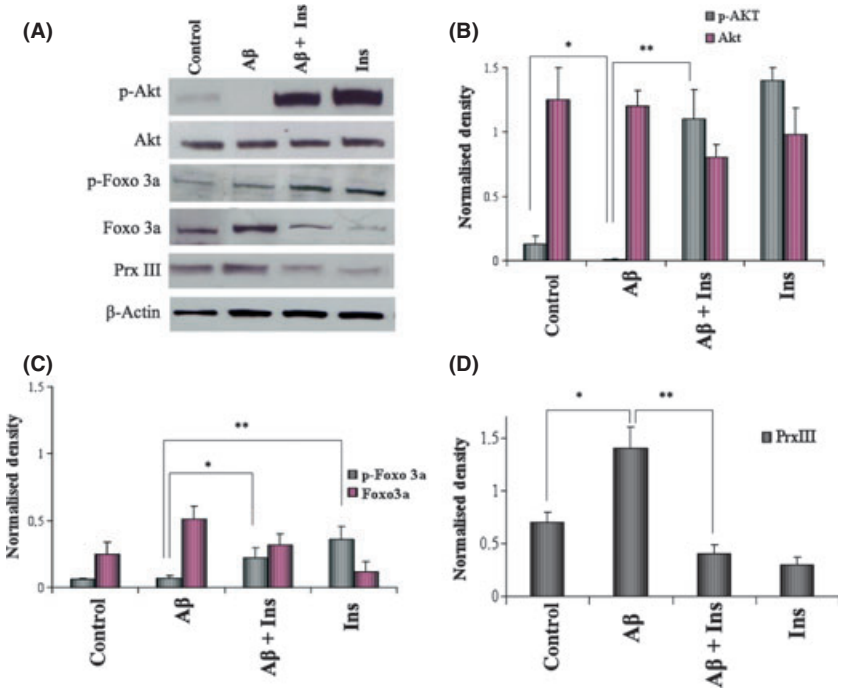

Fig. 4 Active Akt modulates phosphorylation of Foxo3a regulating its gene expression. (A) Western blot of protein extracted from LAN5 was untreated (control) or treated with rAβ42 oligomers (Aβ) or with rAβ42 oligomers and insulin (Aβ + Ins) or with insulin alone (Ins) and incubated with anti-Akt, anti-phospho-Akt (p-Akt), anti-Foxo3a, anti-phospho-Foxo3a (p-Foxo3a), anti-peroxiredoxin (PrxIII) and anti-β-actin. (B–D) Quantification of immunoreactivity was performed using densitometric analysis; uniformity of gel loading was confirmed with β–actin utilized as standard. (B) $*P < 0.02$, $**P < 001$, vs. indicated groups. (C) $*P < 0.01$, $**P < 0.003$, vs. indicated groups. (D) $*P < 0.02$, $**P < 0.05$, vs. indicated groups.

Insulin restores mitochondrial membrane potential

Reactive oxygen species generation is tightly correlated with mitochondrial membrane depolarization. To have further evidence that insulin reduces ROS generation, we performed a JC-1 assay to determine membrane depolarization. Under physiological conditions, membrane potential measured by JC-1 is revealed by red fluorescence emission. On the contrary, stressed cells result in a breakdown of the mitochondrial membrane potential and a prevalent increase in green fluorescence. Consequently, mitochondrial depolarization is indicated by a decrease in the red/green fluorescence intensity ratio. In Fig. 5, we show the mitochondrial membrane depolarization obtained after rAβ42 and CCCP treatment. In contrast, cells treated with insulin after rAβ42 treatment presented an increase in the fluorescence intensity ratio indicating that insulin restored the mitochondrial membrane potential and function by counteracting Aβ activity. Similar results were shown by fluorescence microscopy analysis (Fig. 5B).

pAkt under oxidative stress translocates to mitochondrion

Reactive oxygen species generation leads to mitochondrial depolarization initiating a cell death cascade. The data reported until now provide evidence that insulin protects LAN5 cells after Aβ stimulation through Akt activation. Some studies indicate that Akt under certain stimuli translocates to the mitochondrion (Bijur & Jope, 2003) where it can mediate mitochondrial protection through phosphorylation of hexokinase-II (HK-II) a component of the PT pore (Miyamoto *et al.*, 2008). Further, activated Akt is able by phosphorylation to inhibit Bad, an apoptotic protein (del Peso *et al.*, 1997). Both these proteins are involved in mitochondrial cytochrome C release, a critical early event in the apoptotic cascade (Kennedy *et al.*, 1999). To evaluate the role of activated Akt under oxidative stress on mitochondrial integrity and apoptosis, we investigated whether, under our experimental conditions, it translocates to the mitochondrion where it could increase the presence of HK-II and phosphorylate Bad. We tested this supposed mechanism by fractionating LAN5 cells treated with rAβ42 alone or with insulin into cytosolic and mitochondrial fractions. Moreover, we used H_2O_2 as an oxidative stress control (Fig. 6). The presence of active Akt was detected in the mitochondrial fraction of cells treated with rAβ42 or H_2O_2 and insulin, whereas no signal was detected in samples with rAβ42 or H_2O_2 alone. An intriguing result was obtained with the cells treated with insulin alone in which a very low signal of Akt was detected, suggesting that active Akt translocation to the mitochondrion occurs mainly after oxidative stress conditions probably when rescue from damage is necessary (Fig. 6A,B). Moreover, this result is strengthened by the presence of an increased amount of total HK-II and phosphorylation of Bad only in the sample stimulated earlier with rAβ42 and H_2O_2 and then with insulin, indicating that these modifications were attributed to the presence of active Akt (Fig. 6A–D).

To obtain a clear-cut result about the localization of active Akt within the mitochondrion after Aβ and insulin stimuli, we performed a colocalization experiment. LAN5 cells were previously labelled with MitoTracker Deep Red, a red fluorescent dye that stains mitochondria in live cells and whose accumulation depends on membrane potential, and then immunostained with anti-phospho-Akt. Images obtained by confocal microscopy are shown in Fig. 7. Active mitochondria were observed in control cells. As expected, no fluorescence was detected in Aβ exposed cells. In contrast, the presence of active mitochondria and Akt was instead detected in both samples exposed to insulin. As can be seen in the merged images of Fig. 7(A), a high degree of colocalization between signals in red and in green channels is found for cells treated with Aβ and insulin, whereas a

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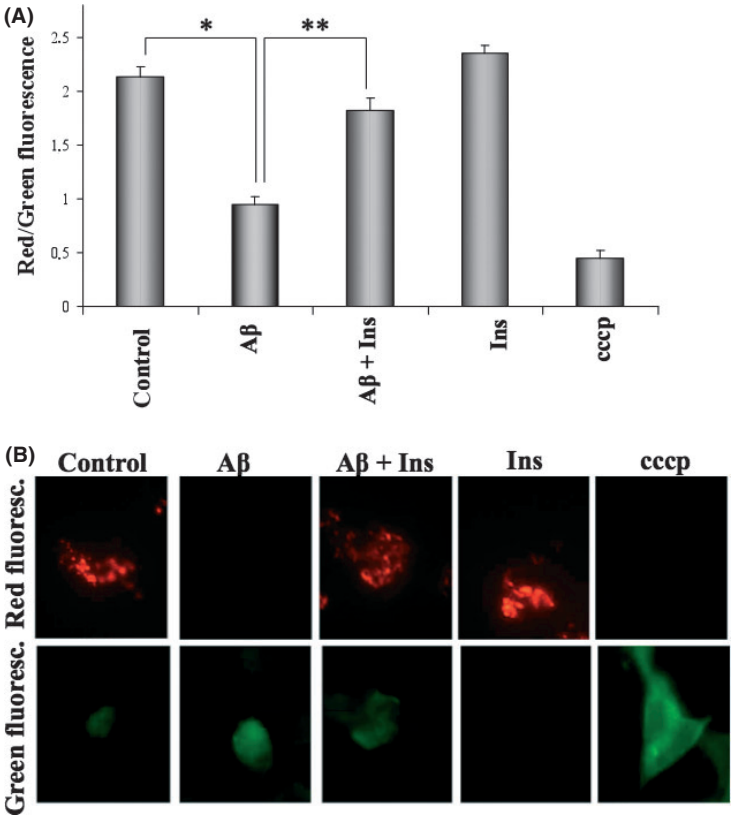

Fig. 5 Insulin restores mitochondrial membrane depolarization. (A) Untreated LAN5 cells (control) or treated with rAβ42 oligomers (Aβ) or with rAβ42 oligomers and insulin (Aβ + Ins) or with insulin alone (Ins) or with CCCP (cccp) as positive control were submitted to JC-1 assay. Histogram represents red/green fluorescence ratio obtained. $*P < 0.01$, $**P < 0.05$, vs. indicated groups. (B) Representative red and green fluorescent macroscopic images of untreated LAN5 cells (control) or treated with rAβ42 oligomers (Aβ) or with rAβ42 oligomers and insulin (Aβ + Ins) or with insulin alone (Ins) or with CCCP (cccp) after JC-1 assay.

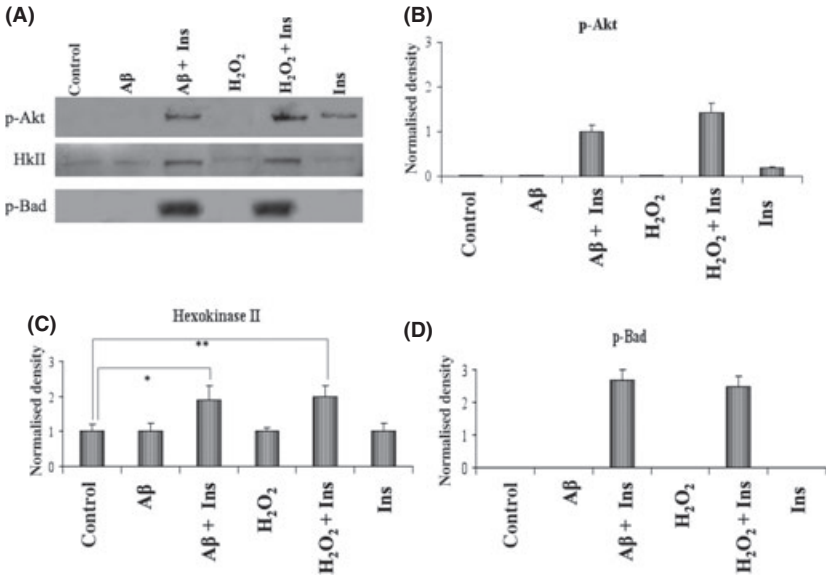

Fig. 6 Active Akt translocates to mitochondrion and modulates HK-II levels and Bad phosphorylation. (A) Western blot of proteins extracted from mitochondria of untreated LAN5 cells (control) or treated with rAβ42 oligomers (Aβ) or with rAβ42 oligomers and insulin (Aβ + Ins) or with H_2O_2 (H_2O_2) or with H_2O_2 and insulin (H_2O_2 + Ins) or with insulin alone (Ins) and incubated with anti-phosphorylated-Akt (p-Akt), anti-HK-II (HKII) and anti-phosphorylated-Bad (p-Bad). (B-D) Uniformity of gel loading was confirmed with β-actin as standard. Quantification of immunoreactivity, where necessary, was performed using densitometric analysis. $*P < 0.01$, $**P < 0.05$, vs. indicated groups.

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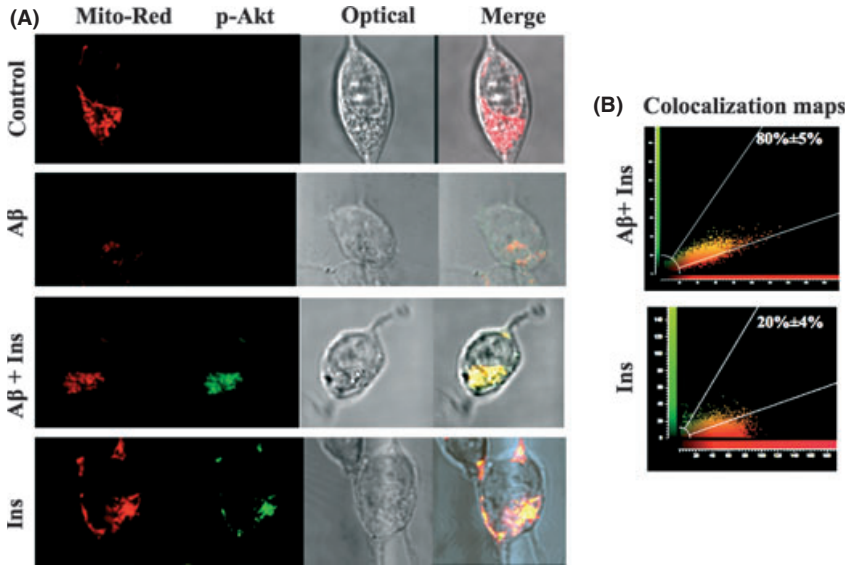

Fig. 7 (A) Active Akt colocalizes with entire mitochondrion. Representative immunofluorescence of untreated cells (control) or treated with rAβ42 oligomers (Aβ), or with rAβ42 oligomers and insulin (Aβ + Ins), or with insulin alone (Ins) and incubated with Mito Red and anti-phosphorylated-Akt (p-Akt), optical and merged images of the same samples are shown. (B) Colocalization map.

definitively lower degree is observable for insulin only. The diagrams produced by colocalization analysis are also presented in Fig. 7(B) showing the distribution of pixels according to the selected channels. Colocalized yellow pixels are located along the diagonal, in the panel relative to insulin alone, the tail shape of the diagram indicated a lower percentage of colocalization. According to the analysis, about 80% of colocalization was detected for the sample exposed to Aβ and insulin, whereas only 20% of colocalization in the cells stimulated with insulin alone was detected, in agreement with the result obtained with Western blots (Fig. 6).

Moreover, with an aim to detect whether Bad localization and its phosphorylation state depend on a received stimulus, we analysed its presence in cytoplasmic and mitochondrial fractions (Fig. 8). Under rAβ42 oligomers and H_2O_2 stimuli, Bad is exclusively present in its unphosphorylated form in the mitochondrial fraction, in agreement with its role as an apoptotic protein. Under insulin stimulus, Bad is present only in its phosphorylated form in the cytoplasm. When LAN5 cells were treated before with rAβ42 oligomers and H_2O_2 and then with insulin as described above, we found the phosphorylated form mainly in the mitochondrion, whereas a minor level of phosphorylated Bad was found in the cytosol suggesting that Bad translocation to the cytoplasm was in progress.

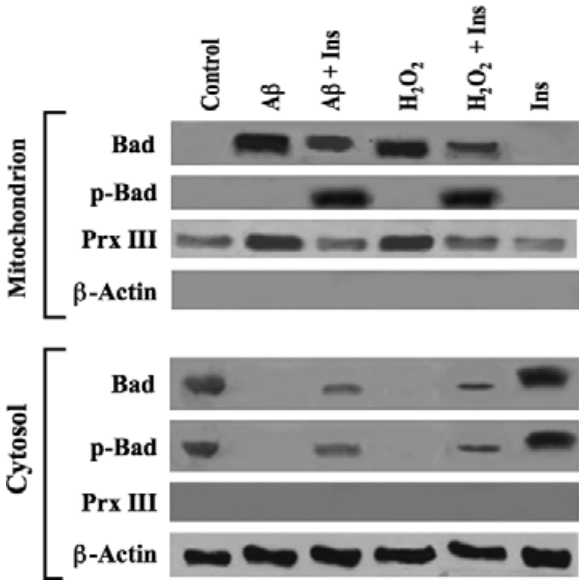

Fig. 8 Bad shuttles between cytoplasm and mitochondrion. Western blot of proteins extracted from mitochondria and cytosol of untreated LAN5 cells (control), or treated with rAβ42 oligomers (Aβ) or with rAβ42 oligomers and insulin (Aβ + Ins) or with H_2O_2 (H_2O_2) or with H_2O_2 and insulin (H_2O_2 + Ins) or with insulin alone (Ins) and incubated with anti-Bad (Bad), anti-phospho-Bad (p-Bad), anti-β-actin and anti-peroxiredoxin (PrxIII).

Discussion

Studies on the mechanisms underlying oxidative cell death constitute a very important aspect of an understanding of the causes leading to neurodegenerative diseases (Beal, 1995). Under physiological conditions, tightly controlled levels of endogenous ROS are utilized by the cell to modulate redox-sensitive processes (Dudek *et al.*, 1997), but imbalanced ROS production leads to cell death. Furthermore, the mechanisms involved in the transition from normal ROS physiology to oxidant-mediated cell death are not fully understood. Brain is especially prone to oxidative stress-induced damage as a result of its high levels of polyunsaturated fatty acids, high oxygen consumption, high transition metal content and poor antioxidant defences (Nunomura *et al.*, 2006). Aβ oligomers, the principal neurotoxin involved in AD, increase ROS levels. Oxidative stress could be a mechanism to induce insulin resistance, which in turn has been often associated with some age-related diseases. Here, we demonstrate that Aβ oligomers, eliciting oxidative stress, cause the disruption of IR signalling, thus leading to cell death. In contrast, insulin stimulation, after exposure to Aβ, through Akt phosphorylation counteracts ROS production and promotes cell survival using two different mechanisms: (i) inhibition of Foxo3a, a protein involved in cell death

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induced by oxidative stress and (ii) translocation of active Akt to the mitochondrion. Here, Akt is able to maintain unimpaired the PT pore, increasing HK-II and blocking apoptosis through Bad phosphorylation.

Aβ and insulin: a balance between cell death and survival

An understanding of the molecular mechanisms by which survival and apoptotic signals integrate is the key for the comprehension of the cell death regulation. Towards this aim, as first step, we performed a viability assay, and we found that insulin inhibits rAβ42-induced cell death in LAN5 neuroblastoma cells in a dose- and time-dependent manner suggesting that this protective role can be played by IR signalling. Moreover, after rAβ42 treatment, morphological analysis showed loss of neurites that were regenerated after insulin administration. Insulin receptors were activated in LAN5 cells in response to insulin administration, providing a CNS model suited for investigating cellular mechanisms involved in insulin resistance potentially associate with AD. By a confocal immunofluorescent assay, we found that insulin is able to activate its receptor, also after rAβ42 oligomer treatment, even if it can only activate the receptors of the cell body and of the growing short neurites. These observations suggest that as the IR is localized mainly in the neurites, loss of them could be one of the causes of insulin resistance in AD patients. Further, neuroprotection of insulin requires activation of IR signalling, as demonstrated by the modulation of levels of phospho-Akt, inhibited by Aβ, on the basis of the stimulus. However, we cannot exclude that when the entire neurites are regenerated, an increasing number of IR could be present and help the survival programme.

Foxo3a is a key player in cell death/life pathways

One of the causes by which Aβ oligomers lead to cell death is an abrupt increase in ROS levels (Reddy, 2006) that upsets the physiological balance between the production of oxidants and the endogenous antioxidant defences in neuronal cells. Here, we give evidence that insulin reduces ROS levels, generated by rAβ42 by a mechanism involving active Akt, as confirmed by the use of wortmannin to restore ROS levels also in the presence of insulin.

Studies on several animal and cellular model systems have established that Foxo3a is a key player in cell death/life pathways being a transcription factor having, as a target, genes involved in stress resistance, metabolism, cell cycle arrest and apoptosis. Because these activities are in some cases antagonistic, the activity of Foxo3a is differentially controlled in response to various types and intensities of stimuli (Calnan & Brunet, 2008). Moreover, Foxo3a activity is modulated by targeted phosphorylation, acetylation, mono- and poly-ubiquitination and interaction with other transcription factors. Further, Foxo3a plays its role transiting between the cytoplasm and nucleus depending on its phosphorylation state (Brunet *et al.*, 2004). In the presence of insulin, activated Akt translocates to the nucleus where directly phosphorylates Foxo at distinct sites stimulating interaction with 14-3-3 protein (Greer & Brunet, 2005). This chaperone protein promotes the nuclear export and inhibits the nuclear import of Foxo proteins, driving the cells towards survival (van der Heide *et al.*, 2004). In contrast, Foxo proteins, under conditions of oxidative stress, are phosphorylated by other protein kinases, including Mst1 and JNK. They are able to disrupt Foxo interaction with 14-3-3, promoting its nuclear import and thereby inducing cell death in neurons and thus opposing Akt's action (Gilley *et al.*, 2003; Sunayama *et al.*, 2005). Moreover, the presence of a competitive interplay between Akt and JNK in regulating Foxo3a after oxidative stress has also been explained through activation of a two-arm pathway (Davila & Torres-Aleman, 2008). In our experimental system, rAβ42 oligomers generate a chronic increase in ROS and, as suggested by the results, Foxo3a can upregulate genes responsible for cell death. In contrast, insulin administration counteracts oxidative stress via Akt activation and inhibition of Foxo3a activity. Based on the mechanism discussed above, we have argued that phosphorylated Foxo3a is exported from the nucleus to the cytoplasm. Even if we do not show direct evidence for nuclear/cytoplasmic shuttling of Foxo3a, we have indirectly demonstrated the presence of Foxo3a in the nucleus by the increased expression of PrxIII protein, a target of Foxo3a (Chiribau *et al.*, 2008), and by the decreased expression of PrxIII when insulin is added after rAβ42 treatment. To help explain that Foxo3a regulates stress-resistant genes, such as PrxIII, and pro-apoptotic genes, it has been proposed that the result might depend on the intensity of the received stimulus or by chromatin remodelling (Greer & Brunet, 2005; Calnan & Brunet, 2008). The data here reported support the idea that the insulin pathway regulates cell survival or death by controlling the sequestering of Foxo3a into the cytoplasm through phosphorylation.

Active Akt moves from cytoplasm to mitochondrion and promotes cell survival by acting on Bad and HK-II

Mitochondria play a critical role in the regulation of both cell survival and death and have emerged as a pivotal 'convergence point' for neurodegeneration (Moreira *et al.*, 2009, 2010). Dysfunction of mitochondrial energy metabolism culminates in exacerbated ROS generation and activates the apoptotic cascade (Beal, 2005; Petrozzi *et al.*, 2007). Our previous study has demonstrated that Aβ oligomers trigger an intrinsic apoptotic pathway by cytochrome C release and caspases 9 and 3 activation (Di Carlo *et al.*, 2010). Upon Aβ treatment, as expected, mitochondrial dysfunction occurs, as indicated by membrane potential depolarization and ROS generation, whereas insulin treatment is able to revert this damage using a mechanism involving active Akt. These data are supported by the evidence that active Akt translocates to the mitochondrion only under oxidative stress conditions, as clearly demonstrated by Western blot of proteins extracted from fractionated cells and immunolocalization experiments. Here, it regulates the presence and post-translation modification of HK-II and Bad, two proteins involved in cell death. Our experiments reveal that the total amount of HK-II found in the mitochondrial fraction is increased by Akt activation, and we cannot exclude that Akt here phosphorylates HK-II. This phosphorylation has, indeed, been indirectly demonstrated using phospho-Akt substrate (PAS) antibody against Akt phosphorylation consensus sequences (Miyamoto *et al.*, 2008). However, this result, together with the restored mitochondrial membrane potential, suggests that active Akt prevents the dissociation of HK-II from the mitochondrion, an event that induces cytochrome C release and subsequent apoptosis (Majewski *et al.*, 2004). Phosphorylation of Bad disrupts its association with the anti-apoptotic bcl-2 protein and favours the formation of pores and the release of cytochrome C. Moreover, regarding Bad, the data suggest that under oxidative stress stimuli (Aβ or H_2O_2), unphosphorylated Bad translocates from the cytoplasm to the mitochondrion to activate the apoptotic programme but, after insulin administration and Akt translocation to mitochondrion, the present Bad molecules are phosphorylated and apoptosis inhibited. These data are in agreement with the fact that growth factors activate Akt, which in turn phosphorylates Bad in Ser 136 and blocks the Bad-induced death in a site-specific manner (Datta *et al.*, 1997). Further, phosphorylated Bad can be present in the cytoplasm bound to 14-3-3, as occurs for Foxo3a. These associations make cells more prone to survival signals. In contrast, phosphorylation of 14-3-3 by JNK releases Bad and Foxo3a, antagonizing the effects of Akt signalling (Sunayama *et al.*, 2005). Here,

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we demonstrated that after phosphorylation, the Bad molecules were subsequently transported to cytoplasm, but we cannot exclude that they were sequestered by 14-3-3 protein. Thus, active Akt shuttles among different subcellular compartments to carry out its survival programme.

Moreover, we suppose that the neuroprotective effects of insulin here described may be integrated with other signalling events. It has been demonstrated that the neurotrophic factor IGF-I protected against Aβ-induced neuronal death via the activation of ERK and Akt and the inhibition of JNK activity (Cheng & Feldman, 1998; Wei *et al.*, 2002). On the basis of these results, it has been hypothesized that a downstream point of these molecules could exist, where survival and death/stress signalling converge. Recently, it has been demonstrated that the inhibition of PI3K/Akt and MEK/ERK pathways activates Foxo transcription factors (Roy *et al.*, 2010), and we cannot exclude that the downstream point could just be Foxo3a.

We propose a model (Fig. 9) in which under oxidative stress induced by Aβ, Foxo3a is present in the nucleus where it activates several genes, including the proapoptotic ones, whereas Bad is transported to the mitochondrion: these events lead to apoptotic cell death (Fig. 9B). When insulin is added, after Aβ stimulation, oxidative stress is antagonized by the activation of Akt that phosphorylates Foxo3a, which then translocates from the nucleus to the cytoplasm inhibiting the transcription Foxo-dependent genes. At the same time, active Akt translocates to the mitochondrion where it phosphorylates Bad and modulates HK-II expression and perhaps its phosphorylation. Bad and HK-II are in fact two molecules that inhibit activation of the apoptotic pathway and lead to cell survival (Fig. 9C). However, supported by literature, we cannot exclude that when Foxo3a and Bad are sequestered in the cytoplasm, they are bound to 14-3-3, which could be a key protein for maintaining the right balance of these and perhaps other molecules in the different cellular compartments, especially under physiological conditions.

The balance between signals promoting survival and apoptosis is important for determining cell fate. In concert with data shown in the literature, we propose that Akt, activated by insulin and shuttling in different subcellular compartments, plays a key role in recovery from the cell death programme triggered by Aβ oligomers. Translocation from the cytoplasm to the nucleus induces negative regulation of gene expressions via Foxo3a phosphorylation. Translocation from the cytoplasm to the mitochondrion mediates the protection of this organelle through phosphorylation of Bad and probably HK-II, two proteins involved in cell death.

Insulin or insulin-like growth factors (IGF-I) have been shown to protect cells against numerous stress agents and aggregation-prone peptides such as amylin and α-synuclein; probably, the same molecules, here described, and their transport can also be at the basis of the

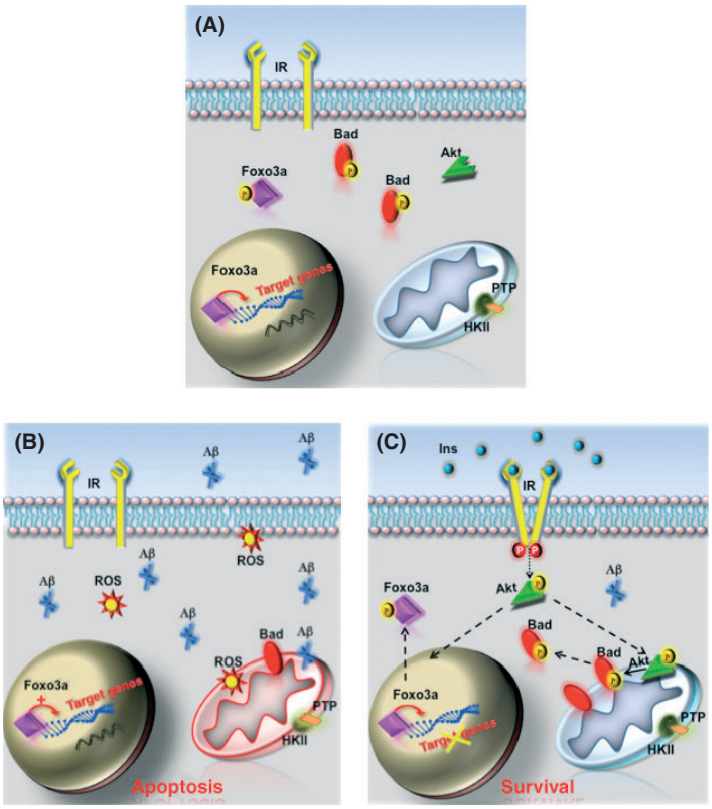

Fig. 9 A model for antagonism between Aβ (apoptotic) and insulin (survival) signals. (A) Physiological condition of a cell is maintained by the presence of Akt mainly in unphosphorylated state; phosphorylated Foxo3a and Bad are present in the cytoplasm probably bound to 14-3-3 protein; some unphosphorylated Foxo3a molecules are present in the nucleus, which activate the genes involved in normal cell homeostasis; in the mitochondrion, the permeability transition (PT) pore is closed. (B) Exposure to Aβ oligomers induces reactive oxygen species generation; unphosphorylated Foxo3a activates the proapoptotic genes in the nucleus; unphosphorylated Bad is translocated to the mitochondrion, which together with the PT pore opening, causes the cell death. (C) Exposure of a cell to insulin signals, after Aβ stimulus results in inhibiting apoptosis and promoting cell survival. Binding of insulin to insulin receptor activates Akt that in turn reduces ROS generation and inhibits Foxo3a by phosphorylation and translocation to the cytoplasm. Moreover, active Akt translocates to the mitochondrion where it phosphorylates Bad molecules that were translocated after Aβ stimulus, promoting their return to the cytoplasm. At the same time, mitochondrial active Akt increases HK-II levels and probably phosphorylates it, avoiding opening of the PT pore. P shown in yellow circles represents Akt-mediated phosphorylation.

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neuroprotection mechanisms (Dore *et al.*, 1997; Kao, 2009). Further, the activity of glycogen-synthase kinase-3 (Gsk3β), a protein involved in tau phosphorylation, can be downregulated in response to insulin or IGF-I through the activation of the PIK3/Akt pathway (Hong & Lee, 1997). As AD is a multifactorial pathology, finding protective molecules having more targets can be important in addressing therapeutic strategies.

In summary, the molecules and pathways here described have already been shown to participate in cell death by oxidative stress or survival under different circumstances. Their integration into a unique process that decides between neuronal death and survival related to AD and diabetes was not well documented before. Moreover, we would like to emphasize that the same molecule, depending on its state of phosphorylation, and its presence in a particular cellular environment can decide between life and death of a cell. Aβ and insulin activate two opposite cell signals using some common downstream molecules. Depending on their post-translational modifications and perhaps amounts, these molecules create a competitive balance between cell survival and degeneration. Because insulin signalling in the brain is known to decline with age, the outcome of this balance represents a risk factor for AD that is well suited for therapeutic intervention. By restoring the balance to favour neuron survival, new drugs designed to specifically enhance CNS insulin signalling would provide a new and potentially significant class of AD therapeutics.

Materials and methods

Preparation and characterization of rAβ42 oligomers

The recombinant Aβ42 peptide (rAβ42) was produced, purified and prepared in oligomeric form according to Carrotta *et al.*, 2006;. We utilized recombinant Aβ42 with the aim of obtaining oligomers of controlled size starting with monomers, avoiding the possibility of finding aggregate species that are present sometimes in synthetic batches. To assess the size of oligomers during toxicity assays, small rAβ42 oligomers, prepared at pH 7.2 (Carrotta *et al.*, 2006), were diluted in RPMI (Picone *et al.*, 2009). After 15, 30, 60 and 120 min of incubation at 37°C, aliquots were loaded on a nondenaturing PAGE and Coomassie blue stained. Here, a mixture of rAβ42 monomer and small aggregates of about 25 kDa as the larger species are referred to as oligomers.

Cell cultures and treatment

Cells were cultured with RPMI 1640 medium (Celbio srl, Milan, Italy) supplemented with 10% foetal bovine serum (Gibco-Invitrogen, Milan, Italy) and 1% antibiotics (50 mg mL^{-1} penicillin and 50 mg ml^{-1} streptomycin). Cells were maintained in a humidified 5% CO_2 atmosphere at 37 ± 0.1°C. For time course and concentration effects on Akt activation, LAN5 cells were treated with 20 or 40 μM of oligomeric rAβ42 for 15, 30, 60 and 120 min. For all the other experiments, LAN5 cells were treated with 40 μM of oligomeric rAβ42 for 1 h. After this treatment, rAβ42 was removed, and the cells were incubated without or with insulin at different concentrations (100 and 200 μM) in serum-free medium at 37°C for 4 or 20 h. The treated cultured cells and the controls were morphologically analysed by microscopy inspection; laser scanning confocal microscope (LSCM) was utilized for specific assays. For the ROS, JC-1 and Western blot experiments, we utilized rAβ42 at 40 μM for 1 h and insulin at 100 μM for 4 h.

Determination of cell viability

Cell viability was measured by MTS assay (Promega Italia, S.r.l., Milan, Italy). MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium] was utilized according to the manufacturer's instructions. After cell treatments, 20 μL of the MTS solution was added to each well, and the incubation was continued for 4 h at 37°C, 5% CO_2. The absorbance was read at 490 nm on the Microplate reader WallacVictor 2 1420 Multilabel Counter (PerkinElmer, Inc. Monza, Italy). Results were expressed as the percentage MTS reduction in the control cells.

Analysis of ROS and mitochondrial transmembrane potential generation

To assess ROS generation, the cells were incubated as mentioned above. Afterwards, cells were incubated with 1 mM dichlorofluorescein diacetate (DCFH-DA) in PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na_3PO_4, pH7.4) for 10 min at room temperature in the dark. After washing with PBS, the cells were analysed by fluorescence microscope (Axio Scope 2 microscope; Zeiss, Oberkochen, Germany) and fluorimetry (Microplate reader WallacVictor 2 1420 Multilabel Counter; PerkinElmer, Inc.) for the quantization of fluorescence intensity. The mitochondrial membrane potential was measured directly using a MitoProbe JC-1 Assay kit (Molecular Probes, Eugene, OR, USA). After treatment, the cells were incubated with 2 mM JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide) fluorescent dye in PBS for 30 min at 37°C. CCCP (carbonyl cyanide 3-chlorophenylhydrazone) (50 μM), a mitochondrial membrane potential disrupter, was used as a control to perform standard compensation. Fluorescence emission shift of JC-1 from red (590 nm) to green (529 nm) was evaluated by fluorimeter (Microplate reader WallacVictor 2 1420 Multilabel Counter; PerkinElmer, Inc.) and fluorescence microscope, (Axio Scope 2 microscope; Zeiss) with 488-nm excitation laser.

Total protein extraction and Western blotting

Total proteins were prepared by dissolving in solubilizing buffer (50 mM Tris–HCl pH 87.4, 150 mM NaCl, 0.5% Triton X-100, 2 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM DTT, 0.1% SDS with protease inhibitor; Amersham Biosciences (Milan, Italy) and phosphatase inhibitor cocktail II; Sigma-Aldrich, Milan, Italy), LAN5 cells untreated (control) or treated with oligomers, alone or with insulin. Protein samples (20 μg) were electrophoretically separated using 10% SDS–PAGE gel and transferred onto nitrocellulose filters for immunoblotting. After blocking in 3% BSA in TBST, the Western blot was incubated with anti-phosphorylated Akt (S473) (1:1000; Invitrogen), anti-Akt (1:1000; Cell Signaling), anti-phosphorylated-Foxo 3a (S253) (1:500; Cell Signaling), anti-Foxo 3a (1:1000; Cell Signaling), anti-peroxiredoxin III (PrxIII) (1:3000; Sigma-Aldrich) anti-β-actin (1:1000; Sigma-Aldrich). Primary antibodies were detected using the ECL chemiluminescence kit (Amersham Biosciences, Milan, Italy) according to the manufacturer's instructions and using secondary antibodies conjugated to horseradish peroxidase (1:2000; Cell Signaling, Boston, USA). Band intensities were analysed with a gel documentation system (Bio-Rad, Milan, Italy), and expression was adjusted to β-actin expression. The protein levels were expressed as densitometry and percentage of controls.

Mitochondrial and cytoplasmic protein extraction and Western blotting

Cytosol and mitochondria fractions of the cells untreated (control), treated with oligomers, alone or with insulin and H_2O_2, were prepared using Mitochondria Isolation kit (Pierce, Rockford, USA) according to the

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manufacturer's instructions. Lysates of both the fractions were separated by SDS–PAGE gel electrophoresis and transferred onto nitrocellulose filters for immunoblotting. The Western blot was incubated with anti-phosphorylated-Akt (S473) (1:1000; Cell Signaling, Boston, USA), anti-hexokinase-II (1:1000; Cell Signaling), anti-phosphorylated-Bad (S136) (1:500; Cell Signaling) and anti-Bad (C-7) (1:1000; Santa Cruz Biotechnology, Inc., Heidelberg, Germany). Anti-β-actin (1:1000; Sigma-Aldrich) and anti-PrxIII, a mitochondrial protein, (1:3000; Sigma-Aldrich) were used as markers of purified cytoplasmic and mitochondrial fractions, respectively. Primary antibodies were detected using the ECL chemiluminescence kit (Amersham) according to the manufacturer's instructions and using secondary antibodies conjugated to horseradish peroxidase (1:2000; Cell Signaling).

Confocal imaging: immunofluorescent analysis

LAN5 cells were cultured on Lab-Tek™ II Chambered Coverglass (Nunc, Roskilde, Denmark) 180 000 cells per cm^2 and treated as mentioned above. After treatment, the cells were washed in PBS and fixed in freshly prepared 4% paraformaldehyde in PBS for 30 min and kept at 4°C. After three washes in PBS, the samples were incubated with PBS 0.2% Triton X-100 for 10 min and then with 3% bovine serum albumin/PBS. The cells were then immunostained with anti-phosphorylated-Akt (S473) (1:1000; Cell Signaling, Boston, USA), or anti-phosphorylated-IR 1:500 (p-IR) (Calbiochem Italia, Milan, Italy) antibody at 4°C overnight. After three washes in PBS, the samples were incubated with anti-rabbit FITC-conjugate secondary antibody (1:300; Sigma-Aldrich, Milan, Italy). Mitochondria were stained by MitoTracker Deep Red (Sigma-Aldrich, Milan, Italy) 20 nM for 15 min in a humidified 5% CO_2 atmosphere at 37 ± 0.1°C before fixing the cells. Fluorescence images were acquired in 512 × 512 format in two channels by means a Leica TCS SP5 LSCM (inverted) using a 40× oil objective NA = 1.25 (Leica Microsystems, Leica, Wetzlar, Germany); the pinhole size was 45 μm. We used a 488-nm Argon laser to excite FITC dye and 543-nm He-Ne laser for Mito Red. The emission spectral range was set to 495–530 nm for FITC fluorescence (green channel) and 560–640 nm for Mito Red Fluorescence (red channel). In double colour experiments, images were sequentially acquired in the red and green channel with a scanning frequency of 400 Hz. For colocalization diagrams, images were processed with the colocalization tool of Leica Application Suite software. Regions of spatial overlap can be easily identified by drawing regions of interest in the cytofluorogram.

Statistical analysis

All experiments were repeated at least three times, and each experiment was performed in triplicate. The results are presented as mean SD. Statistical evaluation was conducted by ANOVA, followed by Student's *t*-test for analysis of significance. Results with a *P*-value<0.05 were considered statistically significant.

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Authorship contributions

Pasquale Picone: conception and design, data analysis, collection and/or assembly of data; Daniela Giacomazza: provision of study material, data interpretation, manuscript writing; Valeria Vetri: conception and design, data analysis, collection and/or assembly of data; Rita Carrotta: provision of study material, data interpretation; Valeria Militello: provision of study material, data interpretation, final approval of manuscript; Pier Luigi San Biagio: provision of study material, data interpretation, final approval of manuscript; Marta Di Carlo: conception and design, data analysis and interpretation, manuscript writing.

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