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Insulin-like growth factor-1 prevents A$\beta_{[25-35]}$/(H_2O_2)- induced apoptosis in lymphocytes by reciprocal NF-κB activation and p53 inhibition via PI3K-dependent pathway

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Abstract

The role of insulin-like growth factor (IGF-1) as neural survival factor for the treatment of Alzheimer's disease has recently gained attention. The present study shows that IGF-1 protects lymphocytes from (10, 30 μM) A$\beta_{[25-35]}$ and (25, 50, 100 μM) H_2O_2-induced apoptosis through NF-κB activation and p53 down regulation involving the phosphoinositide 3-kinase (PI-3K)–dependent pathway as demonstrated by using either (25 μM) LY294002 (PI-3K inhibitor), (10 nM) ammonium pyrrolidinedithiocarbamate (PDTC; NF-κB inhibitor), 50 nM pifithrin-α (PFT; p53 inhibitor) or by using immunocytochemistry detection of NF-κB and p53 transcription factors activation. Importantly, IGF-1, PDTC and PFT were able to protect and rescue lymphocytes pre-exposed to 10 μM A$\beta_{[25-35]}$, even when the three compounds were added up-to 12 h post- A$\beta_{[25-35]}$ exposure. Altogether these results suggest that survival/rescue of lymphocytes from A$\beta_{[25-35]}$ toxicity is determined by p53 inactivation via IGF-1/ PI-3K pathway.

Keywords: *Beta-amyloid, H_2O_2, IGF-1, NF-κB, PI-3K, p53*

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by insoluble amyloid-β [Aβ_{1-42}] protein deposits, tau-containing neurofibrillary aggregates and severe neuronal loss (reviewed by Esiri 2001). The hypothesis that postulate Aβ and metals (e.g., iron, copper) as mediators of oxidative stress for neurodegeneration in AD is supported by ample evidence from both *in vivo* and *in vitro* studies (reviewed by Cotman et al. 2001; Huang et al. 2004; Butterfield and Boyd-Kimball 2004). Indeed, our group have demonstrated that A$\beta_{[25-35]}$ (i.e., the cytotoxic domain of Aβ_{1-42}) and iron promote apoptosis—a type of programmed cell death—in peripheral blood lymphocytes (PBL) by a mechanism involving Aβgeneration of H_2O_2, ensuing activation and/or nuclear translocation of nuclear factor (NF)-κB, p53, c-jun transcription factors, mitochondrial depolarization and caspase-3 activation (Velez-Pardo et al. 2002). In accordance with these observations, H_2O_2 and free radicals have been detected in *in vivo* and *ex vivo* in mouse AD brains (McLellan et al. 2003) as well as NF-κB, p53 and c-Jun transcription factors have been shown *in situ* from AD brains (Garcia et al. 2003 and references therein). These data highlight the potential to use lymphocytes as a cellular model to directly monitor intracellular signalling mechanism(s) leading to death and/or survival responses to different oxidant stress stimuli.

During the last few years, neurotrophic factors have come into focus as potential therapy in AD (Siegel and Chauhan 2000). Specially, insulin-like growth factor-I (IGF-1) has been demonstrated to protect against

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$A\beta_{[25-35]}$ insult (Dore et al. 1997), though the exact molecular mechanism of action involved in neuroprotective properties of IGF-1 remains to be clarified. One clue of its neuroprotective capability comes from the fact that IGF-1 is able to activate NF-κB against H_2O_2 oxidative stress (Heck et al. 1999). However, it has also been shown that NF-κB activation is involved in H_2O_2-induced apoptosis (Kutuk and Basaga 2003). Therefore, the molecular mechanism(s) that may explain the dual role of NF-κB as an attenuator or promoter of apoptosis still remains to be established. The importance to reveal how these antagonist functions are accomplished by NF-κB is valuable for two main reasons. Firstly, NF-κB induces expression of several anti-apoptotic proteins and mitochondria membrane stabilizers, but also induces expression of pro-apoptotic proteins (reviewed by Burstein and Duckett 2003). Thus, cell death and survival signalisation may converge on NF-κB activation. Secondly, NF-κB is amenable to therapeutic intervention (Bremner and Heinrich 2002). In this regard, the relationship between NF-κB mediated p53 up-regulation in death/survival cell decision upon IGF-1 exposure has not yet been fully determined.

Because human PBL express IGF-1 receptors (Tapson et al. 1988; Kooijman et al. 1992) and IGF-1 appears to be of potential therapeutic use against AD (Carro et al. 2002), the present work was thus aimed at a better understanding of the molecular events that are thought to be downstream of IGF-1, in relation to the role played by NF-κB in survival and death-signalisation against $A\beta[_{25-35}]$ and H_2O_2 in lymphocytes, as a single cell model.

Materials and methods

Amyloid β-protein fragment 25–35 (Cat # A4559; $A\beta_{25}GSQKAIIGLM_{35}$), and other reagents if not otherwise specified were purchased from Sigma (St. Louis MO, USA) and were of analytical grade or better. Ammonium pyrrolidinedithiocarbamate (PDTC) was obtained from Calbiochem (Cat 548000). Recombinant human Insulin-like growth factor-I (IGF-1) was acquired from ProSpec-Techno-Gene (Weizmann Science Park, Rehovot, Israel). $DiOC_6(3)$ was purchased from Molecular Probes (Eugene, OR, USA, cat#D-273).

Isolation of lymphocytes

Peripheral blood lymphocytes (PBL) from venous blood of healthy adult male (range age 30–40 years old) were obtained by gradient centrifugation (Lymphocyte separation medium, density: 1.007 G/M; Bio-Whittaker). Isolated PBL were washed thee times with PBS (10 mM sodium phosphate, 160 mM NaCl, pH = 7.4) and finally suspended in RPMI 1640 (GIBCO laboratories, NY, USA) plus 10% foetal calf serum (FCS, GIBCO laboratories). The PBL in suspension were cultured in RPMI1640 supplemented with 10% foetal calf, 2 mM L-glutamine, 100-U/ml penicillin and 100-μg/ml streptomycin. The PBL were plated in 24-wells (1×10^6 cells/ml/well).

Experiments with peripheral blood lymphocytes

Assessment of apoptotic indexes. PBL were pre-incubated for 30 min at 37°C in culture medium containing 50, 100 nM IGF-1 and then (10, 30 μM) $A\beta_{[25-35]}$ fragment, or (25, 50, 100, 200 μM) H_2O_2 in the absence or presence of other products of interest for 24 h. PBL was then used for either parallel microscopic examinations such as viability studies, mitochondrial transmembrane potential ($\Delta\Psi_m$), rescue experiments or Immunocytochemical staining. Since Aβ promotes cellular clusters avoiding an accurate morphological evaluation, PBL cells treated with Aβ were disaggregated by gentle mechanical up-and–down micropipetting. Then, to perform viability studies 95μl either untreated (control) or treated cells were mixed with 5μl (0.1 mg/ml) acridine orange/ethidium bromide (AO/EB) and 5μl of the suspension was placed onto a slide and examined under fluorescence microscope (Nikon, Japan). Based on the differential uptake of the fluorescent DNA binding dyes AO/EB, normal PBL cells (NL, bright green chromatin) can be differentiated from early apoptotic cells (EA, bright green highly condensed or fragmented chromatin), late apoptotic cells (LA, bright orange highly condensed or fragmented chromatin) and necrotic cells (N, bright orange chromatin) (Leite et al. 1999). Quantification of apoptotic morphology was done by counting a minimum of 250 total cells as follows: % apoptotic cells $= 100 \times$ (total number of early and late apoptotic cells/ total number of cells counted). Necrotic cells were not detected under the present experimental conditions. Assessment of apoptotic indexes was repeated 3 times in independent experiments.

Assessment of mitochondrial transmembrane potential ($\Delta\Psi_m$) indexes. PBL were treated as described above for 24 h. After this time, PBL were incubated for 15 min with the cationic lipophilic $DiOC_6(3)$ (1 μM, final concentration) to evaluate $\Delta\Psi_m$. The quantification of non-fluorescent cells (reflecting low membrane potentials) under the fluorescence microscope was performed by counting a minimum of 250 total cells as follows: % non-fluorescent cells $= 100 \times$ (number of non-fluorescent cells) / total number of cells counted (non-fluorescent cells + fluorescent cells, reflecting high normal membrane potentials) compared with untreated control. In parallel, we used AO/EB staining to assess the apoptosis index.

Immunocytochemistry detection of NF-κB, and p53 transcription factor proteins

Immunocytochemistry was performed according to suppliers' protocol (Santa Cruz Biotechnology, goat Immunocruz staining System (cat # sc-2053) using the primary goat poly-clonal antibodies NF-κB p65 (C-20)-G (Santa Cruz Biotechnology cat#sc-372-G), and p53 (FL-393) (Santa Cruz Biotechnology cat #sc-6243-G). After treatments, cells were plated on poly-L-lysine coated cover slip and fixed in 4% methanol in 0.1 M phosphate buffer, pH 7.4 for 25 min and then washed with phosphate-buffered saline (PBS). Slides were exposed to 1% hydrogen peroxide in PBS for 10 min. After several washes, cells were permeabilized with Triton X-100 solution in PBS for 5 min. Cells were incubated with primary antibodies (10 μg/ml) for 2 h at room-temperature (RT) and subsequently incubated with biotinylated antibody at RT for 1 h. Finally, the specimens were stained with the Immunocruz enzyme kit. After staining, they were cover-slipped with cover glasses. Pictures were obtained using a Zeiss (Axiostart 50) microscope equipped with a Canon PowerShot G5 digital camera.

IGF-1 rescue experiments against Aβ$_{[25-35]}$

PBL were incubated with 50 nM IGF-1 immediately or at 1, 3, 6, 12 h of 10 μM Aβ [25–35] post-exposure for 24 h. After this time, treated PBL were evaluated in parallel for apoptotic indexes and mitochondrial transmembrane potential ($\Delta\Psi_m$) indexes as described in Experiments with Peripheral blood lymphocytes section. Additionally, immunocytochemistry detection of NF-κB, and p53 transcription factor proteins were performed when IGF-1 was added immediately or at the mentioned intervals of time as described in "Immunocytochemistry detection of NF-κB, and p53 transcription factor proteins" section.

Statistical analysis

Data are means ± S.E. of three independent experiments. The difference between two groups was statistically analyzed by Student's *t*-test. A *p*-value of < 0.05 versus control was considered significant.

Results

IGF-1 protects against Aβ[$_{25-35}$]-induced apoptosis via PI-3K/Akt

To assess whether IGF-1 protects lymphocytes against Aβ toxicity, PBL were exposed to 10–30μM Aβ[$_{25-35}$] alone or in the presence of IGF-1 for 24 h. As shown in Table I, both concentrations of Aβ[$_{25-35}$] induce apoptosis and their noxious effect is almost completely suppressed by either 10, 50 or 100 nM IGF-1 to control vehicle-treated cell values according to AO/EB staining technique, one the most reliable method to evaluate cell death (Leite et al. 1999). Since mitochondria have been demonstrated to play an important role in cellular fate decision (Green and Kroemer 2004), we evaluated mitochondrial depolarization using the lipophilic cationic $DiOC_6(3)$ compound in parallel to apoptosis. We found that 10–30μM Aβ[$_{25-35}$] induce disruption of mitochondrial transmembrane potential ($\Delta\Psi_m$) concomitantly with apoptotic morphology in PBL, but when co-incubated with IGF-1, mitochondria damage and apoptotic morphology were diminished to control values (Table I). Since 50 nM IGF-1 concentration was the effective minimal concentration in protecting lymphocytes from 10–30 μM Aβ, we used IGF-1 (50 nM) and Aβ[$_{25-35}$] (10μM) concentrations to perform further experiments.

Because it is known that IGF-1 elicits multiple signaling pathways in protection from apoptosis involving the phosphoinositide-3-OH kinase (PI3K)-Akt/ protein kinase B, and mitogen-activated protein kinase pathways (Peruzzi et al. 1999), we were interested to determine which of these pathways were operative in PBL. Cells were incubated with either 25μM LY294002, a specific PI-3K inhibitor, or 25μM PD98059, a specific MEK-1 inhibitor, alone and/or in the presence of 50 nM IGF-1/10 μM Aβ[$_{25-35}$] for 24 h. As expected, no apoptotic morphology or mitochondrial depolarization was observed when LY294002 and PD98059 were co-incubated with IGF-1 alone, and did not affect the Aβ[$_{25-35}$]-induced apoptosis and ($\Delta\Psi_m$) when compared with untreated or control values. Noticeably, while LY294002 blocked the protective effect of IGF-1, PD98059 was ineffective when Aβ[$_{25-35}$] was present in the reaction mixture (Table I).

IGF-1 protects lymphocytes from Aβ[$_{25-35}$]-induced apoptosis by activation of NF-κB and inactivation of p53

To further characterize the IGF-1 survival pathway, we investigated the role of NF-κB and p53 in IGF-1 cytoprotection mechanism. Thus, PBL were incubated with 10 nM PDTC, a specific NF-κB inhibitor, and 50 nM pifithrin-α (PFT), a reversible inhibitor of p53, in the presence of 50 nM IGF-1 and/or 10 μM Aβ[$_{25-35}$] for 24 h. As shown in Table I, both PDTC and PFT not only abridged apoptosis and ($\Delta\Psi_m$) in presence of IGF-1 or Aβ alone, but also reduced both indices when co-incubated with IGF-1 and Aβ to control values (Table I).

Given that NF-κB can be activated via Akt/ PKB kinase (Kane et al. 1999) or by Aβ (Velez-Pardo et al. 2002), and since p53 is a factor transcribed downstream by NF-κB (Wu and Lozano 1994; Hellin et al. 1998), it is likely that IGF-1 inactivates p53.To test

Table I. Effect of IGF-1, and PI3K, MEK-1, NF-κB, p53 inhibitors on PBL under Aβ[25–35] exposure.

Treatment	APO (%)	$\Delta\psi_m$ (%)
Untreated	<1 ± 0	<1 ± 0
IGF-1 (1–nM)	0	0
IGF-1 (10 nM)	0	0
IGF-1 (50 nM)	0	0
IGF-1 (100 nM)	0	0
Aβ(10 μM)	13 ± 2*	16 ± 3*
Aβ(30 μM)	32 ± 3*	33 ± 4*
IGF-1 (1 nM) +Aβ(10 μM)	3 ± 1*	2 ± 1*
IGF-1 (10 nM) +Aβ(10 μM)	<1 ± 0*	<1 ± 0*
IGF-1 (50 nM) +Aβ(10 μM)	<1 ± 0*	<1 ± 0*
IGF-1 (100 nM) +Aβ(10 μM)	0	0
IGF-1 (10 nM) +Aβ(30 μM)	20 ± 2*	23 ± 3*
IGF-1 (50 nM) +Aβ(30 μM)	4 ± 1*	5 ± 1*
IGF-1 (100 nM) +Aβ(30 μM)	0	0
LY294002 (25 μM)	<1 ± 0	<1 ± 0
LY294002 (25 μM) + IGF-1 (50 nM)	0	0
LY294002 (25 μM) +Aβ(10 μM)	15 ± 2	18 ± 2
LY294002 (25 μM) + IGF-1 (50 nM) +Aβ(10 μM)	11 ± 2	14 ± 2
PD98059 (25 μM)	<1 ± 0	<1 ± 0
PD98059 (25 μM) + IGF-1 (50 nM)	0	0
PD98059 (25 μM) +Aβ(10 μM)	12 ± 2	12 ± 2
PD98059 (25 μM) + IGF-1 (50 nM) + Aβ(10 μM)	<1 ± 0*	<1 ± 0*
PDTC (10 nM)	<1 ± 0	<1 ± 0
PDTC (10 nM) + IGF-1 (50 nM)	0	0
PDTC (10 nM) +Aβ(10 μM)	<1 ± 0*	<1 ± 0*
PDTC (10 nM) + IGF-1 (50 nM) +Aβ(10 μM)	0*	0*
PFT (50 nM)	<1 ± 0	<1 ± 0
PFT (50 nM) + IGF-1 (50 nM)	0	0
PFT (50 nM) +Aβ(10 μM)	<1 ± 0*	<1 ± 0*
PFT (50 nM) + IGF-1(50 nM) +Aβ(10 μM)	0	0

PBL were incubated for 24 h with (10, 30 μM) Aβ[25–35], (1, 10, 50, 100 nM) IGF-1, (25 μM) LY294002, (25 μM) PD98059, (10 nM) PDTC and (50 nM) PFT alone or in combination as indicated. Notice that when used in combination the reagents were used at the same concentration when they were used by themselves. The evaluation of apoptosis and ($\Delta\Psi_m$) indexes were performed as described in "Materials and Methods" section. Quantification of apoptosis and ($\Delta\Psi_m$) are expressed as a mean of percentage ±S.E. from three independent experiments. *p-value of <0.05 versus control was considered significant.

this hypothesis, PBL were incubated under IGF-1 and/or Aβ exposure to determine whether NF-κB and p53 were activated. As shown in Figure 1, both IGF-1 and Aβ[25–35]-induced activation/nuclear translocation of NF-κB (Figure 1C,E). In contrast, p53 was only activated/translocated under Aβ[25–35] stimuli (Figure 1F) as compared with untreated cells (Figure 1B). Strikingly, when PBL were incubated with IGF-1 in presence of Aβ, p53 was undetectable (Figure 1H) but NF-κB (Figure 1G) was clearly visible. Similar results were observed when PBL were treated with PFT in presence of Aβ (Figure 1I–J).

IGF-1 protects and rescues lymphocytes from Aβ[25–35]-induced apoptosis

To examine whether IGF-1 was capable to rescue lymphocytes from Aβ[25–35]-induced apoptosis, cells were exposed to 50nM IGF-1 immediately (0 h) or at 1, 3, 6 and 12 h of 10 μM Aβ[25–35] post-treatment. Whereas Aβ alone induced apoptosis and mitochondrial depolarization after 24 h incubation (12 ± 2% AO/EB apoptotic index), IGF-1 was able to protect and rescue lymphocytes against Aβ toxicity to untreated control values (i.e., <1 ± 0% AO/EB apoptotic index) at 0, 1, 3, 6 h tested or even if added up-to 12 h post-Aβ treatment. We further tested whether the pharmacological inhibitors PFT and PDTC could afford a similar rescue and protective effect as IGF-1. Effectively, both inhibitors were able to rescue and protect lymphocytes from Aβ[25–35] treatment (<1% AO/EB apoptotic index at any time tested). Additionally, immunohistochemical staining clearly showed the activation / nuclear translocation of NF-κB when IGF-1 was added upto 12 h post-Aβ treatment, but p53 was undetectable (Figure 2).

IGF-1 protect against H_2O_2-induced apoptosis in a concentration dependent fashion

We further wanted to determine whether IGF-1 was able to protect lymphocytes from the Aβ[25–35] by-product H_2O_2, and if the protective effect was mediated by PI3K. Thus, PBL cells were exposed to

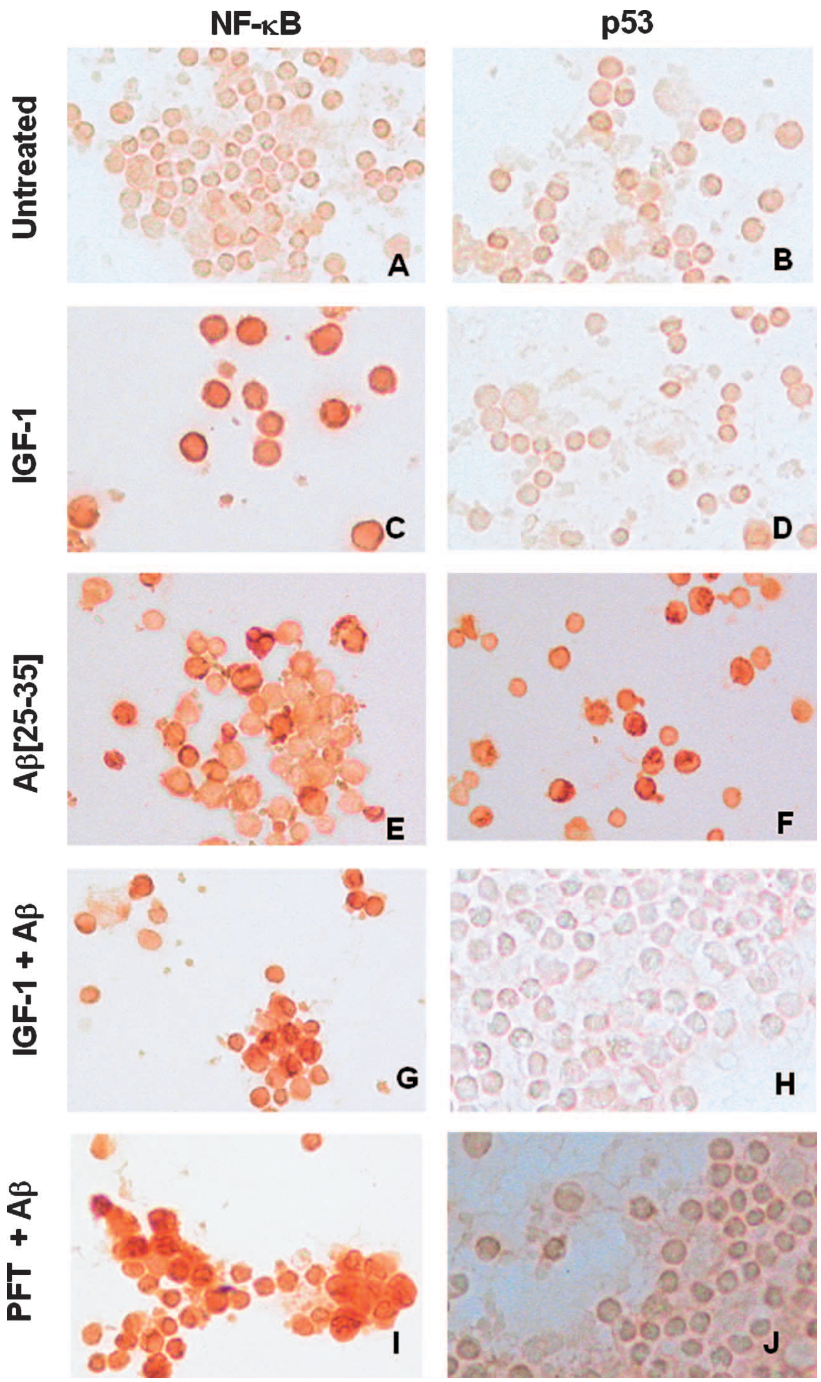

Figure 1. IGF-1 and $A\beta_{25-35}$ induce the activation of the transcription factors in PBL. PBL cells were left untreated (A, B), exposed to 100 nM IGF-1 (C, D), 10 μM $A\beta_{25-35}$ (E, F), co-incubated with both 100 nM IGF-1 and 10 μM $A\beta_{25-35}$ (G, H), or with 50 nM PFT plus 10 μM $A\beta_{25-35}$ (I, J) for 24 h. Notice that when used in combination the reagents were used at the same concentration when they were used by themselves. After this time of incubation, cells were stained with anti-NF-kB-p65 (A,C,E,G,I), and anti-p53 (B,D,F,H,J) antibodies according to procedure described in Materials and methods. Notice that NF-κB, and p53 positive-nuclei (dark brown colour) reflect their activation/ nuclear translocation. PBL cells treated with 50 nM PFT alone showed similar results as in untreated cells. Magnifications × 400 (A–J).

increasing (25, 50, 100, 200 μM) concentrations of H_2O_2. As shown in Figure 3, H_2O_2-induced apoptosis concomitantly with mitochondrial depolarization in a concentration dependent manner. While IGF-1 (50–100 nM) was ineffective to reduce apoptotic morphology and mitochondrial damage-induced by 200 μM H_2O_2, IGF-1 was either moderately effective diminishing both apoptotic morphology/ $\Delta\Psi_m$ at low concentrations (50–100 μM) or completely abolished apoptosis and $\Delta\Psi_m$ in PBL cells (e.g., 25μM H_2O_2). PDTC and PFT treatment had similar effects as IGF-1. Of notice, LY294002 inhibited the IGF-1 survival

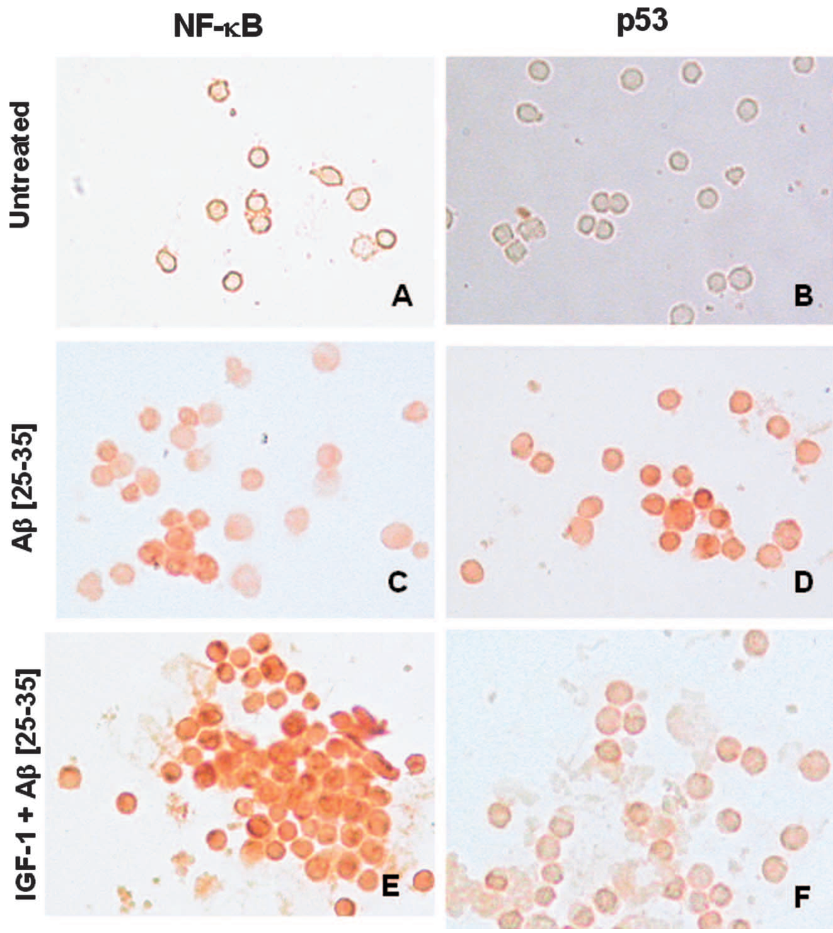

Figure 2. IGF-1 protects and rescues PBL from $A\beta_{25-35}$ cytotoxicity PBL cells were exposed to 50 nM IGF-1 at 12 h of 10 μM $A\beta[_{25-35}]$ post-treatment. After 24 h of incubation (A, B, E, F), or 12 h of incubation with 10 μM $A\beta[_{25-35}]$ alone (C, D), cells were stained with anti-NF-κB-p65 (A,C,E), and anti-p53 antibodies (B,D,F) according to procedure described in Materials and methods. Notice that NF-κB positive-nuclei (dark brown colour, C and E) and p53 positive-nuclei (D) reflect their activation/ nuclear translocation compared with untreated cells (A and B) and Magnification ×400 (A–B).

outcome of PBL when co-incubated with IGF-1 plus 100 μM H_2O_2.

IGF-1 protects PBL from $A\beta[_{25-35}]$, and H_2O_2-induced apoptosis when iron ion is present in the incubation mixture

To investigate the IGF-1 survival effect on PBL under hydroxyl radicals stress as a result of Fenton reaction (i.e., $H_2O_2 + Fe^{2+} \cdots {}^{\cdot}OH + Fe^{3+} + {}^{-}OH$), cells were co-incubate with 50nM IGF-1 and 25 μM Fe^{2+} in the presence or absence of 10 μM Aβ, or 25 μM H_2O_2. Unexpectedly, IGF-1 was able to prevent cell death from injurious combination of Aβ, H_2O_2 with Fe^{2+} (Table II).

Discussion

The major finding of the present study relates to the cell survival and rescue mechanism of IGF-1 against $A\beta[_{25-35}]$/ (H_2O_2)-induced toxicity in lymphocytes and its connection with the activation of NF-κB and down-regulation of p53 transcription factors. We found that a 30 min pre-treatment with 10, 50, 100nM of IGF-1 caused almost 100% survival (i.e. absence of apoptotic morphology and mitochondrial depolarization) after 10–30 μM $A\beta[_{25-35}]$ challenge in PBL cells of 24 h in vitro cultured (Table I). Similar results in earlier studies have shown (10–100 nM) IGF-1 as a potent and effective neuroprotective agent in hippocampal neurons (Dore et al. 1997) as well as in SH-SY5Y neuroblastoma cells line (Wei et al. 2002) against (10–50μM) $A\beta[_{25-35}]$ stimuli, but the signalling mechanisms involved have not been fully established. Thus, the present data highlight the reliability of using lymphocytes as a non-neural cell model to disentangle the IGF-1 survival/death signalling mechanism in response to oxidative stress insults. In this regard, PBL shares several biochemical and functional features with neurons: both are post-mitotic cells, synthesize proteins involved in cell survival /death machinery (Rathmell and Thompson 1999), expresses IGF-1R (Tapson et al. 1988; Kooijman et al. 1992), DA_{1-5}-receptors (Ricci et al. 1994, 1995, 1997; Amenta et al.

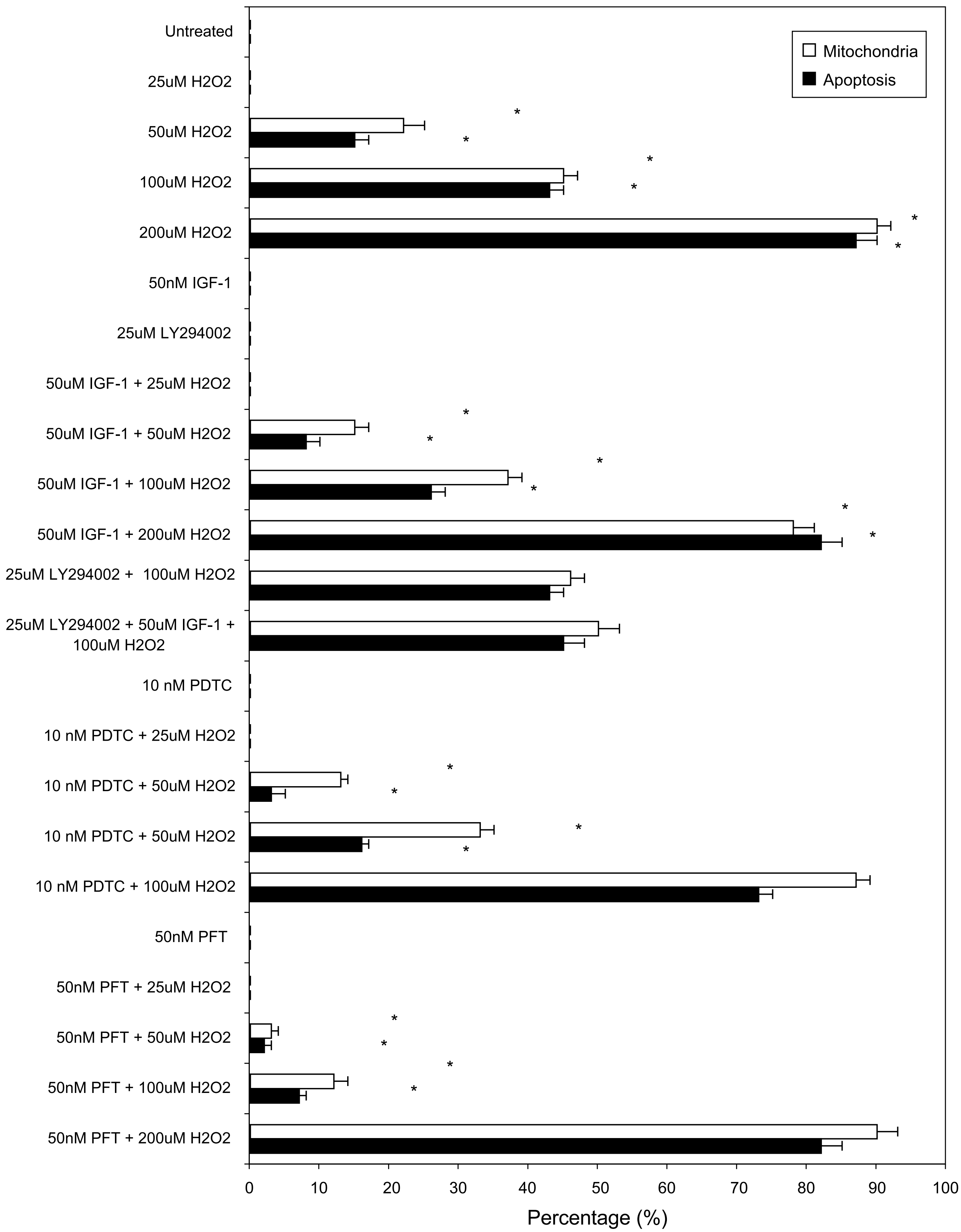

Figure 3. IGF-1 protects PBL from H_2O_2 in a PI3K-dependent pathway. PBL were left untreated or treated with (25, 50, 100, 200 µM) H_2O_2, (50 nM) IGF-1, (25 µM) LY294002, (10 nM) PDTC or (50 nM) PFT alone or in combination as illustrated for 24 h. Notice that when used in combination the reagents were used at the individual concentration when they were used by themselves. After this time of incubation, cells were evaluated for apoptotic morphology and ($\Delta\Psi_m$) as described in Materials and methods. Quantification of apoptosis and ($\Delta\Psi_m$) are expressed as a mean of percentage ± S.D. from three independent experiments. **p*-value of < 0.05 versus control was considered significant.

1999), dopamine transporter (Amenta et al. 2001) and transferrin (iron) receptor (Lum et al. 1986), but most importantly, both cells respond similarly (i.e., morphologically and biochemically) to oxidative stress stimuli (e.g., H_2O_2, DA, metals: Jimenez Del Rio and Velez-Pardo 2001, 2002, 2004a, b; Jimenez Del Rio et al. 2004 and references within).

IGF-1 has been shown to inhibit apoptosis via the phosphoinositide-3-OH kinase (PI3K)-Akt (protein kinase B), and mitogen-activated protein kinase pathways in differentiated PC12 cells (Parrizas et al. 1997a) probably via Ras-MEK-Rsk1-Bad, (Shimura et al. 2000). Specifically, we demonstrated that IGF-1 protects against Aβ$[_{25-35}]$-induced apoptosis, and by using pharmacological inhibition of PI3K, we found that LY294002 abolishes IGF-1 protection effect (Table I). In contrast, the specific MEK-1 inhibitor PD98059 did not block IGF-1 suppression of Aβ$[_{25-35}]$-evoked cell death. These results suggest that while IGF-1 inhibited Aβ$[_{25-35}]$-induced apoptosis via PI3K-dependent pathway, Ras-Bad pathway might play not a major role as a survival signalization or it might not be operational in PBL under the present experimental conditions. Taken together our data comply with the notion that PI3K-Akt dependent pathway is sufficient for the growth factor-induced PBL cell survival (Miller et al. 1997; Philpott et al. 1997; Crowder et al. 1998; Eves et al. 1998). Interestingly, the PI3K-Akt promotes cell survival via targets in addition to Bad such as caspase-9 (Cardone et al. 1998), Forkhead (FH) transcription factors (Brunet et al. 1999), induction of NF-κB through phosphorylation and activation of IκB kinase (IKK; Kane et al. 1999; Sizemore et al. 2002). Because FH transcription factors have been implicated in expression of the Fas ligand (Brunet et al. 1999), and caspase-9 phosphorylation by Akt has not been probed yet in vitro (Fujita et al. 1999), we focused our attention on the important link between PI3K-Akt and Aβ$[_{25-35}]$-induced activation/ nuclear translocation of NF-κB. Effectively, we found that IGF-1, and Aβ$[_{25-35}]$ alone (Figure 1C, E) or in combination (Figure 1G)-induced activation /translocation of NF-κB, and LY294002 inhibitor in the presence of IGF-1 completely abridges NF-κB activation (similar observation as Figure 1A, as determined by immunocytochemistry staining). These findings indicate that NF-κB transcription factor is activated by both pro-and anti-apoptotic stimulus, and that NF-κB activation involves the PI3K under IGF-1 stimulus (Heck et al. 1999). Moreover, by using the inhibitor PDTC, it is evidently shown that NF-κB is essential for apoptosis (Velez-Pardo et al. 2002; Song et al. 2004) as well as for survival process (Table I). Taken together, these results comply with the notion that activation of NF-κB in the same cell type (e.g., PBL (this work), cerebellar granule cells (Kaltschmidt et al. 2002)) result in different outcomes: apoptosis or citoprotection depending on stimulus (Kaltschmidt et al. 2000).

We have previously shown that Aβ$[_{25-35}]$/(H_2O_2)-induce apoptosis through activation and/or nuclear translocation of NF-κB and p53 transcription factors, and caspase-3 activation (Velez-Pardo et al. 2002). Interestingly, NF-κB has been reported to activate transcription of the p53 gene (Wu and Lozano 1994; Hellin et al. 1998; Jimenez Del Rio and Velez-Pardo 2002; Velez-Pardo et al. 2002), which in turn activates the expression of several genes that directly control or regulate the process of apoptosis such as Bax (a pro-apoptotic Bcl-2 protein family, Miyashita and Reed 1995; Deng et al. 2000). In this work, it is shown that IGF-1 was also able to activate NF-κB (Figure 1C). Therefore, these observations prompted us to examine the role of p53 in IGF-1 protection against Aβ$[_{25-35}]$. We were able to demonstrate that p53 is totally absent

Table II. Effect of Aβ, H_2O_2, DA and metal on PBL cells under IGF-I exposure.

Treatment	APO (%)	$\Delta\psi_m$ (%)
Untreated	$<1 \pm 0$	$<1 \pm 0$
IGF-1 (50 nM)	0	0
Aβ(10 μM)	13 ± 2*	16 ± 3*
Fe^{2+}(25 μM)	$<1 \pm 0$	$<1 \pm 0$
H_2O_2 (25 μM)	$<1 \pm 0$	$<1 \pm 0$
IGF-1 (50 nM) + Aβ (10 μM)	0*	0*
IGF-1 (50 nM) + Fe^{2+}(25 μM)	0	0
IGF-1 (50 nM) + H_2O_2 (25 μM)	0	0
Fe^{2+}(25 μM) + Aβ (10 μM)	21 ± 3*	23 ± 2*
Fe^{2+}(25 μM) + H_2O_2 (25 μM)	14 ± 2*	10 ± 2*
IGF-1 (50 nM) + Fe^{2+}(25 μM) + Aβ (10 μM)	$<1 \pm 0$	$<1 \pm 0$
IGF-1 (50 nM) + Fe^{2+}(25 μM) + H_2O_2 (25 μM)	$<1 \pm 0$	$<1 \pm 0$

PBL were left untreated or treated with 10μM Aβ$[_{25-35}]$, 25μM H_2O_2, 25μM iron, 50nM IGF-1, or in combination as indicated for 24 h. Notice that when used in combination the reagents were used at the same concentration when they were used by themselves. Afterwards, cells were evaluated for apoptotic morphology and mitochondrial transmembrane potential ($\Delta\Psi_m$) as described in Materials and methods. Quantification of apoptosis and ($\Delta\Psi_m$) are expressed as a mean of percentage ± S.D. from three independent experiments. *p-value of <0.05 versus control was considered significant.

when PBL were incubated with IGF-1 alone (Figure 1D) or when co-incubated with Aβ[$_{25-35}$] (Figure 1H) concomitantly with absence of apoptotic morphology and mitochondrial depolarization (Table I). These data suggest that p53 is a critical molecule in Aβinduced cell death. In support of this view, it is shown for the first time that the specific p53 inhibitor pifithrin-α (PFT) completely suppressed Aβ[$_{25-35}$]-evoked apoptosis in PBL (Table I). Moreover, PFT (Figure 1J) was able to mimic IGF-1 protective effect without affecting NF-κB activation (Figure 1I). These results suggest that p53 might be an essential molecule in cellular fate (Bargonetti and Manfredi 2002) under IGF-1 regulation.

Which molecular mechanism(s) explain the dual role of NF-κB as an attenuator or promoter of apoptosis?. One prevailing model proposes that when the molecular ratio of pro-survival (e.g., Bcl-2, Bcl-xL, Bcl-w) to pro-death Bcl-2 family members (e.g., Bax, Bad, Bak, Bid) is biased towards pro-death Bcl-2 family members (either through changes in expression level, localization or activity), the outer mitochondrial membrane becomes permeable to apoptogenic proteins resulting in the activation of a cascade of effector caspases, such as caspase-3, that kill the cells by irreversible proteolysis of critical nuclear and cytoplasmic constituents (for a review, see Adams and Cory 2001). In this vein, our data suggest that IGF-1 might promote gene transcription of survival genes via NF-κB activation and suppresses gene transcription of pro-apoptotic proteins through p53 inactivation. This notion is supported by the fact that PBL under IGF-1 stimuli protects mitochondria from Aβ[$_{25-35}$]-induced mitochondrial membrane depolarization (Table I), and IGF-1 inhibition of apoptosis is associated with an up-regulation of bcl-xL mRNA and proteins levels (Parrizas and Leroith 1997b). How then p53 turn-off could be related with IGF-1 citoprotection? One piece of information comes from the work by Ogawara and colleagues (2002) showing that Akt enhances the ubiquitinization-promoting function of Mdm2 (murine double minute) by phosphorylation of S^{186}, which results in reduction of p53 protein. Moreover, Feng and co-workers (2004) have recently shown that PKB/Akt induces phosphorylation of Mdm2 at Ser^{166} and Ser^{188} resulting in Mdm2 protein stabilization. Based on our present data and this information, it is reasonable to assume that p53 is modulated by IGF-1 through PI3K-Akt pathway. In fact, our findings reveal that p53 but not NF-κB is the critical transcription factor that may possibly balances the expression of pro-death proteins towards intracellular death decision under noxious stimuli. However, further investigation is needed to corroborate this hypothesis.

Previous data by Dore and co-workers (1999) have found that IGF-1 not only protects but also rescues hippocampal neurons against 30μM Aβ[$_{25-35}$]. However, no satisfactory explanation has been provided for these significant IGF-1 properties. Here, we confirmed that IGF-1 is able to protect and rescue lymphocytes from Aβ[$_{25-35}$]-induced apoptosis, even when IGF-1 is added up-to 12 h post- Aβ[$_{25-35}$] exposure for 24 h of incubation. Moreover, Immunocytochemical staining shows sustained activation of NF-κB and absence of p53 at any interval of time evaluated (Figure 2). Strikingly, PDTC and PFT inhibitors were also able to afford a similar protection and rescue effects as IGF-1 did. Taken together these results suggest that the protection/rescue of PBL from Aβ[$_{25-35}$] toxicity is determined by p53 inactivation under IGF-1 control.

H_2O_2 has been shown to mediate amyloid β-protein toxicity (Behl et al. 1994) and it has been implicated as a pivotal molecule in AD (McLellan et al. 2003; Jimenez Del Rio and Velez-Pardo 2004a, Milton 2004), we tested the ability of IGF-1 to protect PBL against H_2O_2 noxious stimulus. We found that IGF-1 effectively abolishes both apoptotic morphology and mitochondrial depolarization at low concentrations of (25μM) H_2O_2, but reduced apoptosis up to 50% when using mild concentrations (50–100μM H_2O_2, Figure 3). These results are in agreement with previous reports wherein IGF-1 protects rat primary cerebellar neurons and immortalized hypothalamic GT-1 cells against 60μM H_2O_2 (Heck et al. 1999). Moreover, we observed that PDTC and PFT inhibitors show a similar protective effect as IGF-1. Taken together these results confirm that both NF-κB and p53 are involved in H_2O_2-mediated signalization. Therefore, natural and/or synthetic NF-κB and p53 inhibitors are potential clinical agents in the treatment of neurological disorders related with oxidative stress (Bremner and Heinrich 2002). Interestingly, LY294002 blocked the IGF-1 survival effect under H_2O_2. Taken together our data suggest that IGF-1 protects PBL from oxidative stress by activation of NF-κB and blockage of p53 via IGF-1/PI3K-mediated mechanism. Consequently, these findings are consistent with the above suggestion that IGF-1/PI3K-mediates protection against Aβ toxicity. As expected, however, IGF-1, PDTC and PFT were not capable to reduce apoptosis/$\Delta\psi_m$ in cells when the highest concentration of H_2O_2 was used (e.g., 200μM H_2O_2). This result is consistent with previous reports indicating that high concentration of H_2O_2 may directly damage mitochondria resulting in a NF-κB- and p53-independent cell death mechanism (Takeyama et al. 2002; Jimenez Del Rio et al. 2004).

Iron is a redox-active transition metal associated with the neuropathology of Alzheimer (for a review sees Castellani et al. 2004). We found for the first time that IGF-1 completely abolishes apoptotic and $\Delta\psi_m$ indexes when co-incubate with 25μM Fe^{2+} plus 10μM Aβ[$_{25-35}$], or 25μM H_2O_2 (Table II). These results indicated that IGF-1 is a potent inhibitor of

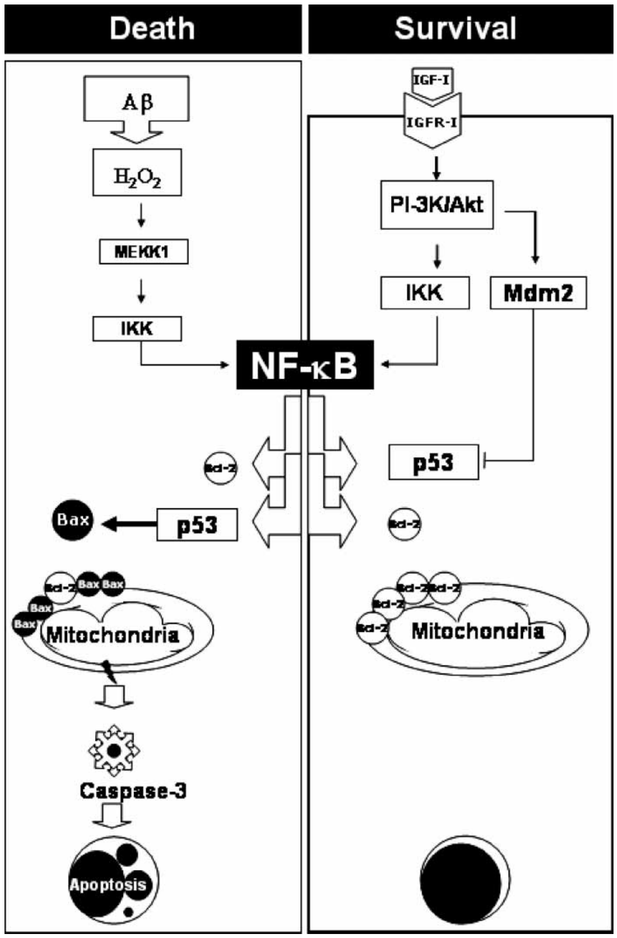

Figure 4. Schematic model of $A\beta_{25-35}$-induced apoptosis and IGF-1 survival signaling in lymphocytes. Aβ25–35 (10 μM) generates H_2O_2, which in turn indirectly activates NF-κB through activation of the multisubunit IkB Kinase (IKK) by p21-ras protein and MEKK1/ MAPKK kinase, respectively. Hence, the released of active NF-B dimmer (p50, p63) translocates into the nucleus and transcribes p53 transcription protein. Consequently, this protein activates the pro-apoptotic Bax protein. By out-numbering Bcl-2 proteins (e.g., Bcl-xL), Bax which is able to permeabilize mitochondria, promotes the release of the apoptogenic cytochrome c. As a result, cyt C together with Apaf 1, dATP, and procaspase-9 (i.e., apoptosome complex) elicits caspase-3 protease activation leading to typical nuclei apoptotic morphology (Jimenez Del Rio and Velez-Pardo 2004a and references within). IGF-1 can also activate NF-κB through IKK activation via PI-3K/Akt pathway (Kane et al. 1999; Sizemore et al. 2002). Then, NF-κB translocates into the nucleus and transcribes both anti-apoptotic Bcl-2 proteins and p53. Interestingly, p53 proteins could be indirectly regulated by PI-3K/Akt which phosphorylated mdm2 at S^{186}, thus enhancing its binding to p53 (Ogawara et al. 2003) and promoting p53 degradation. In fact, Mdm2 is an ubiquitin ligase that binds to the N-terminus and transfers ubiquitin moieties to several sites of p53. Ubiquitinated p53 is then rapidly exported from the nucleus and degraded by the ubiquitin proteosome system (UPS, Pickart 2001). As a result, p53 dependent pro-apoptotic proteins are not transcribed (e.g., Bax), and the ratio of anti-apoptotic versus pro-apoptotic is balanced in favor of the former proteins, providing mitochondrial membrane stability. Hence, normal nuclei morphology and cellular functions are preserved.

apoptosis and mitochondrial damage against oxidative stress generated by low $A\beta_{[25-35]}$/ H_2O_2 / and Fe^{2+} ions (i.e., Fenton reaction: $Fe^{2+} + H_2O_2 \cdots Fe^{3+} + {}^{\cdot}OH + {}^{-}OH$).

In summary, the significance of all together our data is twofold. First, we propose that IGF-1-PI3K/Akt activity might lead to increase anti-apoptotic NF-κB and decreased pro-apoptotic p53 transcriptional functioning to protect against Aβinduced apoptosis as shown in Figure 4. Thus, this data may contribute to a better understanding of the intracellular molecular mechanism by which IGF-1 promotes cell survival against oxidative stress. Secondly, pharmacological manipulation of specifically NF-κB activation and p53 switch-off by inhibitors mimicking the IGF-1-survival effect may provide some hints in the design of therapeutic strategies to prevent, delay, or ameliorate the treatment of genetically population at high risk of suffering from AD neurodegenerative disorder (Lopera et al. 1997) as encounter in Antioquia, Colombia.

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