Low-Power Laser Irradiation Inhibiting $A\beta_{25-35}$ -induced PC12 Cell Apoptosis via PKC Activation

Lan Zhang, Da Xing, Debin Zhu and Qun Chen

MOE Key Laboratory of Laser Life Science & Institute of Laser Life Science, South China Normal University, Guangzhou

Key Words

Low-power laser irradiation • FRET • Real-time quantitative RT-PCR • $A\beta_{25-35}$ • Protein kinase C • Bax/bcl-xl • MRNA ratio • Apoptosis

Abstract

Apoptosis is a contributing pathophysiological mechanism of Alzheimer's disease (AD). Recently, low-power laser irradiation (LPLI) has been applied to moderate AD, but the underlying mechanism remains unknown. In this study, the techniques of fluorescence resonance energy transfer (FRET) and real-time quantitative RT-PCR were used to investigate the anti-apoptotic mechanism of LPLI. Rat pheochromocytoma (PC12) cells were treated with amyloid beta 25-35 ($A\beta_{25-35}$) for induction of apoptosis before LPLI treatment. The cell viability assays and morphological examinations show that low fluence of LPLI (0.156 J/cm²-0.624 J/cm²) could inhibit the cells apoptosis. An increase of PKC activation was dynamically monitored in the cells treated with PMA (specific activator of PKC), LPLI only or $A\beta_{25-35}$ followed by 5 min LPLI treatment, respectively. However, the effect of LPLI activating PKC could be inhibited by Gö 6983 (specific inhibitor of PKC). Similar results were obtained by using Western blot analysis.

Furthermore, LPLI involved an increase in mRNA of the cell survival member *bcl-xl* and a decrease in the up-regulation of cell death member *bax* mRNA caused by $A\beta_{25-35}$. Further data show that low fluence of LPLI could reverse the increased level of *bax/bcl-xl* mRNA ratio caused by $A\beta_{25-35}$ treatment. In addition, Gö 6983 could inhibit the decreased level of *bax/bcl-xl* mRNA ratio. Taken together, these data clearly indicate that LPLI inhibited $A\beta_{25-35}$ -induced PC12 cell apoptosis via PKC-mediated regulation of *bax/bcl-xl* mRNA ratio.

Copyright © 2008 S. Karger AG, Basel

Introduction

Low-power laser irradiation (LPLI) can modulate various cellular processes such as proliferation, differentiation and apoptosis [1-8]. It has been reported that laser irradiation has a stimulatory effect on cell proliferation at relatively low fluence, but an inhibitory effect at higher fluence [9, 10]. There are several literature reports on anti-apoptosis of LPLI [8]. Duan et al have reported that within 24 h, cell apoptosis induced by amyloid beta 25-35 ($A\beta_{25-35}$) was significantly diminished with light irradiation [11]. It is well established that $A\beta_{25-35}$ is a biologically active region that retains the toxicity of a full-

length peptide(s) associated with Alzheimer's disease (AD) [12, 13], LPLI diminishing $A\beta_{25-35}$ -induced apoptosis, thus, may be another means for a neuron-protection. However, the anti-apoptotic mechanism of LPLI is still unclear.

Protein kinase Cs (PKCs) are a family of Ser/Thr protein kinases that play important roles in many cellular processes including proliferation, tumor promotion, differentiation, apoptosis and anti-apoptosis [14-18]. Our previous work and other reports show that LPLI could affect PKC signal transduction pathways [3, 4]. Therefore, we speculate that LPLI inhibiting the PC12 cells apoptosis is also via PKC activation.

In order to monitor the dynamics of PKC activation, fluorescence resonance energy transfer (FRET) technique was used. FRET is a nonradiative transfer of energy between 2 fluorophores that are placed in close vicinity and in a proper relative angular orientation [19, 20]. It is a powerful technique that can provide an insight into the spatial and temporal activities of signaling molecules in living cells [21-24]. In this study, C kinase activity reporter (CKAR) is constructed based on the FRET. If PKC is activated, the CKAR conformation will change that leads a gradual decrease in the FRET [25].

It is well established that the members of the Bcl-2 family of intracellular proteins are mediators of cell survival and apoptosis [26]. The interactions between the anti-apoptotic (Bcl-2, Bcl-x_L etc.) and the pro-apoptotic (Bax, Bad etc.) members can alter the permeability of mitochondrial membrane and trigger the release of cytochrome c or cause cascade activation of the caspases [27, 28]. It has been shown that PKC activation can affect cellular *bax* and *bcl-xl* expression [18]. To further investigate the relationship between PKC activation and apoptosis after LPLI treatment, cellular *bax* and *bcl-xl* mRNA levels were quantitatively studied using real-time quantitative RT-PCR (QT-PCR). The method can clearly differentiate the changes of anti-apoptotic Bcl-x_L and pro-apoptotic Bax, with better temporal resolution and higher sensitivity than that of conventional techniques.

Materials and Methods

Chemicals

RPMI 1640 medium was purchased from Gibco BRL (Grand Island, NY). Fetal bovine serum and horse serum were purchased from Hyclone (Logan, Utah, USA). $A\beta_{25-35}$, Hoechst 33258 and Phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Cell Counting Assay Kit-8 (CCK-8) was purchased from Dojindo

Laboratories (Kumamoto, Japan). LipofectamineTM 2000 reagent was purchased from Invitrogen (Carlsbad, CA, USA). Gö 6983 was purchased from Merck (Darmstadt, Germany). All reagents were of analytical grade unless otherwise stated. $A\beta_{25-35}$ stock solution of 1 mM was prepared in distilled and deionized water and stored at -20°C. Prior to a treatment, peptides were pre-incubated at 37°C for 5 days to promote aggregation and then diluted with medium to desired concentrations. The concentrations of PMA and Gö 6983 used in our experiments were 200 nM and 1 μ M, respectively.

Cell culture and treatment

The PC12 cells were cultured in a humidified (5% CO₂, 37°C) incubator in RPMI 1640 culture medium supplemented with 10% fetal bovine serum, 5% horse serum, penicillin (100 U/ml), and streptomycin (100 μ g/ml). The medium was refreshed every other day, and the cells were plated at appropriate densities according to each experimental protocol. Before transfection, the cells were cultured in a custom-built dish. When the cells came to 70-80% confluence, plasmid DNA of CKAR (a gift from Prof. Alexandra C. Newton of California University) was transfected with LipofectamineTM 2000 reagent into the PC12 cells.

Low-power laser irradiation

The light source of LPLI was a He-Ne laser (5 mW, 632.8 nm, model HN-1000, Guangzhou, China). In each experiment, sub-cultured monolayer cells (1 $\times$ 10⁶ cells/dish) were incubated for 24 h, then the cells were treated with different chemicals and/or irradiated at a fluence rate of 0.52 mW/cm² for a fluence of 0.156 J/cm²-1.248 J/cm². The chemicals were added to the culture medium 30 min before a LPLI treatment. The entire procedure was carried out at room temperature. Throughout each experiment, the cells were kept either in a complete dark or a very dim environment, except when subjected to the light irradiation, to minimize the ambient light interference.

Cell viability assays

Cell viability was measured with CCK-8 according to the manufacture's protocol. PC12 cells were plated on 96-well plates (100 μ l, 1 $\times$ 10⁴/well). To identify the proper dosage of $A\beta_{25-35}$ for apoptosis induction, the cells were co-incubation with $A\beta_{25-35}$ of various concentrations for 48 h. The CCK-8 solution (10 μ l) was added to each well and the cells were incubated for another 2 h. The optical absorbance at 450 nm for each sample was measured using a standard plate-reader (DG5032, Hua Dong, Nanjing, China). In the preliminary study, we found that the cell proliferation was decreased significantly with 25 μ M $A\beta_{25-35}$ treatment (data not shown). Thus, the concentration was chosen as a standard protocol to induce PC12 cells apoptosis.

In the subsequent experiments, each well was treated as indicated and incubated for an additional 48 h. CCK-8 assay was then used to measure the corresponding cell viability.

Morphological examination of apoptotic cells

As a direct confirmation, cell apoptosis was morphologically evaluated with Hoechst 33258. PC12 cells

(1×10^6 cells) were treated as indicated and incubated for an additional 48 h in 35 mm dish. Hoechst 33258 (10 $\mu\text{g/ml}$) was added to each dish and the cells were incubated at 37°C for an additional 30 min in the dark. Fluorescence images of the normal and apoptotic cells were examined with a modified commercial microscope system equipped with a mercury lamp (band-pass filter: 352–461 nm), a 395 nm dichroic mirror and a long-pass filter 397 nm emission filter (LSM510/ConfoCor2, Zeiss, Jena, Germany). The fluorescence images were collected via a Zeiss C-Apochromat objective (40x, NA=1.3).

FRET analysis of PKC activation

To investigate the potential mechanism of LPLI inhibiting $\text{A}\beta_{25-35}$ -induced apoptosis, PKC activation was studied using FRET technique. The CKAR comprises CFP, a FHA2 phosphopeptide-binding domain, a PKC substrate sequence, and YFP. Before PKC activation, CFP and YFP are in proximity. Energy can be transferred directly from CFP to YFP, so the fluorescence emitted from YFP can be detected when CFP is excited. Once PKC is activated, CFP will separate from YFP. This results in a gradual decrease in YFP/CFP emission intensity ratio.

PC12 cells expressing CKAR were treated with different chemicals and/or LPLI after serum starvation for 12 h. A real-time monitoring of PKC activation was performed on a commercial laser scanning microscopes combination system (LSM510/ConfoCor2, Zeiss, Jena, Germany) equipped with a Plan-Neofluar 40x/1.3 NA Oil DIC objective.

To measure the FRET effect, cells were excited by the 458 nm line of an Ar-Ion Laser. The emission fluorescence was split by a second dichroic mirror (secondary beam splitter NPT545) into two separate channels: the 470–500 nm band-pass (CFP channel) and the 530 nm long-pass (YFP channel). The CFP and YFP emission intensity distributions were mapped with Zeiss Rel3.2 image processing software (Zeiss, Jena, Germany). YFP/CFP emission ratios were obtained by dividing the average fluorescence intensity values of single cells after background subtraction. For direct comparisons, the YFP/CFP ratio at the first time point was normalized to 1.

For time-lapse imaging, culture dishes were mounted onto the microscope stage equipped with a temperature-controlled chamber (Zeiss, Jena, Germany).

Western blot analysis

After different treatment, PC12 cells were washed once in cold PBS and lysed in a buffer containing 50 mM Tris HCl pH 8.0, 150 mM NaCl, 1% TritonX-100, 100 $\mu\text{g/ml}$ PMSF and Protease Inhibitor Cocktail Set I (Calbiochem, San Diego, CA).

Lysates were separated by SDS-PAGE, and transferred to a PVDF membrane (Roch). The membrane was first blocked with 5% skim milk for 1 h, followed by incubation with primary antibodies overnight at 4°C. β -actin antibody (Santa Cruz Biotechnology) was used at 1:500 dilution, p-PKC (pan) antibody (Cell Signaling Technology) was used at 1:1000 dilution. Primary antibody incubation was performed in 5% bovine serum albumin in TBS-T (Tris base saline, pH 7.4, 0.1% Tween 20). A designated secondary antibody diluted at the recommended ratio, in TBS-T and 5% bovine serum albumin

for 60 min at room temperature. Washing with TBS-T was performed between all steps. Detection was performed using the Odyssey Scanning Infrared Fluorescence Imaging System (LI-COR, U.S.A.).

RNA isolation and quantitative real-time RT-PCR (QT-PCR)

PC12 cells were treated with different chemicals and/or LPLI, then incubated for 6 h. *bax* mRNA, *bcl-xl* mRNA and GAPDH mRNA were amplified from the cells. Total RNA was isolated using the High Pure RNA Isolation Kit (catalog no. 1828665; Roche Applied Science, Mannheim, Germany) and treated with RNase-free DNase. The cDNA synthesis and amplification by QT-PCR were carried out using the LC RNA Master SYBR Green I kit (catalog no. 3064760; Roche Applied Science, Mannheim, Germany). Briefly, for cDNA synthesis and QT-PCR, each capillary contained 0.8 μl (0.8 μg) of total RNA template, 0.65 μl $\text{Mn}(\text{OAc})_2$ stock solution (3.25 mM), 3.75 μl LightCycler RNA Master SYBR Green I, and 0.75 μl (0.3 μM) each of the primers in a total volume of 10 μl . PCR primers were 5'-CTG CAG AGG ATG ATT GCT GA-3' (forward) and 5'-GAG GAA GTC CAG TGT CCA GC-3' (reverse) for *bax* (accession no. U32098); 5'-AGG CTG GCG ATG AGT TTG AA-3' (forward) and 5'-CGG CTC TCG GCT GCT GCA TT-3' (reverse) for *bcl-xl* (accession no. X82537); and 5'-TGG TGC TGA GTA TGT CGT GGA GT-3' (forward) and 5'-AGT CTT CTG AGT GGC AGT GAT GG-3' (reverse) for GAPDH (accession no. M17701) [29]. In PC12 cells, the expected sizes of the generated fragments are 207 bp for *bax*, 337 bp for *bcl-xl*, and 292 bp for GAPDH, respectively. The cDNA synthesis and QT-PCR reactions were performed using LightCycler (Roche Applied Science, Mannheim, Germany) for reverse transcription of the template RNA at 61°C for 20 min, denaturation of the cDNA/RNA hybrid at 95°C for 1 min, amplifying the target DNA for 45 cycles at 95°C for 5 s, annealing temperature at 55°C for 8 s, extension at 72°C for 15 s. A negative control (H_2O) was included to check for cross contamination in every run. Amplification specificity was checked using melting curve, according to the manufacturer's instruction. Since each specific product melts at a higher temperature than the primer dimers, the melting curves thus allow discrimination between the primer dimers and a specific product.

Creation of the Coefficient File and Quantification

The total RNA was diluted serially from 5000 ng to 1.6 ng using five points with RNase-free H_2O . Then added different concentrations of total RNA template, 0.65 μl $\text{Mn}(\text{OAc})_2$ stock solution (3.25 mM), 3.75 μl LightCycler RNA Master SYBR Green I, 0.75 μl (0.3 μM) each of the primers in a total volume of 10 μl . The cDNA synthesis and QT-PCR reactions were performed as described above. The experimental standard curves were analyzed with Real Quant Software (Roche Applied Science, Mannheim, Germany) to create three coefficient files (*bax*, *bcl-xl* and GAPDH).

The results were quantified using coefficient files and the Real Quant Software (Roche Applied Science, Mannheim, Germany). The levels of *bax* mRNA and *bcl-xl* mRNA were expressed as the target/reference ratio of

the sample divided by the target/reference ratio of the control. In all experiments, *bax* mRNA was target 1, *bcl-xl* mRNA was target 2 and GAPDH mRNA was reference.

Statistical analysis

Each data point is from three independent experiments and represented as mean $\pm$ SEM. For statistical comparison, Student's t-test was used and significance was defined as $*P < 0.05$.

Results

Effects of LPLI on PC12 cell proliferation measured with CCK8

The results show that laser irradiation had a profound effect on the proliferation of $A\beta_{25-35}$ -treated PC12 cells. Compared with that of non-treated cells, LPLI treatment in the range from 5 to 20 min could promote the proliferation of $A\beta_{25-35}$ -treated PC12 cells (Fig. 1). However, the proliferation was significantly decreased when the cells were treated with $A\beta_{25-35}$ -only, $A\beta_{25-35}$ followed by 40 min LPLI or $A\beta_{25-35}$ plus Gö 6983 followed by 5 min LPLI.

Morphological examination of LPLI inhibiting apoptosis

In order to evaluate the cell apoptotic status directly, morphological examinations were performed. With Hoechst 33258 staining and fluorescence microscopy, chromatin condensation was observed in the cells treated with $A\beta_{25-35}$ -only, $A\beta_{25-35}$ followed by 40 min LPLI or $A\beta_{25-35}$ plus Gö 6983 followed by 5 min LPLI, respectively. This indicates the occurrence of apoptosis (Figs. 2B, D and E). The nuclei of the PC12 cells treated with $A\beta_{25-35}$ followed by 5 min LPLI (Fig. 2C) showed no difference with that of the control (Fig. 2A). The results are in agreement with that from the CCK8 assay that, low fluence of LPLI could inhibit $A\beta_{25-35}$ -induced PC12 cell apoptosis and the specific PKC inhibitor Gö 6983 could inhibit the effect of LPLI.

Real-time monitoring of PKC activation in single living cell

To explore the mechanism of the LPLI inhibiting apoptosis, the effect of LPLI on PKC activation in living single cell was monitored using FRET technique. The typical time-course images of CFP and YFP after 5 min LPLI treatment are shown in Fig. 3A. The fluorescence images show the emission in the CFP channel increased, and the emission in the YFP channel decreased. The ratios

Fig. 1. Effects of LPLI on $A\beta_{25-35}$ -induced PC12 cell apoptosis. Cell proliferation measured with CCK8 assay. The data is normalized to that of control (no treatment) ($n = 3$; $*P < 0.05$ versus control).

of YFP/CFP emission are confirmed by quantitative analysis of fluorescence intensities in the selected region. The ratio of YFP/CFP emission decreased when the cells were subject to LPLI only or $A\beta_{25-35}$ followed by 5 min LPLI treatment (Fig. 3B). Similar result was observed when the cells were treated with PMA (PKC activator). But the cells treated with $A\beta_{25-35}$ -only, $A\beta_{25-35}$ followed by 40 min LPLI or $A\beta_{25-35}$ plus Gö 6983 followed by 5 min LPLI did not result in a change in YFP/CFP emission ratio. These results indicate that low fluence of LPLI can effectively activate PKC in the cells pre-treated with $25 \mu M$ $A\beta_{25-35}$. These results were also confirmed by Western blot analysis (Fig. 3C).

Confirmation of specific amplification and creation of the coefficient file

To further investigate the mechanism of LPLI inhibiting apoptosis, we examined the changes in *bax* mRNA and *bcl-xl* mRNA. Firstly, melting curves of the amplifications were obtained to check the specific amplification of *bax* mRNA, *bcl-xl* mRNA and GAPDH mRNA of samples isolated from cells treated with $A\beta_{25-35}$ and the non-treated cells as control. Primer dimers are also shown as a negative sample (Fig. 4). Secondly, the standard coefficient files were created. A total RNA concentration range of 3 orders of magnitude $1.00E+1 \mu l$ to $3.20E-3 \mu l$ was included in the dilution series. This covered the expected concentration range of all samples. The standard curves were analyzed with Real Quant Software to create the three coefficient files, which were later used for relative quantification analysis (Fig. 5).

Fig. 2. Hoechst 33258 morphological examination of LPLI inhibiting apoptosis. (A) Control. (B) $A\beta_{25-35}$ (25 μ M, 48 h). Condensation fragmentation (as the arrow indicates). (C) $A\beta_{25-35}$ + 5 min LPLI: similar to that of control. (D) $A\beta_{25-35}$ + 40 min LPLI: typical apoptotic form similar to that form $A\beta_{25-35}$ treatment. (E) $A\beta_{25-35}$ + 5 min LPLI + 1 μ M Gö 6983: typical apoptotic form similar to that form $A\beta_{25-35}$ treatment.

LPLI inhibiting apoptosis detected with *bax/bcl-xl* mRNA ratio

PC12 cells were treated with 25 μ M $A\beta_{25-35}$ and/or LPLI (irradiation time: 5 to 40 min, 0.52 mW/cm²), then *bax* mRNA, *bcl-xl* mRNA and GAPDH mRNA were amplified from the cells. The results show that, when the cells were treated with 25 μ M $A\beta_{25-35}$ for 6 h, the *bax* mRNA increased (Fig. 6A) and the *bcl-xl* mRNA decreased significantly (Fig. 6B). When the LPLI irradiation time was in the range from 5 to 20 min, the *bax* mRNA fell to the control level gradually and the *bcl-xl* mRNA either increased significantly or showed no change, in comparison to that of the control group. However, when the irradiation time was prolonged to 40 min, the *bax* mRNA raised and the *bcl-xl* mRNA decreased to that as in the $A\beta_{25-35}$ only group.

The pro-apoptotic *bax* mRNA to the anti-apoptotic *bcl-xl* mRNA ratio was evaluated as a cell apoptotic marker. This ratio was found to increase significantly in the $A\beta_{25-35}$ treatment only group or treated with $A\beta_{25-35}$ followed by 40 min LPLI group (Fig. 6C). The ratio increase corresponds well to the cellular apoptosis evaluated with morphology as described earlier. These results clearly indicate that a controlled LPLI treatment can have anti-apoptotic effect and the process can be early monitored with *bax/bcl-xl* mRNA ratio.

Fig. 3. PKC activation induced by LPLI. (A) Fluorescence images of CFP and YFP after 5 min LPLI treatment. The pseudo-color scale at rights represents relative fluorescence intensity. (B) Fluorescence intensity ratio of the YFP to CFP channel after various treatments. (C) The activations of PKC were analyzed by Western blot. β -actin as a loading control. Results represent one of three replicates.

Fig. 4. Melting curve analysis of the amplified samples. GAPDH A, *bax* A and *bcl-xl* A are samples of the cells treated with 25 μ M $A\beta_{25-35}$ for 6 h. GAPDH B, *bax* B and *bcl-xl* B are samples from the cells without $A\beta_{25-35}$ treatment. As a negative, the template RNA was replaced by PCR-grade water.

Fig. 5. Creation of the coefficient file. (A) Melting curve analysis of the specificity of amplified GAPDH RNA. As a negative, the template RNA was replaced by PCR-grade water (B) Amplification plots of GAPDH RNA. Five fold serial dilutions of GAPDH RNA were amplified by real-time QT-PCR using LightCycler. (C) Standard curve constructed with the GAPDH RNA serially diluted standards from 1.00E+1 μ l to 3.20E-3 μ l. The data were fit using least squares linear regression analysis (n = 3).

Anti-apoptotic effect of LPLI diminished by the inhibition of PKC

To further confirm that the changes of *bax/bcl-xl* ratio after LPLI treatment are caused by the PKC activation, *bax/bcl-xl* ratio was measured in PC12 cells co-treated with 1 μ M Gö 6983 and 25 μ M A β_{25-35} followed by 5 min LPLI. This ratio was found to decrease similar to that in the A β_{25-35} treatment only group (Fig. 7). The result demonstrates that the anti-apoptotic effect of LPLI was diminished by the inhibition of PKC.

Fig. 6. LPLI inhibiting apoptosis detected with *bax/bcl-xl* mRNA ratio. (A) Expression of *bax* mRNA. (B) Expression of *bcl-xl* mRNA. (C) The *bax/bcl-xl* mRNA ratio. Data were normalized to that of control (no treatment) (n = 3; *P < 0.05 versus control).

Fig. 7. The *bax/bcl-xl* mRNA ratio after co-treatment with 1 μ M Gö 6983 and 25 μ M A β_{25-35} followed by 5 min LPLI. Data were normalized to that of control (no treatment) (n = 3; *P < 0.05 versus control).

Discussion

The objective of this study is to investigate the mechanism of LPLI inhibiting apoptosis. Our previous work and other reports show that PKC signal transduction pathway is very important in cell proliferation induced by LPLI. The possible mechanism of LPLI activating PKC has been suggested. One possibility is that tyrosine protein kinases (TPKs)/phospholipase C (PLC)-gamma/PKC pathway mediates PKC activation. Another possibility is the increases of Ca²⁺ concentration and diacylglycerol (DG) induced by LPLI can activate the PKC [3, 4, 11, 15]. Since PKC can be activated by LPLI, we speculate that LPLI inhibiting the PC12 cells apoptosis is also via PKC activation. To demonstrate the role of PKC activation in potential anti-apoptotic mechanism of LPLI, the real-time FRET technique in a single living cell was used. The results (Fig. 3) clearly support that PKC activation was required in the process of LPLI inhibiting apoptosis. Furthermore, it has been established that PKC activation can affect cellular *bax* and *bcl-xl* expression [18]. In the current study, we have examined the changes in cell-death/survival-related genes using real-time quantitative RT-PCR. The results (Figs. 6 and 7) demonstrate that the inhibition of apoptosis by LPLI not only involves the PKC pathway but also an increase in mRNA of the cell survival member *bcl-xl* and a decrease in the up-regulation of cell death member *bax* mRNA caused by A β_{25-35} . The data show that a short time or low fluence of LPLI could reverse the increased level of *bax/bcl-xl* mRNA ratio caused by A β_{25-35} treatment. When the irradiation time was prolonged to 40 min, LPLI

Fig. 8. A model of LPLI inhibiting A β_{25-35} -induced PC12 cell apoptosis.

could not reverse the increased level of *bax/bcl-xl* mRNA ratio caused by the A β_{25-35} treatment. The amount of LPLI fluence, thus, can affect the responses in cell *bax* mRNA and *bcl-xl* mRNA levels. This is expected as cell apoptosis is a complex process and is controlled by multiple signal pathways. By comparing with the results from the CCK8 assay and the Hoechst 33258 morphological examination (Figs. 1 and 2), the *bax/bcl-xl* mRNA ratio is a more efficient and sensitive apoptosis marker.

In summary, a controlled low dose LPLI can have anti-apoptotic effect on cells. By monitoring the dynamics of PKC activation and the change in the *bax* to *bcl-xl* ratio after Gö 6983, A β_{25-35} and LPLI treatment, we conclude that A β_{25-35} can induce cell apoptosis by increasing the level of *bax* mRNA, and the anti-apoptotic effect of LPLI is dependent on the up-regulation of *bcl-xl* and down-regulation of *bax* via PKC activation pathway (Fig. 8). The PKC-mediated regulation of *bax* and *bcl-xl* mRNA, as reflected by their corresponding ratio, plays an important rule in inhibiting A β_{25-35} -induced PC12 cell apoptosis.

Acknowledgements

This research is supported by the National Natural Science Foundation of China (30470494; 30627003) and the Natural Science Foundation of Guangdong Province (7117865). We thank Professor Alexandra C. Newton at Department of Pharmacology, University of California for providing C kinase activity reporter plasmid.

References

- 1 Wu Y, Xing D, Luo S, Tang Y, Chen Q: Detection of caspase-3 activation in single cells by fluorescence resonance energy transfer during photodynamic therapy induced apoptosis. *Cancer Lett* 2006;235:239-247.
- 2 Wang F, Chen TS, Xing D, Wang JJ, Wu YX: Measuring dynamics of caspase-3 activity in living cells using FRET technique during apoptosis induced by high fluence low-power laser irradiation. *Lasers Surg Med* 2005;36:2-7.
- 3 Gao X, Chen T, Xing D, Wang F, Pei Y, Wei X: Single cell analysis of PKC activation during proliferation and apoptosis induced by laser irradiation. *J Cell Physiol* 2006;206:441-448.
- 4 Ben-Dov N, Shefer G, Irintchev A, Wernig A, Oron U, Halevy O: Low-energy laser irradiation affects satellite cell proliferation and differentiation in vitro. *Biochim Biophys Acta* 1999;1448:372-380.
- 5 Conlan MJ, Rapley JW, Cobb CM: Biostimulation of wound healing by low-energy laser irradiation. A review. *J Clin Periodontol* 1996;23:492-496.
- 6 Wu Y, Xing D, Chen WR: Single cell FRET imaging for determination of pathway of tumor cell apoptosis induced by photofrin-PDT. *Cell Cycle* 2006;5:729-734.
- 7 Zhang X, Jiang F, Zhang ZG, Kalkanis SN, Hong X, Decarvalho AC, Chen J, Yang H, Robin AM, Chopp M: Low-dose photodynamic therapy increases endothelial cell proliferation and VEGF expression in nude mice brain. *Lasers Med Sci* 2005;20:74-79.
- 8 Shefer G, Partridge TA, Heslop L, Gross JG, Oron U, Halevy O: Low-energy laser irradiation promotes the survival and cell cycle entry of skeletal muscle satellite cells. *J Cell Sci* 2002;115:1461-1469.
- 9 Karu TI: Effects of visible radiation on cultured cells. *Photochem Photobiol* 1990;52:1089-1098.
- 10 Lubart R, Wollman Y, Friedmann H, Rochkind S, Laulicht I: Effects of visible and near-infrared lasers on cell cultures. *J Photochem Photobiol B* 1992;12:305-310.
- 11 Duan R, Liu TC, Li Y, Guo H, Yao LB: Signal transduction pathways involved in low intensity He-Ne laser-induced respiratory burst in bovine neutrophils: a potential mechanism of low intensity laser biostimulation. *Lasers Surg Med* 2001;29:174-178.
- 12 Forloni G, Chiesa R, Smioldo S, Verga L, Salmona M, Tagliavini F, Angeretti N: Apoptosis mediated neurotoxicity induced by chronic application of beta amyloid fragment 25-35. *Neuroreport* 1993;4:523-526.
- 13 Pike CJ, Walencewicz-Wasserman AJ, Kosmoski J, Cribbs DH, Glabe CG, Cotman CW: Structure-activity analyses of beta-amyloid peptides: contributions of the beta 25-35 region to aggregation and neurotoxicity. *J Neurochem* 1995;64:253-265.
- 14 Musashi M, Ota S, Shiroshita N: The role of protein kinase C isoforms in cell proliferation and apoptosis. *Int J Hematol* 2000;72:12-19.
- 15 Braun DC, Garfield SH, Blumberg PM: Analysis by fluorescence resonance energy transfer of the interaction between ligands and protein kinase Cdelta in the intact cell. *J Biol Chem* 2005;280:8164-8171.
- 16 Han YS, Zheng WH, Bastianetto S, Chabot JG, Quirion R: Neuroprotective effects of resveratrol against beta-amyloid-induced neurotoxicity in rat hippocampal neurons: involvement of protein kinase C. *Br J Pharmacol* 2004;141:997-1005.
- 17 Maher P: How protein kinase C activation protects nerve cells from oxidative stress-induced cell death. *J Neurosci* 2001;21:2929-2938.
- 18 Weinreb O, Bar-Am O, Amit T, Chillag-Talmor O, Youdim MB: Neuroprotection via pro-survival protein kinase C isoforms associated with Bcl-2 family members. *Faseb J* 2004;18:1471-1473.
- 19 Fields S, Song O: A novel genetic system to detect protein-protein interactions. *Nature* 1989;340:245-246.
- 20 Sekar RB, Periasamy A: Fluorescence resonance energy transfer (FRET) microscopy imaging of live cell protein localizations. *J Cell Biol* 2003;160:629-633.
- 21 Chen M, Xing D, Chen T, Zhang L: BimL involvement in Bax activation during UV irradiation-induced apoptosis. *Biochem Biophys Res Commun* 2007;358:559-565.
- 22 Wu Y, Xing D, Chen WR, Wang X: Bid is not required for Bax translocation during UV-induced apoptosis. *Cell Signal* 2007;19:2468-2478.
- 23 Pei Y, Xing D, Gao X, Liu L, Chen T: Real-time monitoring full length bid interacting with Bax during TNF-alpha-induced apoptosis. *Apoptosis* 2007;12:1681-1690.
- 24 Chen T, Wang J, Xing D, WR C: Spatio-temporal dynamic analysis of bid activation and apoptosis induced by alkaline condition in human lung adenocarcinoma cell. *Cell Physiol Biochem* 2007;20:569-578.
- 25 Violin JD, Zhang J, Tsien RY, Newton AC: A genetically encoded fluorescent reporter reveals oscillatory phosphorylation by protein kinase C. *J Cell Biol* 2003;161:899-909.
- 26 Adams JM, Cory S: The Bcl-2 protein family: arbiters of cell survival. *Science* 1998;281:1322-1326.
- 27 Selznick LA, Zheng TS, Flavell RA, Rakic P, Roth KA: Amyloid beta-induced neuronal death is bax-dependent but caspase-independent. *J Neuropathol Exp Neurol* 2000;59:271-279.
- 28 Polster BM, Fiskum G: Mitochondrial mechanisms of neural cell apoptosis. *J Neurochem* 2004;90:1281-1289.
- 29 Zhou LJ, Zhu XZ: Reactive oxygen species-induced apoptosis in PC12 cells and protective effect of bilobalide. *J Pharmacol Exp Ther* 2000;293:982-988.