

Overexpression of 5-Lipoxygenase Increases the Neuronal Vulnerability of PC12 Cells to $A\beta_{42}$

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(Received June 20, 2011; Accepted September 17, 2011)

5-Lipoxygenase (5-LOX), which is believed to be a major source of oxidative stress, participates in somatostatin-receptor transmembrane signaling in the central nervous system. We used the Tet-On inducible expression system in PC12 cells to obtain cell lines with reproducible, stable 5-LOX expression levels to study its function. Cell apoptosis rates induced by $A\beta_{42}$ were determined using an apo-BrdDU kit. Lipid peroxide, antioxidant enzyme, and caspase-3 activities were evaluated with respective commercial kits. The expression of 5-LOX, bcl-2, and bax were detected by immunoblotting. A subclone of PC18 with Tet-On inducible expression of 5-LOX was selected from PC12 transfectants. Expression of 5-LOX had no significant inhibitory effect on the cell viability of the PC18 clone. In contrast, compared with the control group, the cell viability of clone PC18 was significantly reduced after the induction of 5-LOX during $A\beta$ exposure. The differences in cell viability before and after the induction of 5-LOX during $A\beta$ insult were significantly offset by AA861. Overexpression of 5-LOX only slightly improved the activities of superoxide dismutase (SOD). The levels of intracellular peroxides, SOD and caspase-3 activity, and Bax expression were significantly upregulated, and the levels of glutathione peroxidase and catalase were downregulated correspondingly in clone PC18 during $A\beta$ exposure. These results indicate that constitutive expression of 5-LOX is not detrimental *per se*, but overexpression of 5-LOX may become problematic during $A\beta$ exposure.

Key words—Alzheimer's disease; 5-lipoxygenase; amyloid β protein; peroxide; apoptosis

INTRODUCTION

Alzheimer's disease (AD) is a common, complicated neurodegenerative disorder characterized by progressive cognitive decline. Accumulated evidence suggests that $A\beta$ activates microglial and astrocyte cells and leads to the activation of inflammatory factors that contribute to the process of degeneration.^{1,2} Slowing the progression of AD can be achieved with certain antiinflammatory drugs.³

Arachidonate 5-lipoxygenase (5-LOX), one of the three major types of mammalian lipoxygenase, plays a central role in leukotriene (LT) biosynthesis. Overproduction of LTs contributes to a variety of inflammations, including asthma and allergic responses.⁴ The expression of 5-LOX is greater in developing than in mature neurons.³ In addition, 5-LOX is also strongly expressed in aging rats and mice, especially in the hippocampus, and some authors suggested that the 5-LOX system might be a promising target for neuroprotection.^{4,5} However, current evidence for this remains controversial. 5-LOX is constitutively ex-

pressed in neurons in various regions of the brain including the hippocampus, cerebellum, superficial neocortex, thalamus, hypothalamus, and brainstem, with the most prominent expression in the hippocampus and cerebellum.³ In addition, 5-LOX participates in somatostatin-receptor transmembrane signaling in the central nervous system.⁶ In the nervous system, an inborn error in LT metabolism has been found that results in a fatal developmental and neurological syndrome.⁷ Therefore it is important to elucidate the relationship (if any) between 5-LOX and the progression of AD.

The aim of this study was to investigate and assess the extent to which the expression of 5-LOX affects cell viability. A number of papers reported the biological activities of 5-LOX using metabolites or enzyme inhibitors in the central nervous system.^{4,5} To evaluate more directly the role of intracellular 5-LOX in neuronal survival and death, we used the Tet-On inducible expression system in PC12 cells to obtain cell lines with reproducible, stable 5-LOX expression levels to study its functions.

MATERIALS AND METHODS

Materials The apo-BrdDU kit and all compo-

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nents of the Tet-On expression system (PC12 Tet-On cells, doxycycline (DOX), Tet System-approved fetal bovine serum, the DNA vector pBI, pBI-G bidirectional Tet vector, and pTK-Hyg vector) were purchased from BD Biosciences Clontech (Palo Alto, CA, U.S.A.). DMEM, G418 sulphate, hygromycin B, and horse serum were products of Gibco (Logan, Utah, USA). FuGENE 6 transfection reagent was purchased from Roche (Indianapolis, IN, USA). DOX, arachidonic acid, 5-HETE, 5-HPETE, X-gal, and ONPO were obtained from Sigma (St Louis, Missouri, USA). *Escherichia coli* strain DH5 α , T4 DNA ligase, and PCR-reverse transcriptase system kits were obtained from Takara Bio Inc (Kyoto, Japan). The mammalian expression vector pEGFP-5-LOX encoding a green fluorescent protein (GFP-5-LOX fusion protein) was kindly provided by Dr. Xin-Sheng Chen (The Second Military Medical University, Shanghai, China). A rabbit polyclonal antibody against 5-LOX and monoclonal mouse anti- β -actin were purchased from Abcam plc (Cambridge, U.K.). PstI, SallI, and HindIII restriction enzymes, pfu DNA polymerase, Taq DNA polymerase, and primers were purchased from Sangon Company (Shanghai, China). A β ₄₂ was purchased from Sigma. Lactate dehydrogenase (LDH), superoxide dismutase (SOD), catalase, glutathione peroxidase (GSH-Px), and lipid peroxidation (malondialdehyde, MDA) assay kits were obtained from Jiancheng Bioengineering Institute (Nanjing, P. R. China). The fluorimetric CasPACE Assay System was from Promega (Madison, WI, U.S.A.).

Tet-On Inducible Mammalian Expression System and DNA Construction The Tet-On expression system permits tight DOX regulation of the gene of interest (Gene X) expression under the control of the reverse tetracycline-controlled transactivator (rtTA) protein and the Tet operator DNA sequence (tet O).⁸⁾ pTRE is a vector expressing Gene X under the

control of the tetracycline response element (TRE), which consists of seven direct repeats of a 42-bp sequence containing tet O. The rtTA binds to TRE and activates transcription of downstream genes in the presence of DOX. Bidirectional Tet expression vectors can be used with Tet-On cell lines to allow the simultaneous expression of two genes under the control of a single TRE. In this paper, the bidirectional vector pBI-G, containing an available multiple cloning site in one direction and a *LacZ* gene in the other, was used. Since there are only three cloning sites (SallI, NotI, and PstI) within the minimal CMV promoter in the pBI-G Tet vector, we created a modified version of the bidirectional Tet vector pBIG, called pBIZL, which contains the 200 bases of cDNA and the HindIII site. A ZL cDNA fragment encoding the amino acid was generated by the PCR technique from pEGFP-5-LOX using a forward primer containing the PstI and HindIII restriction enzyme sites and a reverse primer containing a SallI restriction site. After digestion with PstI and HindIII, the ZL fragment was ligated into the pBIG vector to form a pBIZL vector. Full-length 5-LOX cDNA was isolated in PCR from pEGFP-5-LOX using the forward primer 5-LOX F and reverse primer 5-LOX R (Table 1), and an 1800-bp fragment was amplified and ligated into the pBIZL vector pre-cut with SallI and HindIII to generate the expression vector pBI-5-LOX (Fig. 1). Ligation boundaries and PCR-generated DNA sequences in the above constructs were verified using automated DNA sequencing.

Plasmid Transfection and Stable Cell Line Screening PC12 cells were used for transfection since they have been widely used for the investigation of neuronal survival and differentiation.⁹⁾ PC12 Tet-On cells were maintained in G418 100 μ g/ml until transfection. The cells were co-transfected with pBI-5-LOX and the selection plasmid pTK-Hyg in 10 : 1 and 20 : 1 ratios using the FuGENE 6 transfection reagent.

Table 1. Primers Used in pBI-5-LOX Construction and 5-LOX Detection

Primers	Primary sequence	Base pairs
Primer 1	5'-CCCAAGCTTATGCCCTCCTACACGGTCACCGTGG-3'	34 mer
Primer 2	5'-TTTGTCTGACTCAGATGGCCACACTGTTCCGGAATCCGG-3'	37 mer
Primer 4	5'-CCCCCTGCAGAAGCTTATGGTGGACACGCTGCCC-3'	34 mer
5-LOX F	5'-CCCGGGGCATGGAGAGCA-3'	18 mer
5-LOX R	5'-GCGGTCGGGCAGCGTGTC-3'	18 mer

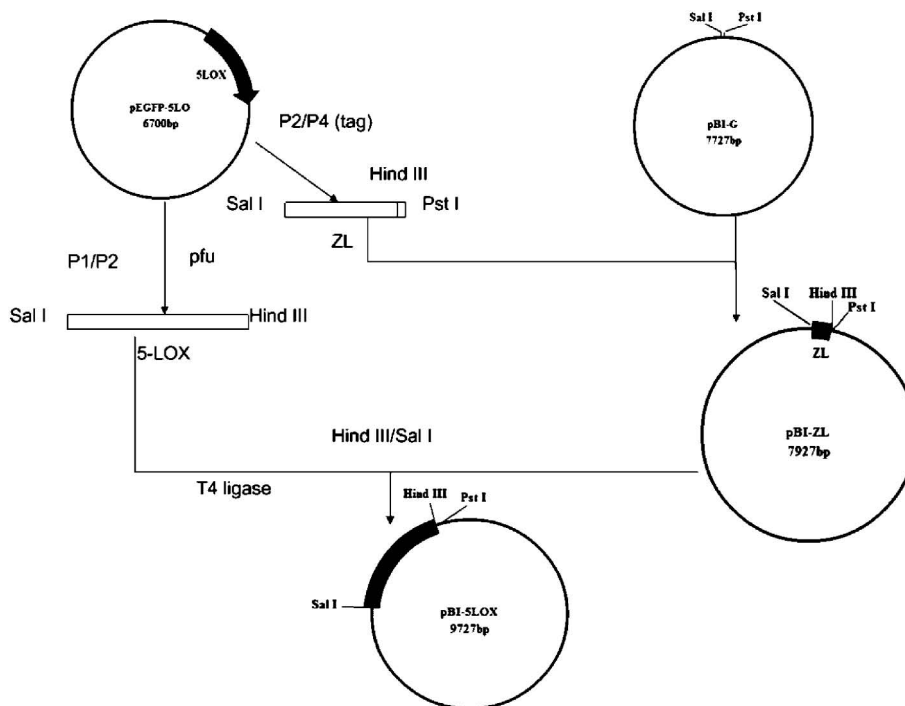


Fig. 1. Construction of pBI-5LOX Plasmid

gent. Stably double-transfected clones were selected in medium containing 100 mg/l of hygromycin B and 100 mg/l of G418. Following screening, stable cell lines were maintained in 50 mg/l of hygromycin B and 100 mg/l of G418, and 5-LOX expression was induced with DOX for 48 h. 5-LOX protein expression was detected by Western blotting and lipoxygenase activity analysis was performed as described previously.¹⁰ After centrifugation of cytosolic proteins for 10 min at 10000 g, the supernatants were collected and incubated in a buffer containing ATP 5 mM, CaCl₂ 2.2 mM, arachidonic acid 100 μ M, and Tris 40 mM (pH 8.0) at 37°C for 10 min. Reactions were terminated with 2 volumes of stop solution (acetonitrile : methanol : acetic acid, 350 : 150 : 3) and then centrifuged for 15 min at 10000 g. Metabolites of arachidonic acid (5-HETEs and 5-HPETEs) were separated by reverse-phase HPLC using a mobile phase consisting of acetonitrile : water : acetic acid (60 : 40 : 0.1) with UV detection at 235 nm and a flow rate of 1 ml/min.^{10,11}

A β -induced Cell Toxicity in 5-LOX-overexpressing Clone PC18

A β ₄₂ was dissolved in distilled water and incubated at 37°C for 7 days to form aggregates. Cell viability was evaluated using a colorimetric assay with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), an indicator of mitochondria

drial respiratory chain activity. Briefly, cells were pretreated with DOX for 48 h and then exposed to A β ₄₂ 5 μ M for 2 days. MTT solution 5 g/l was added to each well and incubated for a further 4 h at 37°C. The media were then removed, and formazan crystals were solubilized with DMSO. The optical density of each well was measured at 490 nm (OD₄₉₀) using an ELISA reader (Bio-Tek ELX800uv, Bio-Tek Instruments, INC, UK). PC12 Tet-On cells served as negative controls. The proliferation of cells was determined by calculating the absorbance of the test wells as a percentage of the control wells. Apoptotic cells were detected using an apo-BrDU kit (fluorescein-labeled anti-Brdu antibody for labeling DNA breaks and propidium iodide/RNase A solution for counterstaining total DNA). The experiments were carried out using FACS Calibur (Becton Dickinson, Mountain View, CA, U.S.A.) after staining.¹¹ To determine whether the antagonist of 5-LOX could prevent A β ₄₂-induced neurotoxicity, cells were pretreated with DOX 1 mg/l in the presence of AA861 (5 μ M; a selective 5-LOX inhibitor) before being subjected to A β ₄₂ insult.

Lipid Peroxidation and Antioxidant Enzyme Activities

The lipid peroxides were determined by measuring thiobarbituric acid-reactive substances (TBARS). Cells were lysed with 4 ml of fulvic acid

(0.167 M) and 0.5 ml of 10% phosphotungstic acid and then centrifuged at 4000 g for 10 min. The precipitate was resuspended with 1.5 ml of distilled water and 0.5 ml of TBA reagent (1 : 1 (v/v) mixture of 0.67% TBA and acetic acid). The reaction mixture was heated at 95°C for 1 h. After cooling in an ice bath, 2 ml of *n*-butanol was added and the mixture was shaken vigorously for 30 s. The sample was then centrifuged at 3000 g for 10 min, after which the *n*-butanol layer was subjected to fluorometric measurement with λ_{ex} 515 nm and λ_{em} 553 nm using a fluorescence spectrophotometer. The value of fluorescence was calculated by comparison with the standard curve prepared with 1,1,3,3-tetraethoxypropane. The cell-free supernatants were assayed using the fluorescent probe 2,7-dichlorofluorescein diacetate (DCF-DA). After treatment with $A\beta_{42}$ peptide for 48 h, cells were incubated for 45 min with the oxidation-sensitive fluorophore DCFDA (1 mg/l), and then quantitative evaluation of the DCF fluorescence measured with the plate reader with excitation at 485 nm and emission detection at 520 nm was performed.¹²⁾

To evaluate the activities of antioxidant enzymes, the cells were washed with iced PBS buffer and then homogenized. The homogenate was centrifuged at 10000 g for 15 min at 4°C. The supernatant was used as a test sample for the enzyme assay. The protein concentration of the supernatant was determined with the BCA method. Total SOD was measured following the instructions of the kit manufacturer. The control (C) consisted of all the reagents except for the supernatant (2%, w/v), while the blank (B) consisted of buffer and the supernatant without any reagents. The absorbance of T, C, and B was measured at 550 nm, and the enzyme activity was expressed in units (1 unit = 50% inhibition of the oxidation of oxyimine by the xanthine-xanthine oxidase system). Glutathione (GSH), a tripeptide antioxidant, plays multiple biological functions. It is involved in the detoxification of harmful molecules, such as reactive oxygen species (ROS) (hydrogen peroxide (H_2O_2) and hydroperoxides) through GSH-Px. The activity of GSH-PX was determined by quantifying the rate of H_2O_2 -induced oxidation of reduced glutathione (GSH) to oxidized glutathione (GSSH). A yellow product with absorbance at 412 nm was formed as GSH reacted with dithiobisnitrobenzoic acid. One unit of GSH-Px was defined as the amount that reduced the level of

GSH by one micromole per minute per milligram of protein. The assay of catalase activity was based on its ability to decompose H_2O_2 . The absorbance of the supernatant at 254 nm changed when the H_2O_2 solution was injected into the cuvette. The disappearance of H_2O_2 was measured at 240 nm for 60 s at 1-min intervals. The change in the absorbance reflected catalase activity.¹³⁾

Effects of $A\beta_{42}$ on the Expression of 5-LOX and Bax/Bcl-2 Cells were plated at the concentration of 4×10^5 /ml in the presence or absence of DOX 1 mg/l for 48 h. Then the cells were then incubated with $A\beta_{42}$ 5 M for an additional 48 h. Immunoblotting analysis was carried out as previously described,¹⁰⁾ using the anti-5-LOX antibody. Immunoblotting analysis was carried out as previously described using the following antibodies: poly-antibody anti-5-LOX (Biorworld, Minneapolis, MN, USA, 1 : 1000), poly-antibody anti-bax (Cell Signaling, Beverly, MA, USA, 1 : 1000), poly-anti-bcl-2 (Cell Signaling, 1 : 1000), or the primary monoclonal antibody anti- β -actin.

Caspase-3 Activity Assay Cells were lysed in caspase assay buffer containing HEPES 50 mM (pH 7.5), NaCl 100 mM, EDTA 2 mM, 0.1% CHAPS, 10 % sucrose, and DTT 5 mM. Aliquots of 15 μ g of crude cell lysate were incubated with the caspase-3 substrate Ac-DEVD-AMC (Pharmingen, San Diego, CA, U.S.A.) at 37°C for 30 min. The protein concentration was determined using a BCA kit. Caspase-3 activity was quantified in a FLUOstar Optima plate reader (BMG Labtech GmbH, Allmendgruen, Germany) with excitation at 380 nm and emission at 440 nm. Calibration curves were constructed using free AMC. Caspase-3 activity was indicated in pico moles of AMC per min per milligram of protein.

Data Analysis Data collected from 3–6 independent experiments were used to calculate means, which are expressed as mean \pm S.D. SPSS statistical software ver. 10.0 for Windows was used to evaluate statistical significance using one-way ANOVA and the Student-Newman-Keuls test. A *p* value of less than 0.05 was considered to represent a statistically significant difference.

RESULTS

DNA Constructs The plasmid pEGFP-5-LOX containing full-length 5-LOX cDNA was employed as a template. To enable inducible expression of recombinant 5-LOX with a HindIII site, the pBI-ZL vector

was created based on pBI-G by inserting it into the multiple cloning site of a sequence encoding a 200-bp tag containing a HindIII cleavage site. 5-LOX cDNA was subsequently cloned into the corresponding Hind-III and SalI sites of the pBIZL vector to create the modified vector pBI-5-LOX (Fig. 1). The recombinant plasmid we constructed was verified by enzyme digestion, PCR, and sequencing. Its nucleotide sequence completely coincided with the published one.¹⁴⁾

Stable 5-LOX Expressing Tet-On PC12 Cells

Stable transfected cell lines with inducible expression of 5-LOX were screened with β -galactosidase and further confirmed by immunoblotting (Fig. 2). The expression levels of 5-LOX in clones PO13 and PG12 were similar to the level in the control clone transfected with an empty vector. This result suggests that PC12 Tet-On cells endogenously express low levels of 5-LOX, and that these two clones are not desirable stable transfectants for the purposes of our study. Therefore we chose clone PC18 for the subsequent experiments. DOX dose dependently increased the expression of 5-LOX in clone PC18 (Fig. 3). 5-LOX catalyzes the conversion of arachidonic acid to 5-HPETE and further to LT-A4. Since 5-HPETE is unstable in aqueous solution, 5-HPETE can be reduced to the more stable 5-HETE. Therefore the activity of 5-LOX can be evaluated using reverse-phase HPLC by detecting 5-HETE and 5-HPETE. Reverse-phase HPLC showed peaks of 5-HETE and 5-HPETE in clone PC18 (Fig. 4). The retention times of these peaks were the same as those of authentic 5-HETE and 5-HPETE, respectively. We did not find any peaks of 5-HETE and 5-HPETE in clones PC12, PO12, and PG12 (data not shown). These results suggest that the expression of 5-LOX closely parallels

the activity of 5-LOX in clone PC18. After 25 passages, the stably transfected cell line with high levels of 5-LOX expression and activity (clone PC18) was used in the subsequent experiments.

β -induced Cell Toxicity in 5-LOX-expressing Clone PC18

Although 5-LOX transfection had no significant effect on the morphology of PC12 cells, 5-LOX overexpression induced by DOX 1 mg/l prolonged the cell doubling time compared with that in the PC12 clone transfected with vector alone (Figs. 5 and 6). $A\beta_{42}$ 5 μ M was used to induce cell toxicity in

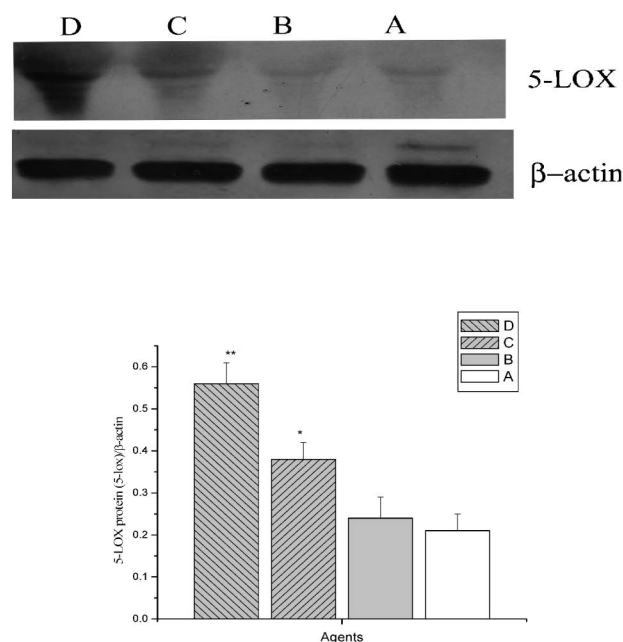


Fig. 3. Inducible Expression of 5-LOX Protein in Clone PC18 after Incubated with Doxycycline for 2 Days

Band intensities were determined and normalized with β -actin. A: clone PC18, B: clone PC18 incubated with 0.1 mg/l doxycycline, C: clone PC18 incubated with 0.3 mg/l doxycycline, D: clone PC18 incubated with 1 mg/l doxycycline.

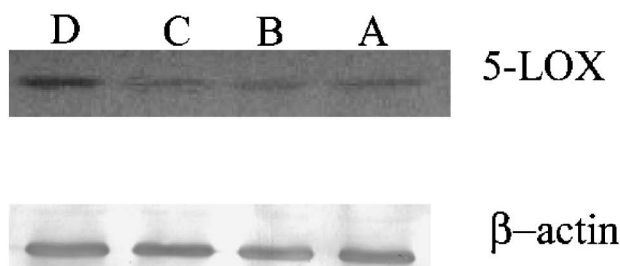


Fig. 2. Selection of Clones That Overexpression of 5-LOX

Protein expression was assessed after cells were incubated with 1 mg/l doxycycline for 2 days. Band intensities were determined and normalized with β -actin. A: control clone transfected with empty vector, B: clone PO13, C: clone PG12, D: clone PC18.

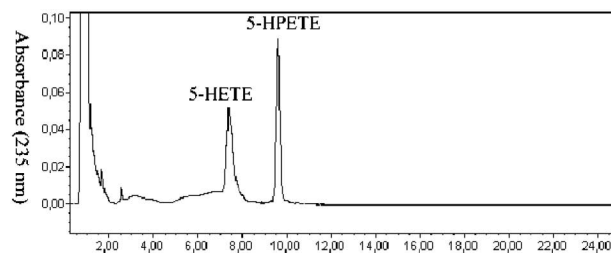


Fig. 4. Reverse-phase HPLC of Metabolic Products from Reaction of 5-LOX in Clone PC18 Extract

Cells were extracted after 48 h incubation with DOX, and were used to analyse the activity of 5-LOX. Thirty microliters of each product was collected and subjected to HPLC detection at 235 nm. The retention time of authentic 5-HETE and 5-HPETE as internal standards are 7.5 min and 9.8 min respectively.

subsequent experiments. Our results showed that $A\beta_{42}$ significantly enhanced the cell death rate after incubation with DOX 1 mg/l for 2 days in clone PC18 ($p < 0.01$, Table 2). These results were further confirmed using the apo-BrdU incorporation assay. After pretreatment with AA861 5 μ M, the cell apoptosis rate induced by 5-LOX and $A\beta_{42}$ was significantly lower in clone PC18 ($p < 0.01$, Fig. 7). However, AA861 failed to block completely the cell damage

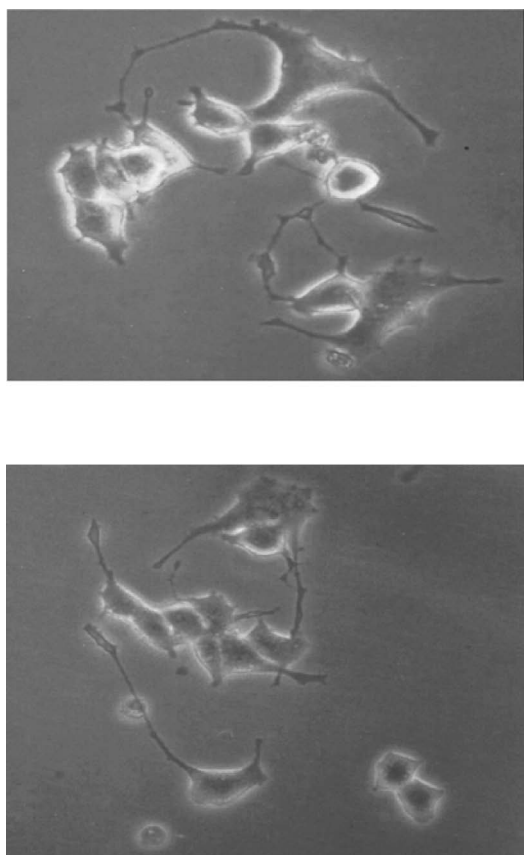


Fig. 5. Effect of 5-LOX Transfection on the Morphology Changes in PC12 Cells

(A) Morphology of PC12-Tet-On cells transfected with empty vector. (B) Morphology of PC12 cells transfected with pBI-5-LOX plasmid (clone PC18). Original magnification is 40 \times .

caused by $A\beta_{42}$ in clone PC18.

Mechanism of 5-LOX in Cell Death $A\beta$ has been shown to induce extensive oxidative stress in the AD brain.¹²⁾ Malondialdehyde (MDA) is one of the most frequently used indicators of lipid peroxidation. Levels of cellular oxidative stress are usually measured with the fluorescent probe DCFDA.¹⁵⁾ Since oxidative stress causes lipid peroxidation and cellular injury, we investigated the effects of 5-LOX on intracellular peroxide and cellular MDA in $A\beta$ -treated cells. Exposure of clone PC18 to $A\beta_{42}$ for 48 h increased the content of cellular MDA and the level of intracellular peroxide. The level of MDA and DCFDA in clone PC18 increased dose dependently with increasing concentrations of DOX during $A\beta_{42}$ exposure (Table 3). We further evaluated the activities of antioxidative enzymes in $A\beta_{42}$ -treated cells. $A\beta_{42}$ markedly increased the activity of SOD and reduced the activities

A

B

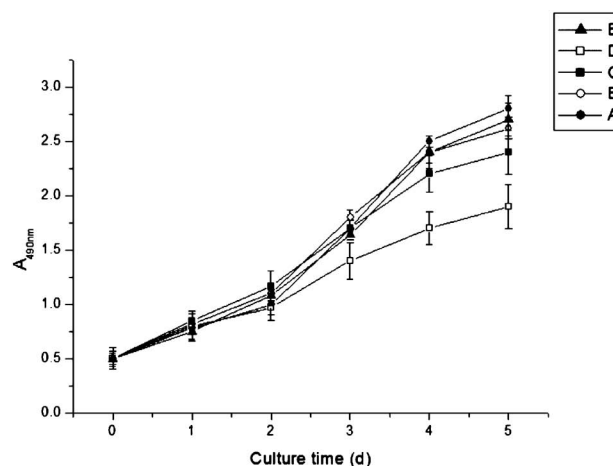


Fig. 6. Effects of 5-LOX on the Growth Curve of Clone PC18

Initial cell concentration was 4×10^4 cell/ml. After incubated with different concentrations of Dox for 48 h, cell viability was evaluated by using MTT method. ($n=6$). A: Clone PC18 incubated with 0 mg/l DOX, B: Clone PC18 incubated with 0.1 mg/l DOX, C: Clone PC18 incubated with 0.3 mg/l DOX, D: Clone PC18 incubated with 1 mg/l DOX, E: PC12-Tet-On cells incubated with 1 mg/l DOX.

Table 2. Effects of 5-LOX on Cell Viability (%) during $A\beta_{42}$ Insult

Group	Dox (0 mg/l)	Dox (0.1 mg/l)	Dox (0.3 mg/l)	Dox (1 mg/l)
PC12	100.0 \pm 4.8%	104.2 \pm 3.7%*	101.7 \pm 4.5%	98.7 \pm 3.3%
PC12+ $A\beta$	82.2 \pm 3.2%*	84.2 \pm 3.7%*	79.4 \pm 3.5%*	80.7 \pm 4.8%*
PC18	102.4 \pm 4.1%	118.2 \pm 8.2%	122.7 \pm 7.5%	103.7 \pm 3.9%
PC18+ $A\beta$	84.7 \pm 3.8%*	90.5 \pm 5.1%*	77.3 \pm 4.6%**	52.7 \pm 3.1%**

Clone PC18 were preincubated with Dox for 48 h, then exposed to 5 μ M $A\beta_{42}$ for additional 48 h; Cell viability was evaluated by using MTT method. PC12-Tet-On cells (PC12) were chosen as control group ($n=6$). * $p < 0.05$; ** $p < 0.01$ vs. PC12 cells; ## $p < 0.01$ vs. PC12 cells insulted by $A\beta_{42}$.

of catalase and GSH-Px after inducible expression of 5-LOX in clone PC18 (Table 4). $A\beta_{42}$ showed a significant increment in oxidative stress in clone PC18 with inducible expression of 5-LOX.

Neuroinflammation can be both a cause and a consequence of the disease process in neurodegenerative disease.¹⁾ Aged animals may show exacerbated cognitive deficits associated with neuroinflammation.¹⁾ Therefore we investigated the effect of $A\beta$ on the expression of 5-LOX in clone PC18. $A\beta_{42}$ enhanced the expression of 5-LOX in clone PC18, although there was no difference in 5-LOX expression before and af-

ter $A\beta_{42}$ treatment when P18 cells were first incubated with DOX 1 mg/l (Fig. 8).

The antiapoptotic protein Bcl-2 and members of the Bcl-2-associated X protein (Bax) family were proposed to play a role in $A\beta$ -induced cell death.¹⁶⁾ These proteins are thought to interfere with the mitochondria-dependent activation of caspase-9, which in turn activates caspase-3.⁷⁾ Caspase activity is essential for $A\beta$ -induced neuronal cell death.¹⁶⁾ Compared with control cell lines, overexpression of 5-LOX significantly upregulated Bax expression (Fig. 9) and enhanced caspase-3 activation (Fig. 10) during $A\beta_{42}$ insult. There were no significant effects of

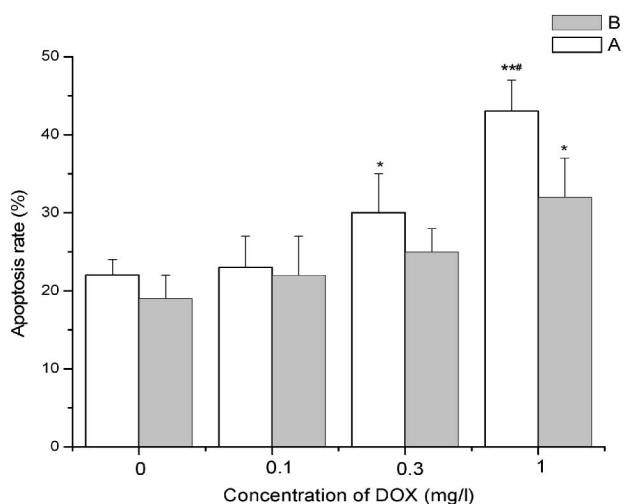


Fig. 7. Effects of Inducible 5-LOX on the Apoptosis Rate of Clone PC18 during $A\beta$ Insult

Cells were preincubated with Dox for 48 h and then exposed to 5 μ M $A\beta_{42}$ for additional 48 h; The apoptosis rate of cell was evaluated by using APOBrdU kit ($n=6$). A: Clone PC18, B: Clone PC18 preincubated with 5 μ M AA861. * $p<0.05$, ** $p<0.01$ vs. clone PC18 insulted by $A\beta$; # $p<0.05$ vs. clone PC18 insulted by $A\beta$ in the presence of AA861.

Table 3. Effects of 5-LOX on Lipid Peroxidation and Cellular Oxidation in PO18 Cells

Groups	DOX (mg/l)	Lipid peroxidation and cellular oxidation (% of control)	
		MDA	DCF fluorescence intensity
PC18	0	100.0 \pm 5.2%	100.0 \pm 6.5%
	0.1	105.4 \pm 6.9%	103.2 \pm 5.5%
	0.3	107.1 \pm 6.2%	104.8 \pm 5.7%
	1	114.8 \pm 7.5%*	107.5 \pm 8.6%
PC18 + $A\beta_{42}$	0	136.4 \pm 9.5%***	125.6 \pm 9.6%**
	0.1	153.5 \pm 7.2%***	137.0 \pm 8.2%**
	0.3	161.5 \pm 10.4%***	141.2 \pm 8.7%**
	1	186.7 \pm 9.5%***	155.1 \pm 9.7%**

Cells were preincubated with Dox for 48 h, then exposed to 5 μ M $A\beta_{42}$ for additional 48 h. Lipid peroxidation and cellular oxidation were measured by commercial kits after homogenization. Clone PC18 was chosen as control group ($n=3$). * $p<0.05$, ** $p<0.01$ vs. clone PC18; # $p<0.05$ vs. clone PC18 insulted by $A\beta_{42}$ group.

Table 4. Effects of 5-LOX on the Activities of Antioxidative Enzymes in Clone PO18 with $A\beta_{42}$

Groups	DOX (mg/l)	Antioxidant enzyme activities (% of control)		
		SOD	Catalase	GSH-PX
PC18	0	100.0 \pm 4.8%	100.0 \pm 4.1%	100.0 \pm 5.2%
	0.1	115.4 \pm 5.2%*	113.7 \pm 4.9%*	110.2 \pm 4.2%*
	0.3	128.8 \pm 3.8%*	118.2 \pm 6.9%*	112.5 \pm 6.4%*
	1	124.3 \pm 6.7%*	111.1 \pm 5.2%*	114.2 \pm 5.3%*
PC18 + $A\beta_{42}$	0	122.7 \pm 5.4%*	82.4 \pm 5.2%*	97.7 \pm 4.1%
	0.1	131.5 \pm 6.8%***	78.2 \pm 4.4%*	91.2 \pm 7.4%
	0.3	129.7 \pm 5.6%***	72.5 \pm 4.9%**	87.4 \pm 6.2%**
	1	152.6 \pm 4.1%***	65.7 \pm 3.2%***	62.5 \pm 3.8%***

Cells were preincubated with 1 mg/l Dox for 48 h, then exposed to 5 μ M of $A\beta_{42}$ for additional 48 h. Cells were homogenized and activities of antioxidant enzymes were measured by commercial kits ($n=3$). * $p<0.05$, ** $p<0.01$ vs. clone PC18; # $p<0.05$, ## $p<0.01$ vs. clone PC18 insulted by $A\beta_{42}$.

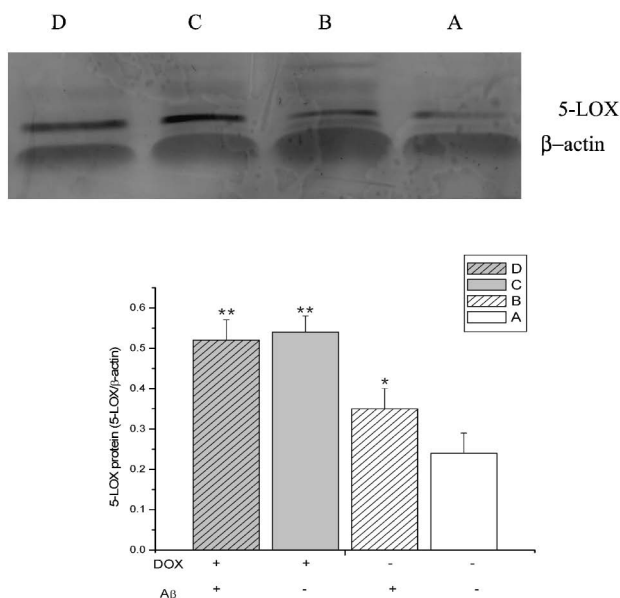


Fig. 8. 5-LOX Expression in Clone PC18 Induced by Aβ₄₂
 *p<0.05, **p<0.01 vs. clone PC18. A: 0 mg/1 DOX, B: Aβ₄₂, C: 1 mg/1 DOX, D: 1 mg/1 DOX with Aβ₄₂.

5-LOX on Bcl-2 expression in clone PC18 during Aβ₄₂ insult.

DISCUSSION

Overexpression of target genes in mammalian cells is one of the most important ways to study gene functions. However, it is difficult to establish overexpression of toxic genes using normal gene expression systems. The Tet-On expression system has been widely used for the overexpression of toxic proteins. Here we chose this system to study the effects of 5-LOX overexpression in clone PC18.

5-LOX plays a central role in LT biosynthesis and contributes to pathophysiological inflammatory processes including asthma and allergic responses.^{17,18)} Inflammation superimposed on neurodegenerative disease exacerbates cognitive and motor symptoms and accelerates disease progression.¹⁹⁾ Our data suggest that under common maintenance conditions, 5-LOX expression induced by DOX 1 mg/1 did not affect the morphology and survival of clone PC18. It appears that single-gene perturbation is not sufficient to determine cell fate, because its function can be replaced or its harmful effects can be ameliorated by the gene regulatory network. It was reported that significant expression of 5-LOX occurs in the central nervous system.^{4,5)} 5-LOX mRNA was expressed not only in mature oligodendrocytes and astrocytes but also in

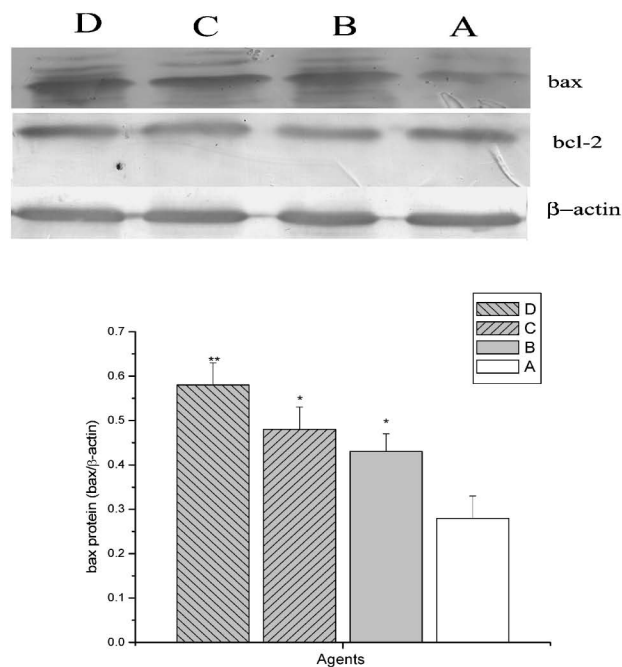


Fig. 9. Effects of Inducible 5-LOX Expression on the Levels of Bax and Bcl-2 in Clone PC18 Insulted by Aβ₄₂
 *p<0.05, **p<0.01 vs. clone PC18 insulted by Aβ₄₂. A: Clone PC18 preincubated with 0 mg/1 DOX, B: Clone PC18 preincubated with 0.1 mg/1 DOX, C: Clone PC18 preincubated with 0.3 mg/1 DOX, D: Clone PC18 preincubated with 1 mg/1 DOX.

cortical neurons.^{20,21)} Overexpression of 5-LOX induced by DOX 0.1 and 0.3 mg/1 can promote cell proliferation, and the expression of 5-LOX induced by DOX 1 mg/1 did not significantly affect the cell viability of clone PC18.

The 5-LOX inhibitor AA861 induces cell apoptosis in clone PC18. These results suggest that 5-LOX is one of the essential regulators of cell survival and apoptosis. It was reported that the activation of 5-LOX is required for nicotine-mediated epithelial-mesenchymal transition and tumor cell growth.²²⁾ Therefore the constitutive expression of 5-LOX in the cen-

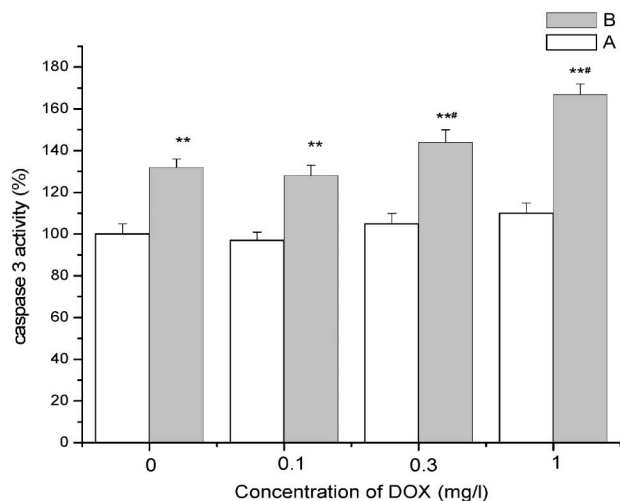


Fig. 10. Effects of Inducible 5-LOX on the Activity of Caspase-3 in Clone PC18 after Insulted by A β ₄₂

Cells were preincubated with Dox for 48 h then exposed to 5 μ M A β ₄₂ for 48 h; The activity of caspase 3 was evaluated by using commercial kits ($n = 6$). A: Clone PC18, B: Clone PC18 incubated with A β ₄₂. * $p < 0.05$, ** $p < 0.01$ vs. Clone PC18, $^{\#}p < 0.05$ vs. Clone PC18 insulted by A β ₄₂.

tral nervous system may essential for neuronal survival.

Increasing evidence suggests that apoptotic biochemical cascades are involved in the dysfunction and death of neurons in neurodegenerative disorders such as AD, Parkinson's disease, and Huntington's disease.²³ Apoptosis can be induced in response to various cytotoxic stimuli, including Fas, A β , tumor necrosis factor- α , *etc.* We found that overexpression of 5-LOX alone was not sufficient to cause cell damage, but it made cells more vulnerable to A β insult after inducible expression of 5-LOX in clone PC18. These effects were confirmed using the MTT assay and apoptosis detection. The 5-LOX inhibitor AA861 did not completely inhibit A β ₄₂-induced apoptosis in clone PC18, suggesting that the inducible expression of 5-LOX would not be sufficient to explain the toxicity of A β ₄₂ in clone PC18.

Oxidative stress plays a major role in cell death and neurodegeneration. 5-LOX may play a role in cytotoxicity by generating toxic metabolites and oxidizing cell membranes.²⁴ Compared with clone PC18 in the absence of DOX, the expression of 5-LOX alone did not change the basal level of reactive oxygen species (ROS). However, the levels of cellular oxidative stress and lipid oxidation during A β treatment were significantly enhanced by overexpression of 5-LOX in clone PC18. Consistent with our findings, increasing evidence supports a signaling link between 5-LOX

and ROS generation.²⁴ Several enzymes (SOD, GSH-Px, and catalase) are important in the antioxidant defense system because they metabolize either free radicals or reactive oxygen intermediates into nonradical products. For example, SOD constitutes the major enzymatic mechanism for O₂- \cdot degradation and catalyzes the conversion of O₂- \cdot into H₂O₂; H₂O₂ is in turn converted into water and molecular oxygen by catalase or GSH-Px. The latter utilizes reduced GSH as a hydrogen donor. Since 5-LOX enhances the activity of SOD and inhibits the activities of catalase and GSH-Px simultaneously in PC12 cells, H₂O₂ accumulates correspondingly in the cell plasma. H₂O₂ has important roles as a signaling molecule in the regulation of a variety of biological processes.²⁶ H₂O₂ can have both positive (signaling) and negative (damaging) effects depending on its level and the cell type under investigation. High doses of H₂O₂ can easily penetrate the lipid membrane and cause lipid, protein, and DNA peroxidation.^{12,25} Low-level 5-LOX expression enhanced the activities of SOD, GSH-Px, and catalase in clone PC18 in the present study, while A β ₄₂ significantly inhibited the activity of GSH-Px and catalase and increased the activity of SOD correspondingly. Therefore A β ₄₂ may significantly promote the level of H₂O₂ during oxidative stress. Taken together, these findings suggest that 5-LOX expression alone is not sufficient to kill the cells but significantly aggravates the oxidative stress induced by A β .

It was reported that 5-LOX co-localizes abundantly in neurofibrillary tangles, neuritic plaques, and glia.²⁰ Therefore we examined the effects of A β on the expression of 5-LOX in PC12 cells. Although we found that A β promoted the expression of 5-LOX in PC12 cells, it failed to enhance the expression of 5-LOX in clone PC18 in the presence of DOX 1 mg/l. Our results suggest that 5-LOX is not the key pathway for A β toxicity in clone PC18, but that the overexpression of 5-LOX significantly exacerbates the cell damage during A β insult.

The balance between proapoptotic (Bax, Bak, and Bad) and antiapoptotic (Bcl-2 and Bcl-xl) proteins may be critical to neuronal survival. Recently, it has been shown that ROS mediates Bcl-2 downregulation by facilitating Bcl-2 degradation²⁶ Our results indicated that 5-LOX did not interfere with the execution of the apoptotic program by inhibiting the expression of bcl-2. Bax and Bcl-2 interaction in mitochondria

regulate apoptosis. Once Bax is released from Bcl-2, there is a Bax-Bax oligomerization that is inserted into the outer mitochondrial membrane and causes mitochondrial permeabilization. The release of cytochrome c from mitochondria and sequential caspase activation induce apoptosis.²⁷⁾ $A\beta_{42}$ significantly increased the expression of bax in clone PC18. Inducible expression of 5-LOX further promotes bax expression in $A\beta$ -treated clone PC18. Neuronal apoptosis is directly linked to the activation of caspase proteases, all of which exist as zymogens and require proteolytic cleavage for activation. Caspase-3 is a key effector in many diverse models of apoptosis.²⁹⁾ Our results suggest that the proapoptotic effects of 5-LOX subjected to $A\beta$ treatment is mediated in part through the activation of caspase-3 in clone PC18. 5-LOX is not detrimental *per se*, but high levels of peroxides and low-level expression of bcl-2 may result in the enhancement of caspase-3 activity. Aging is accompanied by chronic low-grade upregulation of certain proinflammatory responses. Oxidative damage and overactivation of certain proinflammatory responses occur prior to the early stage of AD.¹⁾ The development of specific therapies that target 5-LOX may attenuate neuronal damage and slow the progress of AD but not fully block its process.

In conclusion, constitutive expression of 5-LOX is one of the essential factors in cell proliferation. Overexpression of 5-LOX renders cells more vulnerable to $A\beta_{42}$ -induced cell death. These results indicate possible alternative therapeutic targets for the alleviation of neurodegenerative diseases.

Acknowledgments This study was supported by a China National Outstanding Youth Grant (No. 39925038) and National Comprehensive Technology Platforms for Innovative Drug R&D (Grant No. 2009ZX09301-007).

Conflicts of interest The authors have no conflicts of interest to declare in relation to this article.

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