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Distinct roles of $CysLT_1$ and $CysLT_2$ receptors in oxygen glucose deprivation-induced PC12 cell death

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Abstract

Cysteinyl leukotrienes are involved in ischemic brain injury, and their receptors ($CysLT_1$ and $CysLT_2$) have been cloned. To clarify which subtype mediates the ischemic neuronal injury, we performed permanent transfection to increase $CysLT_1$ and $CysLT_2$ receptor expressions in PC12 cells. Oxygen glucose deprivation (OGD)-induced cell death was detected by Hoechst 33258 and propidium iodide fluorescent staining as well as by flow cytometry. OGD induced late phase apoptosis mainly and necrosis minimally. Over-expression of $CysLT_1$ receptor decreased and over-expression of $CysLT_2$ receptor increased OGD-induced cell death. An agonist LTD_4 (10^{-7} M) also induced apoptosis, especially in $CysLT_2$ receptor over-expressing cells. A selective $CysLT_1$ receptor antagonist montelukast did not affect OGD-induced apoptosis; while non-selective CysLT receptor antagonist Bay u9773 inhibited OGD-induced apoptosis, especially in $CysLT_2$ receptor over-expressing cells. Thus, $CysLT_1$ and $CysLT_2$ receptors play distinct roles in OGD-induced PC12 cell death; $CysLT_1$ attenuates while $CysLT_2$ facilitates the cell death.

Keywords: Cysteinyl leukotriene receptor; Leukotriene; Oxygen glucose deprivation; Rat pheochromocytoma cells (PC12 cells)

Cysteinyl leukotrienes (CysLTs, including LTC_4, LTD_4, and LTE_4), 5-lipoxygenase metabolites of arachidonic acid, are potent inflammatory mediators [1]. The actions of CysLTs are mediated by activating their receptors, cysteinyl leukotriene receptor 1 ($CysLT_1$) and receptor 2 ($CysLT_2$) [2]. In peripheral tissues, $CysLT_1$ and $CysLT_2$ receptors modulate at least four responses: vascular and smooth muscle cell function, immune, inflammation, and tissue repair [3]. In the central nervous system, the roles of CysLTs and 5-lipoxygenase in cerebral ischemic injury have been indicated by a line of evidence [4–10]; however, whether and how $CysLT_1$ and $CysLT_2$ receptors mediate the ischemic neuronal injury remains unknown.

Recently, we found that the expressions of $CysLT_1$ and $CysLT_2$ receptors are induced in the neuron-appearing cells in human brain specimens from patients with traumatic brain injury and brain tumors [11,12]. We also reported that $CysLT_1$ receptor antagonists, pranlukast and montelukast, protect against ischemic brain injury in rats and mice in vivo [13–16]. These findings indicate that $CysLT_1$ and $CysLT_2$ receptors may modulate brain injuries, including ischemic injury. However, since we have not found any protective effects of $CysLT_1$ receptor antagonists on the in vitro ischemic-like injury in the primary cultured neurons (unpublished observations), the roles of $CysLT_1$ and $CysLT_2$ receptors in neuronal injury need to be further studied.

Both $CysLT_1$ and $CysLT_2$ receptors are Gαq protein coupled receptors; but they may play distinct roles. For example, $CysLT_1$ receptor plays an inhibiting role while $CysLT_2$ receptor plays a facilitating role in bleomycin-induced

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pulmonary fibrosis in the receptor gene-knockout mice [17,18]. Whether the two receptors also play distinct roles in ischemic brain/neuron injury is unknown. To explore the possibly distinct roles, we investigated the effects of $CysLT_1$ and $CysLT_2$ receptor gene over-expression and antagonists on oxygen glucose deprivation (OGD)-induced in vitro ischemic injury in a neural cell line, rat pheochromocytoma cells (PC12 cells) [19–21].

Materials and methods

Cell culture and receptor gene transfection. PC12 cells were purchased from the Institute of Cell Biology, Chinese Academy of Sciences, Shanghai, China. The cDNA for mouse $CysLT_1$ or $CysLT_2$ receptor ($mCysLT_1$ and $mCysLT_2$, subcloned into pcDNA3.0) was kindly gifted by Professor C.D. Funk (University of Pennsylvania, USA). The pcDNA3.0 null vector was purchased from Invitrogen (Carlsbad, California, USA). The receptor cDNA expressing vectors and the null vector were lineared by PvuI and transfected into PC12 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, California, USA) according to the manufacturer's instructions. The permanently transfected PC12 cells were selected with 500 μg/ml G418 in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. Single-cell subclones were isolated and plated at low density in 24-well plates, so that only a few clones grew per plate and one clone grew per well. Cells were grown for over 2 months in the selection media. The transfected PC12 cells were defined PC12/WT (transfected with null pcDNA3.0), PC12/$mCysLT_1$ (pcDNA3.0/$mCysLT_1$), and PC12/$mCysLT_2$ (pcDNA3.0/$mCysLT_2$) cells. Before experiment, the cells were photographed with a digital camera (Nikon Coolpix 4500, Japan) under a microscope (Nikon Eclipse TS100, Japan), and their sizes were measured with ImageTool software (University of Texas Health Science Center, San Antonio, USA).

Reverse transcription-polymerase chain reaction (RT-PCR). To determine the mRNA expressions of $CysLT_1$ and $CysLT_2$ receptors, total RNA was extracted using Trizol reagents (Invitrogen, USA) according to the manufacturer's protocol. For cDNA synthesis, 2 μg total RNA was mixed with 1 mM dNTP, 0.2 μg random primer, 20 U RNasin, and 200 U M-MuLV reverse transcriptase in 20 μl reverse reaction buffer. The mixture was incubated at 42 °C for 60 min and then heated at 72 °C for 10 min to deactivate the reverse transcriptase. PCRs were performed on an Eppendorfed Master Cycler. The reaction conditions were set as follows: 1 μl cDNA mixture was reacted in 20 μl reaction buffer containing 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 20 pM primers and 1 U *Taq* DNA polymerase. The mixtures were initially heated at 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s; finally, the reaction was stopped at 72 °C for 5 min. The PCR products (10 μl) were separated by 2% agarose gel electrophoresis.

The primer sequences were designed by using Primer Premier software and the specificity of the oligonucleotide primers was verified using the program BLASTN, as following: mouse $CysLT_1$ receptor forward 5′-(+) CAA CGA ACT ATC CAC CTT CAC C-3′ and reverse 5′-(+) AGC CTT CTC CTA AAG TTT CCA C-3′; mouse $CysLT_2$ receptor forward 5′-(+) GTC CAC GTG CTG CTC ATA GG-3′ and reverse 5′-(+) ATT GGC TGC AGC CAT GGT C-3′; rat $CysLT_1$ receptor forward 5′-(+) TCT CCG TTG TGG GTT TCT-3′ and reverse 5′-(+) TAT AAG GCA TAG GTG GTG-3′; rat $CysLT_2$ receptor forward 5′-(+) AGC GTT AGG AGT GCC TGG AT-3′ and reverse 5′-(+) CAA GTG GAT GGT CCG AAG TG-3′.

Oxygen glucose deprivation (OGD) or LTD_4 treatment. PC12 cells were cultured on poly-L-lysine-coated glass cover slides or flasks, and OGD was carried out 24 h after culture. For OGD treatment, the culture media were changed to a deoxygenated glucose-free or an oxygenated glucose-

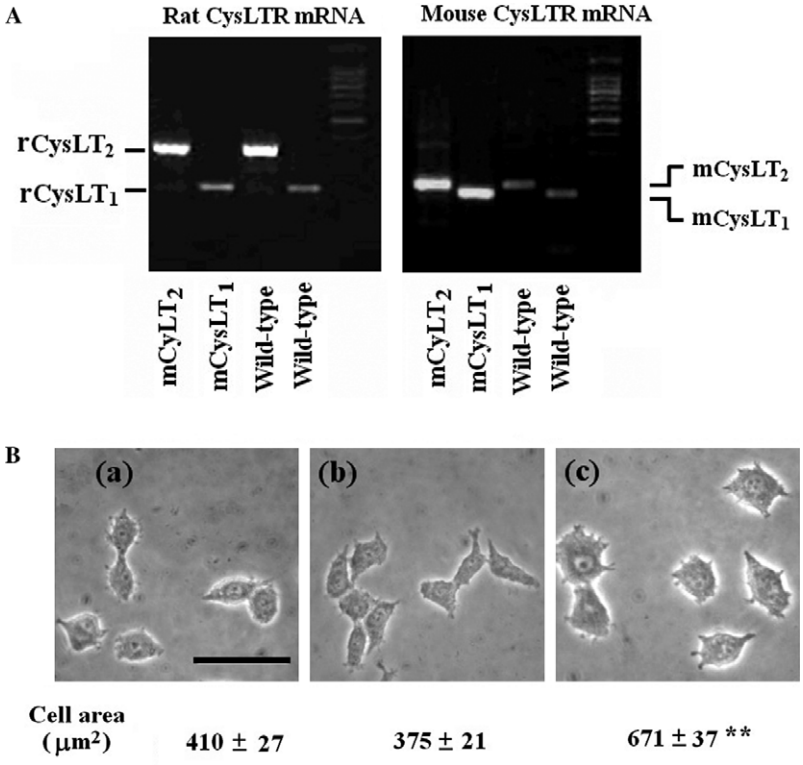

Fig. 1. Expressions of $CysLT_1$ and $CysLT_2$ receptor mRNAs and the morphology of the transfected cells. (A) The mRNA expressions analyzed by RT-PCR show that endogenous $rCysLT_1$ and $rCysLT_2$ receptors are not different among the cells (left panel), and $mCysLT_1$ and $mCysLT_2$ receptors are enhanced in the transfected cells (right). (B) Micrographs show that the cell sizes of PC12/$mCysLT_2$ cells (c) are larger than those of PC12/WT (a) and PC12/$mCysLT_1$ cells (b). Values are summarized as means ± SEM; $n = 30$ cells; $^{**}P < 0.01$ compared with PC12/WT cells, one-way ANOVA. Bar = 50 μm.

containing (for controls) Earle's balanced salt solution at pH 7.4. Then the cells were transferred to a sealed hypoxic box containing a mixture of 95% N_2 and 5% CO_2 at 37 °C, or normal culture conditions (controls) for 1, 3, and 6 h. Montelukast and Bay u9773 were added to the media from 30 min before OGD treatment to the end of OGD to determine the effects of antagonists. LTD_4 (10^{-7} M) was added into the media for 6 or 48 h to determine the effect of an agonist.

Cell death analysis. After OGD or LTD_4 treatment, the cells cultured on glass cover slides were washed with PBS, stained with Hoechst 33258 (0.01 mg/ml) and PI (0.01 mg/ml, Sigma–Aldrich, USA) for 10 min at 37 °C, and then fixed in 4% paraformaldehyde. For each cover slide, 1000–1500 cells were observed under a fluorescence microscope (Olympus BX51, Japan) by an investigator without the knowledge of treatments. After Hoechst 33258 fluorescent staining, normal cells showed a homogeneous staining of their nuclei, and apoptotic cells a deep and asymmetric staining of their nuclei as a result of chromatin condensation and nuclear fragmentation. Necrotic cells were stained with PI (red color). The percentage of necrotic or apoptotic cells was calculated.

To further determine the cell death, the cells in flasks were harvested after OGD or LTD_4 treatment, washed twice with PBS, and evaluated with Annexin V (AV)-FITC apoptosis detection kit I (BD Biosciences Pharmingen, USA) on a flow cytometer (FACSCalibur, Becton–Dickinson, USA). Briefly, 10^6 cells per ml were resuspended in a binding buffer, stained with AV-FITC and PI working solutions in the kit for 15 min at 25 °C in dark, and then analyzed with flow cytometer (totally 10,000 cells). "AV−PI−" represents the normal cells, "AV+PI−" the early phase apoptotic cells, "AV+PI+" the late phase apoptotic cells, and "AV−PI+" the necrotic cells.

Statistical analysis. Values are reported as means ± SEM. Statistical comparisons were made by one-way ANOVA to detect significant difference using SPSS 10.0 for windows. $P < 0.05$ was considered to be statistically significant.

Results

Expressions of $CysLT_1$ and $CysLT_2$ receptor mRNAs

The endogenous mRNA expression of rat $CysLT_2$ receptor ($rCysLT_2$) was higher than that of $rCysLT_1$ in normal PC12 cells. Permanent transfection with $mCysLT_1$ and $mCysLT_2$ receptors increased their mRNA expressions (Fig. 1A). The over-expression of $CysLT_1$ receptor did not alter whereas over-expression of $CysLT_2$ receptor significantly increased the cell size (Fig. 1B).

Hoechst 33258 and PI double staining

OGD mainly induced PC12 cell apoptosis. After 6-h OGD, apoptotic cells were less in PC12/$mCysLT_1$ cells (29.0%, $P < 0.01$) and more in PC12/$mCysLT_2$ cells (48.7%, $P < 0.05$) than in PC12/WT cells (39.8%, Fig. 2A and B). Only few necrotic cells (2.81% and 3.16%) were

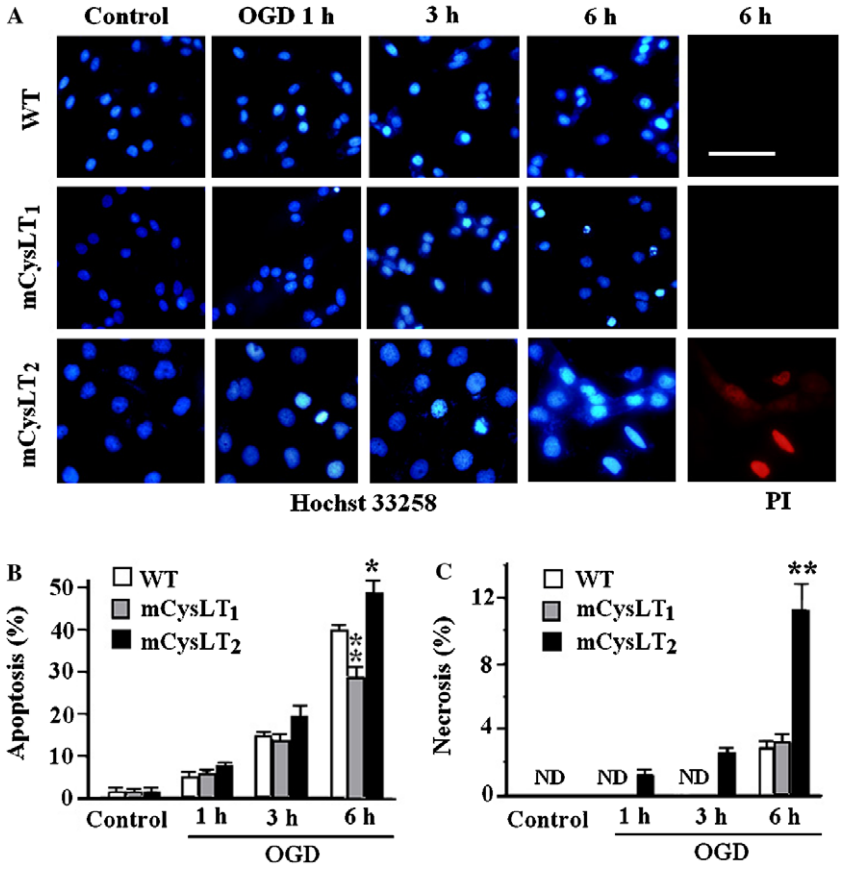

Fig. 2. Cell death after oxygen glucose deprivation (OGD). (A) Representative graphs show cell apoptosis and necrosis 1, 3, and 6 h after OGD as detected by Hoechst 33258 and propidium iodide (PI) fluorescent staining. Apoptotic cells show asymmetric Hoechst 33258 staining of their nuclei as a result of chromatin condensation and nuclear fragmentation, and necrotic cells are stained with PI (red). (B) Percentages of apoptotic cells and (C) necrotic cells are summarized as means ± SEM; $n = 5$–10; $^*P < 0.05$, $^{**}P < 0.01$ compared with PC/WT, one-way ANOVA. ND, not detectable. Bar = 50 μm.

found in PC12/WT and PC12/mCysLT$_1$ cells 6 h after OGD; whereas more necrotic cells (1.12%, 2.53% and 11.3%) in PC12/mCysLT$_2$ cells 1, 3, and 6 h after OGD (Fig. 2A and C). Bay u9773, a non-selective CysLT receptor antagonist, significantly decreased OGD-induced apoptosis, but not necrosis, in all cells; but montelukast, a selective CysLT$_1$ receptor antagonist, did not alter the apoptosis (Fig. 3A and B).

A CysLT receptor agonist LTD$_4$ (10^{-7} M for 6 h) also induced more apoptotic cells in PC12/mCysLT$_2$ cells (15.3%, $P < 0.01$ compared with PC12/WT cells) than in PC12/WT and PC12/mCysLT$_1$ cells (4.0% and 5.6%), indicating LTD$_4$ was able to induce PC12 cell apoptosis and PC12/mCysLT$_2$ cells were more sensitive to LTD$_4$. Bay u9773 (not montelukast) attenuated LTD$_4$-induced cell apoptosis in PC12/mCysLT$_2$ cells (Fig. 3C).

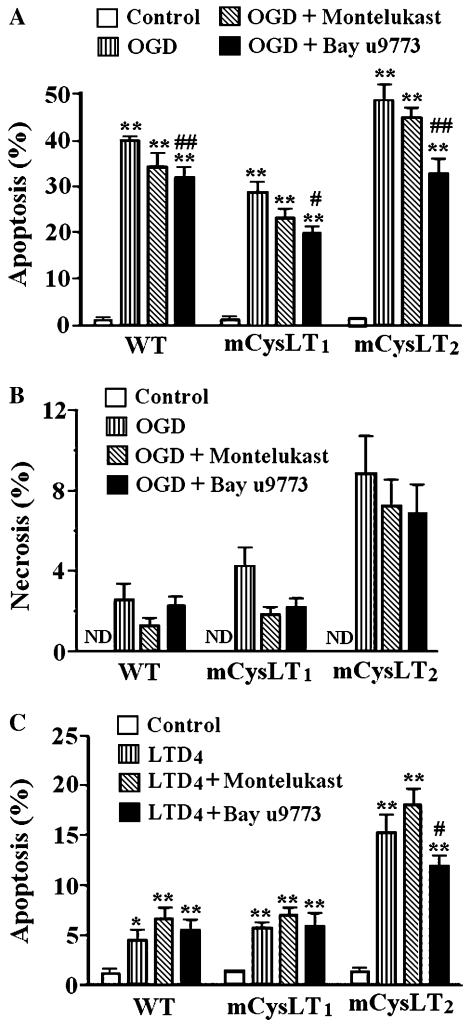

Fig. 3. Effects of antagonists on the cell death induced by OGD or LTD$_4$. A selective CysLT$_1$ receptor antagonist, montelukast (10^{-6} M), or a non-selective CysLT$_1$/CysLT$_2$ receptor antagonist, Bay u9773 (10^{-7} M), was added to the cultures 30 min before 6-h OGD or 6-h LTD$_4$ (10^{-7} M) treatment. Apoptotic or necrotic cells were detected by Hoechst 33258 or PI fluorescent staining. (A) OGD-induced apoptosis was attenuated by Bay u9773 but not by montelukast. (B) OGD-induced necrosis was more obvious in PC12/mCysLT$_2$ cells, which was not significantly inhibited by montelukast and Bay u9773. (C) LTD$_4$-induced apoptosis was attenuated by Bay u9773 but not by montelukast. Values are reported as means ± SEM; $n = 5$–10; $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with control, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with OGD or LTD$_4$ alone, one-way ANOVA. ND, not detectable.

Flow cytometry analysis

OGD mainly increased the late phase apoptotic cells (AV+PI+ in sample graphs, Fig. 4A). Similarly, PC12/mCysLT$_1$ cells were less sensitive and PC12/mCysLT$_2$ cells were more sensitive to OGD injury. After 6-h OGD, the normal cells (AV−PI−) remained more in PC12/mCysLT$_1$ (77.3%) and less in PC12/mCysLT$_2$ cells (22.8%) than those in PC12/WT cells (52.0%); while the late phase apoptotic cells were less in PC12/mCysLT$_1$ (9.3%) and more in PC12/mCysLT$_2$ cells (50.2%) than PC12/WT cells (31.1%, Fig. 4B). In addition, OGD induced more necrotic cells (AV−PI+) in PC12/mCysLT$_2$ cells (18.4%) while much less in PC12/WT and PC12/mCysLT$_1$ cells (Fig. 4B). Bay u9773 (not montelukast) protected PC12 cells from OGD-induced apoptosis only in PC12/mCysLT$_2$ cells (Fig. 4C).

LTD$_4$ (10^{-7} M) treatment for 6 h only decreased the normal cells and increased the late phase apoptotic cells in PC12/mCysLT$_2$ cells; the treatment for 48 h significantly decreased the normal cells in PC12/WT and PC12/mCysLT$_2$ cells, and increased the late phase apoptotic cells in PC12/mCysLT$_2$ cells (Fig. 4D).

Discussion

The most important finding in the present study is that over-expression of CysLT$_1$ receptor reduced but over-expression of CysLT$_2$ receptor increased OGD-induced PC12 cell death, indicating the distinct roles of CysLT$_1$ and CysLT$_2$ receptors in ischemic neuronal injury. The distinct roles of the two receptors have also been reported in mouse studies in which bleomycin-induced pulmonary fibrosis is enhanced in CysLT$_1$ receptor-deficient mice but reduced in CysLT$_2$ receptor-deficient mice [17,18,22]. This distinction may be caused by a difference in the activation of MAPKs that is one of the important signal transduction pathways for CysLT receptors [20,22–24]. In human mast cells, two CysLT receptors initiate different MAPK signaling cascades, CysLT$_1$ receptor activates ERK phosphorylation whereas CysLT$_2$ activates Gαi/o and p38 MAPK [22]. In PC12 cells, OGD activates MAPK isoforms (such as ERK, JNK, and p38) that play an important role in OGD-induced apoptosis [25]. Thus, the different MAPK signaling cascades might be also responsible for the distinct roles of two receptor types in OGD-induced PC12 cell death.

Moreover, we found that exogenous LTD$_4$, a CysLT receptor agonist, induced apoptosis, especially in PC12/mCysLT$_2$ cells. The concentration of LTD$_4$ (10^{-7} M) used here is about 2- to 10-fold higher than the EC$_{50}$ for mouse CysLT$_1$ or CysLT$_2$ receptor [26,27]. Otherwise, a line of evidence has supported the effects of

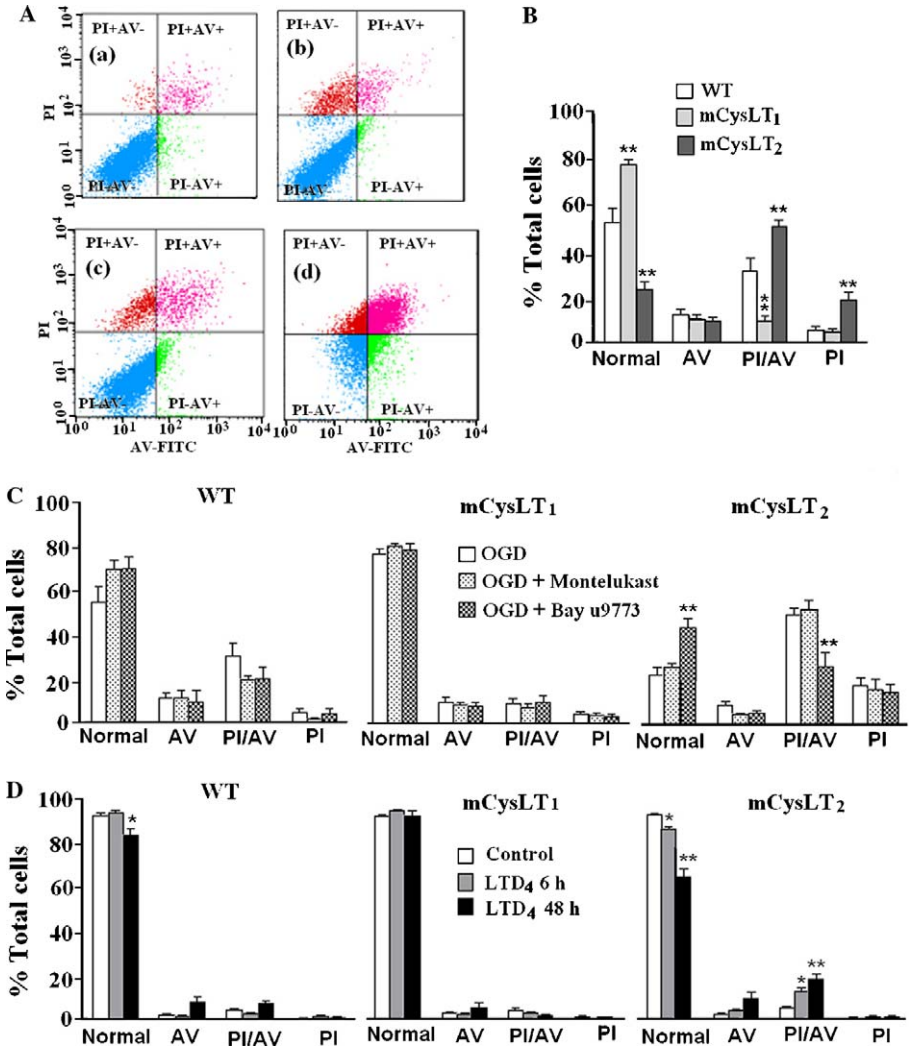

Fig. 4. Cell death analyzed by flow cytometry and the effects of antagonists. PC12 cells were treated with OGD for 6 h or LTD_4 (10^{-7} M) for 6 and 48 h, and cell death was analyzed with a flow cytometer. (A) The dot-plot sample graphs show the normal or dead fractions in control PC12/WT cells (a), and PC12/WT (b), PC12/$mCysLT_1$ (c), and PC12/$mCysLT_2$ cells (d) 6 h after OGD. (B) The percentages of OGD-induced cells death are summarized. (C) OGD-induced cell death in PC12/$mCysLT_2$ cells was attenuated by Bay u9773 (10^{-7} M) but not by montelukast (10^{-6} M). (D) LTD_4-induced cell death was attenuated by Bay u9773 but not by montelukast. Values are reported as means ± SEM; $n = 4$–8, $^*P < 0.05$ and $^{**}P < 0.01$ compared with PC12/WT (B), OGD alone (C) or normal control (D), one-way ANOVA. Normal: PI−AV− in the dot-plot graphs that represents the normal cells; AV: PI−AV+ represents the early phase apoptosis cells; PI/AV: PI+AV+ represents the late phase apoptosis cells; PI: PI+AV− represents the necrotic cells.

endogenous CysLTs on ischemic cerebral/neuronal injury. For example, the production of CysLTs is increased in rat or gerbil brain after cerebral ischemia [5,7], during cerebral ischemia in human [10], and also in the cultured neurons after OGD [28]. In addition, we found that 5-lipoxygenase, a key enzyme for production of CysLTs, is activated after OGD in PC12 cells [29]. In the present study, a longer duration (48 h) was needed for LTD_4 to induce a larger effect and its effect seemed to be less potent than that of OGD. This difference might result from OGD-induced more complex responses that may potentiate the action of CysLTs, such as inducible NOS [19], apoptotic protease activating factor-1 [23], mitochondrial dysfunction, and oxidative stress [21]; while CysLTs may mediate OGD-induced cell death only as one of the modulating systems.

On the other hand, the effects of antagonists further confirmed the distinct roles of the two receptors. The selective $CysLT_1$ receptor antagonist montelukast (10^{-6} M) did not affect OGD-induced apoptosis. In previous studies, we have found the protective effect of $CysLT_1$ receptor antagonists (pranlukast and montelukast) on in vivo cerebral ischemia [13–16], but did not find their protective effects on OGD-induced injury in cultured neurons (unpublished observations). This discrepancy in the effects of $CysLT_1$ receptor antagonists in vivo and in vitro might imply that the in vivo protective effects of pranlukast and montelukast might be due to their anti-inflammatory activities, such as reducing brain–blood barrier permeability and brain edema, inhibiting inflammatory and glial cells. Since no selective $CysLT_2$ receptor antagonist is available, Bay u9773, a

non-selective antagonist with partial agonist property [30], was used to determine the role of $CysLT_2$ receptor. A relatively lower concentration (10^{-7} M) of Bay u9773 was used in the experiments because it had toxic effect on PC12 cells at concentrations over 10^{-6} M (data not shown); but this concentration (10^{-7} M) was higher than that used in the aequorin assay ($0.1–0.5 \times 10^{-7}$ M) for functional activation of the human $CysLT_2$ receptor-expressing HG57 cells by CysLTs [31]. We found that Bay u9773 attenuated OGD- or LTD_4-induced apoptosis in PC12/m$CysLT_2$ cells, and OGD-induced apoptosis in PC12/WT and PC12/m$CysLT_1$ cells (Hoechst 33258 and PI staining). This finding confirmed the roles of $CysLT_2$ receptor in the ischemic-like injury in PC12 cells.

In summary, in the present study we found that $CysLT_1$ receptor attenuates while $CysLT_2$ receptor facilitates OGD-induced PC12 cell death as confirmed by receptor over-expression and antagonism. However, we cannot explain why their roles are distinct and why the cell size is increased in the $CysLT_2$ receptor over-expressing PC12 cells, so much more should be investigated to clarify the implications and the properties of signal transduction of the two receptors in ischemic neuronal injury.

Acknowledgments

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