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Research Report

α-Lipoic acid suppresses 6-hydroxydopamine-induced ROS generation and apoptosis through the stimulation of glutathione synthesis but not by the expression of heme oxygenase-1

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ABSTRACT

We previously reported that the generation of reactive oxygen species (ROS) is the initial event in cell death induced by 6-hydroxydopamine (6-OHDA), an experimental model of Parkinsonism. Since recent studies suggested the important role of antioxidant activity of α-lipoic acid (LA) in the suppression of apoptosis of various types, we studied the effect on 6-OHDA-induced apoptosis of PC12 cells. Biochemical analysis revealed that LA suppressed the 6-OHDA-induced ROS generation, increase of caspase-like activity and chromatin condensation. The suppression of 6-OHDA-induced apoptosis by LA required pre-incubation of PC12 cells with LA for 12–24 h. LA increased the intracellular levels of heme oxygenase-1 (HO-1) and glutathione (GSH) and stimulated the expression of GSH synthesis-related genes such as cystine/glutamate antiporter and γ-glutamylcysteine synthetase (γ-GCS). However, Sn-mesoporphyrin IX, an inhibitor of HO-1, did not attenuate the LA-induced suppression of apoptosis. In contrast, buthionine sulfoximine, an inhibitor of γ-GCS, attenuated the LA-induced suppression of ROS generation and chromatin condensation. In addition, a transcription factor Nrf2, which regulates the

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Abbreviations: Ac-DEVD-MCA, acetyl-Asp-Glu-Val-Asp-MCA; Ac-IETD-MCA, acetyl-Ile-Glu-Thr-Asp-MCA; Ac-LEHD-MCA, acetyl-Leu-Glu-His-Asp-MCA; AMC, 7-amino-4-methyl-coumarin; BSO, buthionine sulfoximine; CMFDA, 5-chloromethylfluorescein diacetate; DHLA, dihydro lipoic acid; FBS, Fetal Bovine Serum; JC-1, 5,5′6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol carbocyanine iodide; γ-GCS, gamma-glutamylcystein synthetase; GSH, glutathione; HE, hydroethidine; HO-1, heme oxygenase-1; LA, α-lipoic acid; Nrf2, Nuclear factor E2-related factor 2; 6-OHDA, 6-hydroxydopamine; PC12, pheochromocytoma 12; ROS, reactive oxygen species; Sn-MP, Sn-mesoporphyrin; SOD, superoxide dismutase

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expression of antioxidant enzymes such as γ-GCS, translocated to the nucleus by LA. These results suggested that LA suppressed the 6-OHDA induced-apoptosis by the increase in cellular glutathione through stimulation of the GSH synthesis system but not by the expression of HO-1.

1. Introduction

Evidence obtained over the past two decades have shown that reactive oxygen species (ROS) are involved in several diseases (Christophe and Nicolas, 2006). Excessive production of ROS may lead to oxidative stress, loss of cell function, and ultimately apoptosis or necrosis (Engel and Evens, 2006). ROS are also known to be mediators of intracellular signaling cascades (Sauer and Wartenberg, 2005). Mitochondria, which have a critical role in cell death or survival, are the primary intracellular source of ROS through the electron transport system and ROS induces huge numbers of oxidation–reduction reactions (Lemasters et al., 1998; Jezek and Hlavata, 2005).

Glutathione (GSH, L-γ-glutamyl-L-cysteinyl-glycine) is one of the low molecular weight thiol-containing molecules (Reliene and Schiestl, 2006). GSH protects cells against ROS-induced oxidative stress and regulates intracellular redox status. GSH is the most abundant intracellular antioxidant, having an important role in protection against ROS toxicity (Anderson, 1998; Wu et al., 2004).

γ-Glutamylcysteine synthetase (γ-GCS) is the rate-limiting enzyme for GSH synthesis (Griffith, 1982). γ-GCS is a heterodimer composed of a heavy subunit (73 kDa) and a light subunit (29 kDa). The heavy subunit exhibits catalytic activity and is subject to non-allosteric inhibition by GSH, while the light subunit (γ-GCS$_L$) regulates the catalytic activity of the heavy subunit (Seelig et al., 1984). L-Buthionine-sulfoximine (BSO) is an irreversible inhibitor of γ-GCS and thereby depletes intracellular GSH. Thus, BSO is often used to investigate the role of GSH under oxidative stress conditions in cells and animals (Griffith 1982; Powell et al., 2001). Cystine/glutamate antiporter also regulates GSH synthesis and apoptosis via cystine uptake into cytosol (Wang et al., 2006). This antiporter is a heterodimeric complex between the ubiquitous CD98 heavy chain, also referred to as 4F2hc, and the xCT light chain responsible for the determination of substrate specificity.

Heme oxygenase-1 (HO-1) has emerged recently as a crucial mediator of antioxidant and tissue-protective actions. Increased HO-1 expression leads to degradation of heme and accumulation of iron, bilirubin, and CO, followed by reduced sensitivity of tissues to oxidant damage (Cheng et al., 2006). Of these metabolites, bilirubin acts as a direct antioxidant (Stocker et al., 1987) whereas CO may exert tissue-protective actions primarily through its vasodilator and antiplatelet effects (Maines, 1997).

It has been widely accepted that γ-GCS, HO-1 and cystine/glutamate antiporter can be transcriptionally regulated by NF-E2-related factor 2 (Nrf2), a central transcription factor that interacts with the antioxidant response elements to activate the antioxidant- and detoxication-related gene transcription in response to oxidative stress (Gail et al., 2004; Jeong et al., 2006; Sasaki et al., 2002).

α-Lipoic acid (LA) and its reduced form, dihydrolipoic acid (DHLA), are components of the multi enzyme complex that

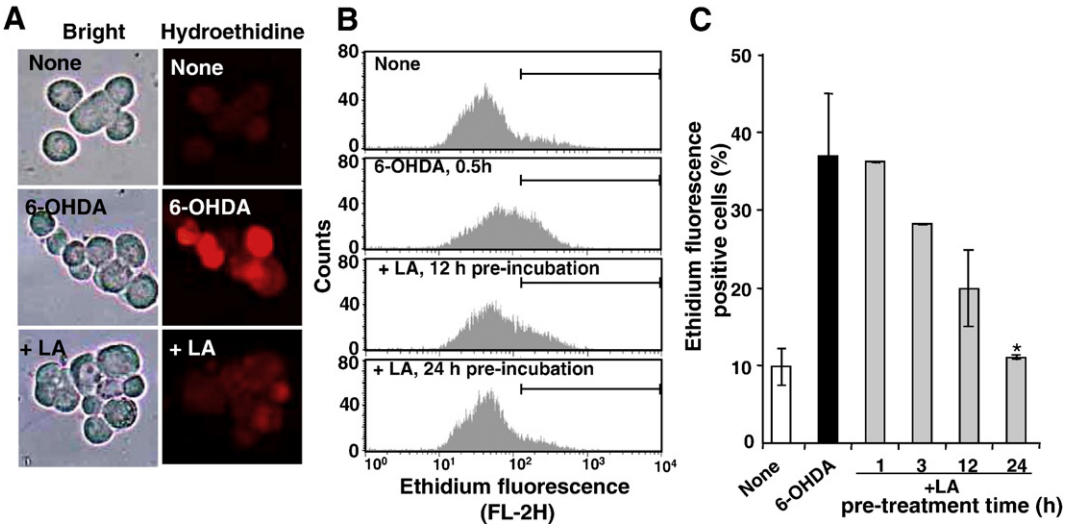

Fig. 1 – Generation of ROS in PC12 cells by 6-OHDA and its suppression by LA in a pre-incubation time-dependent manner. Cells were pretreated with or without 300 μM LA for 1–24 h and then incubated with 75 μM 6-OHDA for 30 min. Cells were stained with 10 μM hydroethidine. PC12 cells generated ROS by the treatment with 6-OHDA. (A) Suppression of ROS generation by LA treatment for 24 h was analyzed by bright field and fluorescence microscopy. Red fluorescence showed the hydroethidine reacted with ROS. (B) The FACScan analyzed patterns of pre-incubation time-dependency by LA. (C) Percentage change of ethidium fluorescence positive cells by the pre-incubation with LA. Ethidium fluorescence positive cells were defined as cells in the gate of panel B. The asterisk indicates that LA significantly suppressed 6-OHDA-induced ROS generation ($p<0.05$). These results are representative of at least three independent experiments.

catalyzes the decarboxylation of α-ketoacids (Smith et al., 2004; Perham et al., 1992; Harris et al., 1975) and are involved in the regulation of carbohydrate and lipid metabolisms (Harris et al., 1975). Aside from their co-enzymatic roles, both LA and DHLA exhibit antioxidant activity (Smith et al., 2004; Bast and Haenen, 2003; Packer, 1998; Smith et al., 2004; Moini et al., 2002; Hagen et al., 2002). Furthermore, LA protects biomembranes from the damage induced by various kinds of oxidative stresses (Persson et al., 2001). In addition, LA protects against programmed cell death in some cells (Persson et al., 2001; Voloboueva et al., 2005) and induces GSH increase and γ-GCS gene expression (Suh et al., 2004a,b; Bharat et al., 2002). However, the anti-apoptotic molecular mechanism of LA has remained unclear.

Previously we described that 6-hydroxydopamine (6-OHDA)-induced apoptosis of PC12 cells was initiated by ROS generation followed by increase of caspase-like activities and chromatin condensation (Fujita et al., 2006). In this study, we examined the effect of LA on the 6-OHDA-induced apoptosis in PC12 cells. We found that LA suppressed the 6-OHDA-induced apoptosis of PC12 cells by up-regulation of the GSH synthesis system including nuclear translocation of Nrf2, up-regulation of γ-GCS_L and 4F2hc mRNA, and intracellular GSH elevation, but not by LA-induced HO-1.

2. Results

2.1. *Suppression of 6-OHDA-induced ROS generation in PC12 cells by LA*

We previously showed that intracellular ROS production initiates apoptotic signal transduction in 6-OHDA-induced PC12 cell apoptosis (Fujita et al., 2006). Intracellular ROS generation was detected by using the fluorescent probe hydroethidine (HE) which ROS oxidize to ethidium. As shown in Figs. 1A and B, intracellular ROS generation was increased by the treatment with 6-OHDA. The intracellular ROS generation was significantly suppressed by pre-incubation with 300 μM LA for 24 h (Figs. 1A and B). Notably, longer pre-incubation with LA resulted in more potent inhibition of 6-OHDA-induced ROS production (Figs. 1B and C).

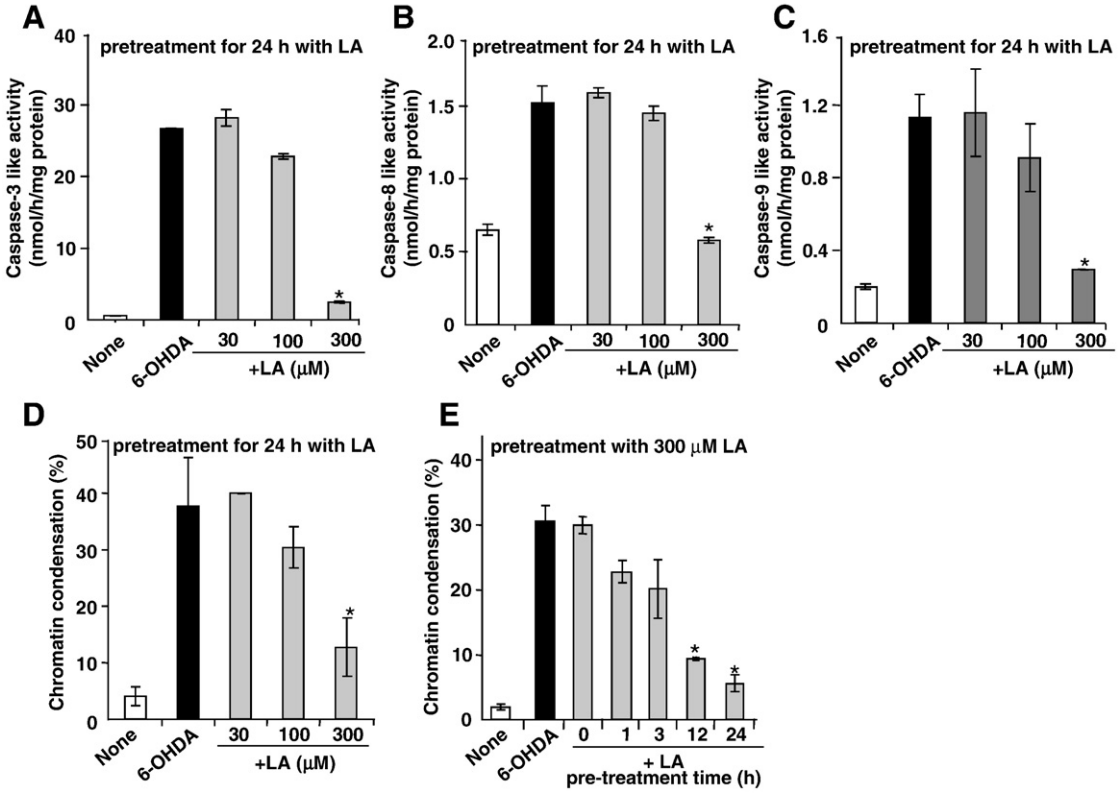

Fig. 2 – Suppression of 6-OHDA-induced apoptotic events by LA. Cells were pre-incubated with the indicated concentration of LA for the indicated time and then incubated with 50 μM 6-OHDA for 12 h. (A–C) Effects of LA on the 6-OHDA-induced caspase-3-like (A), caspase-8-like (B) and caspase-9-like (C) protease activity. The activities of various caspases were measured by using synthetic peptide substrates (Ac-DEVD-MCA for caspase-3-like protease, Ac-IETD-MCA for caspase-8-like protease and Ac-LEHD-MCA for caspase-9-like protease) after treatment with 6-OHDA. (D) Concentration-dependent suppression of 6-OHDA-induced chromatin condensation by LA pretreatment. (E) Pre-incubation time-dependent suppression of 6-OHDA-induced chromatin condensation by LA. Chromatin condensation was detected with Hoechst33342 staining and fluorescence microscopy, and the percentage of chromatin condensation was calculated. The asterisk indicates that LA significantly suppressed 6-OHDA-induced events ($p<0.05$). Data are the means +/− SD derived from three independent experiments.

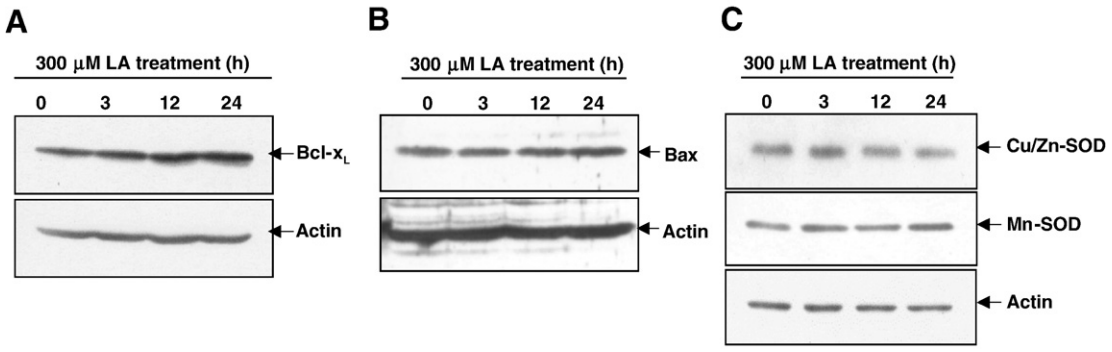

Fig. 3 – Effect of LA on the content of Bcl2 family protein and SOD in PC12 cells. PC12 cells were incubated with 300 μM LA for various times and Bcl-xL, Bax, Cu/Zn-SOD and Mn-SOD were detected by Western blotting each with specific antibodies. (A) Time-dependent change in the content of Bcl-xL after incubation with LA. (B) Effect of LA on the content of Bax. (C) Effect of LA on the content of Cu/Zn-SOD and Mn-SOD. Similar results were obtained in three separate experiments.

2.2. *Suppression of 6-OHDA-induced apoptosis in PC12 cells by LA*

Since LA suppressed 6-OHDA-induced ROS production in PC12 cells, we next investigated the effect of LA on the downstream apoptotic events of the ROS production including increase of caspase-like activity and chromatin condensation (Fujita et al., 2006). As shown in Figs. 2A, B and C, 6-OHDA increased caspase-3, 8 and 9-like activities in PC12 cells, and LA significantly suppressed these increases in a dose-dependent manner. LA suppressed the 6-OHDA-induced chromatin condensation in a concentration-dependent manner (Fig. 2D). Similar to the suppression of intracellular ROS generation (Figs. 1B and C), the suppressive action of LA against 6-OHDA-induced chromatin condensation also depended on the pre-incubation time (Fig. 2E). Pre-incubation with 300 μM LA for 0–3 h did not attenuate the chromatin condensation significantly, and 24 h pre-incubation resulted in a maximal inhibition.

2.3. *Effect of LA on the content of apoptosis-related proteins and antioxidant enzymes in PC12 cells*

Since the sufficient suppressive action of LA required 24 h pretreatment (Figs. 1 and 2) despite LA being rapidly ingested into a cell (Constantinescu et al., 1995), it was considered that the cytoprotective action of LA was indirect. Recently, many reports have shown that LA promotes induction of antioxidant enzymes (Powell et al., 2001; Volobouева et al., 2005; Suh et al., 2004a,b). The ratio between anti-apoptotic Bcl-2 family proteins (Bcl-2, Bcl-xL, etc.) and pro-apoptotic Bcl-2 family proteins (Bax, etc.) regulates apoptotic cell death, and LA increases this ratio in rat endothelial cells (Marsh et al., 2005). To study the involvement of these proteins expression in the antioxidant and anti-apoptotic actions of LA, we investigated the effects of LA on the content of classic antioxidant enzymes (Cu/Zn-SOD and Mn-SOD) and major Bcl-2 family proteins (Bcl-xL and Bax) in PC12 cells. As shown in Figs. 3A and B, LA treatment for 24 h had no significant effect on the expression of apoptosis-related protein Bcl-xL and Bax. Furthermore, LA also had no significant effect on the content of antioxidant enzymes Cu/Zn-SOD and Mn-SOD (Fig. 3C). These results suggested that the expression changes of Bcl-xL, Bax and SODs were not involved in the LA-induced suppression of apoptosis.

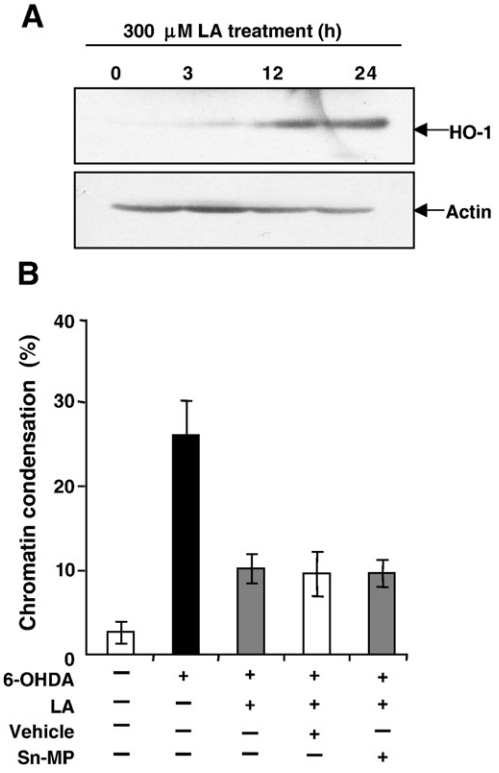

Fig. 4 – Effect of LA on the content of heme oxygenase-1 and effect of Sn-mesoporphyrin IX on the suppression of 6-OHDA-induced apoptosis by LA. (A) Effect of LA on the content of Heme oxygenase-1 (HO-1). Experimental conditions were the same as described for Fig. 3. Similar results were obtained in three separate experiments. (B) Effect of Sn-mesoporphyrin IX (Sn-MP) on the suppression of 6-OHDA-induced chromatin condensation by LA. Cells were pretreated with or without 300 μM LA in the presence or absence of 100 μM Sn-MP for 24 h and then incubated with 50 μM 6-OHDA for 12 h. Data are the means +/− SD derived from three independent experiments.

2.4. *Heme oxygenase-1 was not involved in the LA-induced suppression of 6-OHDA-induced apoptosis*

LA also induced the expression of stress response antioxidant enzyme HO-1 which attenuated the action of intracellular ROS in other cells (Ogborne et al., 2005; Ryter and Choi, 2005). Furthermore, it has been reported that NGF suppressed the 6-OHDA-induced oxidative stress by increasing expression of HO-1 (Salinas et al., 2003). Thus, the effect of LA on the HO-1 content of PC12 cells was examined. As shown in Fig. 6A, LA significantly increased the content of HO-1 in PC12 cells in a time-dependent manner. To obtain further insight into the involvement of HO-1 in LA-induced suppression of apoptosis, we investigated the effect of HO-1 inhibitor Sn-mesoporphyrin IX (Sn-MP) on LA-suppressed chromatin condensation. Contrary to the previous reports, 100 μM Sn-MP pretreatment for 1 h before LA treatment failed to attenuate LA-suppression of the chromatin condensation (Fig. 4B).

2.5. *LA increased the intracellular content of glutathione and the gene expression of γ-glutamylcysteine synthetase light subunit*

The GSH content in various cells is very high in order to maintain the redox state (Dringen, 2000; Takata et al., 2005), and LA promotes GSH synthesis (Suh et al., 2004a,b; Bharat et al., 2002). Thus the effect of LA on the content of GSH in PC12 cells was examined. LA increased the content of GSH in PC12 cells in a time-dependent manner and this increase reached a maximum at 12 h (Fig. 5A). In this case, we confirmed that most of the GSH was reduced form and only 1/100 was GSSG (data not shown). Since intracellular GSH is regulated by γ-GCS (Seelig et al., 1984) and by cystine/glutamate antiporter (Wang et al., 2006; Han et al., 1997) the effect of LA on the gene expression of these proteins was examined. Semi-quantitative RT-PCR analysis showed that LA increased the expression of γ-GCS_L mRNA (Fig. 5B) and 4F2hc mRNA (Fig. 5C). LA had no significant effects on the expression of xCT mRNA (Fig. 5D).

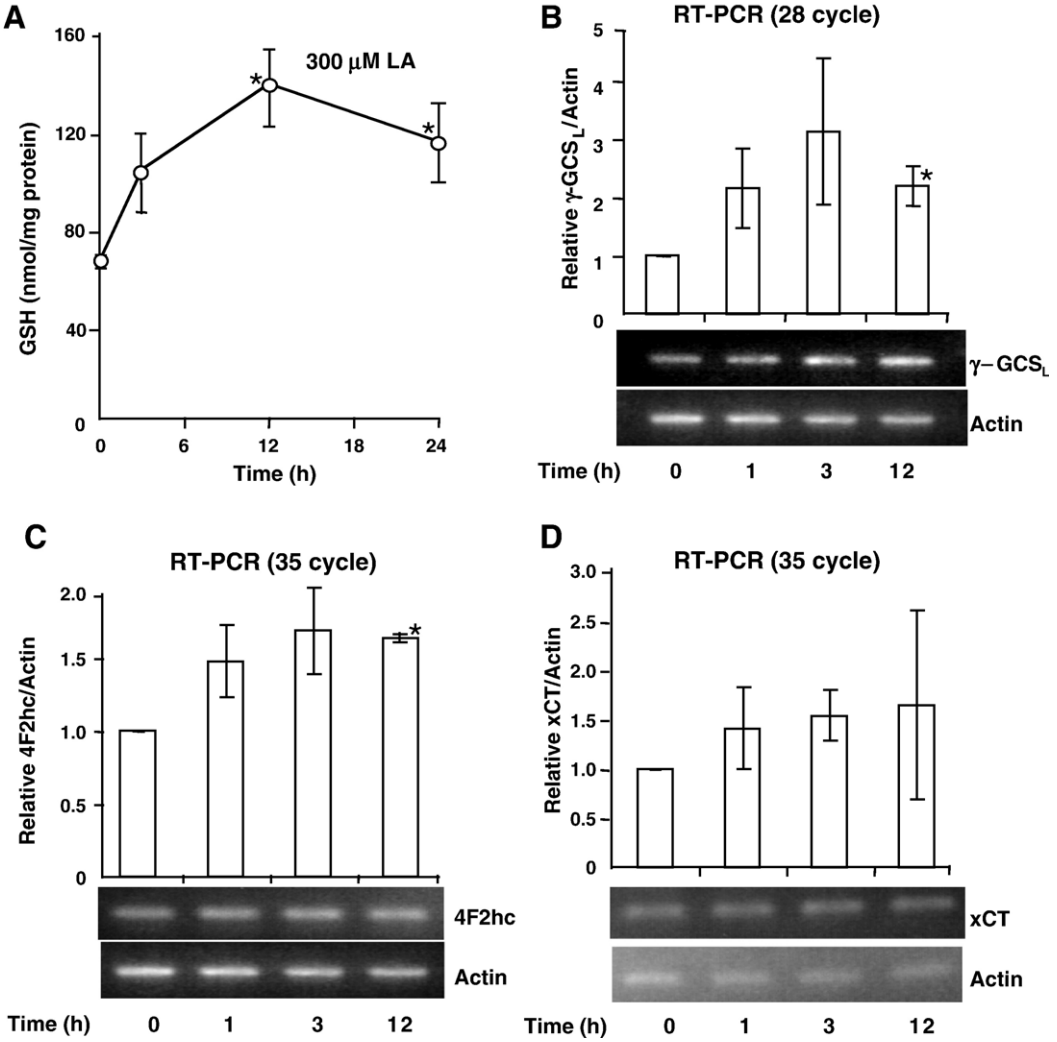

Fig. 5 – Effects of LA on the glutathione synthesis system in PC12 cells. Experimental conditions were the same as described for Fig. 3. PC12 cells were incubated for the indicated time with 300 μM LA. (A) Time-dependent change in the content of GSH after incubation with LA. The content of GSH was determined by the method described in the text. (B–D) Effect of LA on the mRNA expression of γ-glutamylcysteine synthetase L subunit (γ-GCS_L) (B), 4F2hc (C) and xCT (D) in PC12 cells. Expression of the enzyme gene was measured by RT-PCR method as described in the text. The asterisk indicates that LA significantly increased the indicated molecules ($p<0.05$). Data are the means +/– SD derived from three independent experiments.

2.6. GSH depletion by BSO attenuated the LA-induced suppression of apoptosis in PC12 cells

To obtain further insight into the involvement of GSH in LA-induced suppression of apoptosis, we investigated the effect of γ-GCS inhibitor BSO on the LA-suppressed apoptosis. First, we confirmed whether BSO depleted LA-increased GSH in PC12 cells using the enzymatic method and the GSH detectable fluorescent probe 5-chloromethylfluorescein diacetate (CMFDA) (Figs. 6A and B). After incubation of PC12 cells for 24 h with BSO in the presence or absence of 300 μM LA, the LA-increased GSH content was significantly decreased by BSO (Fig. 6B) in a dose-dependent manner (Fig. 6A).

Since BSO markedly depleted the intracellular GSH content, we next investigated the effect of BSO on various LA-suppressed apoptotic events. As shown in Fig. 7, BSO attenuated the LA-induced suppression of caspase-3, 8, and 9-like activities (Figs. 7A, B and C), ROS generation (Fig. 7D), and chromatin condensation (Fig. 7E). It should be noted that the induction of chromatin condensation (Fig. 7E) correlated negatively with the cellular GSH level (Fig. 6A). These results indicated that LA-induced suppression of apoptosis involved the generation of GSH.

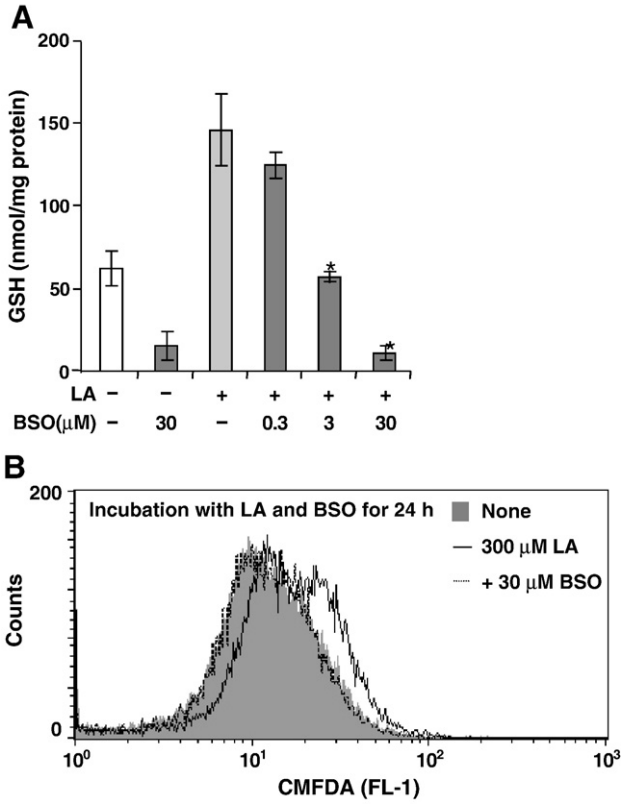

Fig. 6 – Effect of γ-glutamylcysteine synthetase inhibitor on the GSH content in PC12 cells treated with LA. Cells were incubated for 24 h with 0.3–30 μM buthionine sulfoximine (BSO) in the presence or absence of 300 μM LA. (A) Concentration-dependent suppression of LA-induced GSH by BSO was determined with the BIOXYTECH GSH/GSSG-412 Assay kit. The asterisk indicates that BSO significantly suppressed LA-induced GSH ($p<0.05$). Data are the means +/− SD derived from three independent experiments. (B) Suppression of LA-induced GSH by BSO was determined with thiol-reactive fluorescent dye CMFDA. GSH, the intracellular major non-protein thiol in cell, was detected by CMFDA and quantified using a flow cytometer. Similar results were obtained in three separate experiments.

2.7. Effect of LA on the level of nuclear Nrf2 in PC12 cells

Since the transcription factor Nrf2 has been implicated as the central protein that interacts with the antioxidant response element, also referred to as the electrophile response element, to induce γ-GCS (Cheng et al., 2006) and cystine/glutamate antiporter (Sasaki et al., 2002), we investigated the effect of LA on the level of nuclear Nrf2 in PC12 cells. As shown in Figs. 8A and B, the nuclear Nrf2 level was increased by the treatment with LA in a time-dependent manner.

3. Discussion

Although LA suppressed the apoptosis of various cells through antioxidant ability, its molecular mechanism is not fully elucidated. In the previous paper, we demonstrated that ROS generation was an initial step in 6-OHDA-induced apoptosis of PC12 cells (Fujita et al., 2006). In the present study, we showed the molecular mechanism of suppression of 6-OHDA-induced apoptosis by LA in PC12 cells. Our results suggested that LA-stimulated GSH synthesis was a major factor in the suppression of 6-OHDA-induced ROS generation and apoptosis of PC12 cells and that LA-stimulated HO-1 expression was not a major factor.

Short time pre-incubation or no pre-incubation was needed for some antioxidants such as Tiron, *N*-acetylcysteine and GSH to protect PC12 cells from toxicity of 6-OHDA (Fujita et al., 2006; Blum et al., 2001). Although LA is also widely accepted as an antioxidant, short time pre-incubation or no pre-incubation (0–3 h) with LA did not significantly suppress the 6-OHDA-induced apoptosis (Fig. 2E), while long time pre-incubation (12–24 h) with LA significantly suppressed the apoptosis (Fig. 2E). These findings indicate that long time pre-incubation of PC12 is required to mediate the protective effect of LA and suggest that the antioxidant action is indirect.

Accumulated evidence indicate that LA might suppress cell death through GSH synthesis (Packer, 1998; Volobouева et al., 2005). It has been suggested that LA inhibits TNF-alpha-induced ROS generation, GSH reduction and apoptosis in human bone marrow stromal cells (Byun et al., 2005) and potently prevents high glucose-induced oxidative stress and cell death (Vincent et al., 2005). In addition, LA promoted glutathione synthesis via γ-GCS expression and activation (Powell et al., 2001; Suh et al., 2004a,b; Catherwood et al., 2002) and via cystine uptake by cystine/glutamine antiporter (Suh et al., 2004b; Han et al., 1997). In the present experiment, we observed that LA suppressed 6-OHDA-induced ROS generation (Fig. 1) and chromatin condensation (Fig. 2E and F) and induced gene expression of γ-GCS_L and 4F2hc (Fig. 5B and C), and that the LA-suppressed ROS generation and chromatin condensation were attenuated by BSO (Fig. 7D and E). These findings suggested that the LA-suppressed ROS generation and apoptosis depended on GSH synthesis. In this context, the effect of LA on the suppression of ROS generation

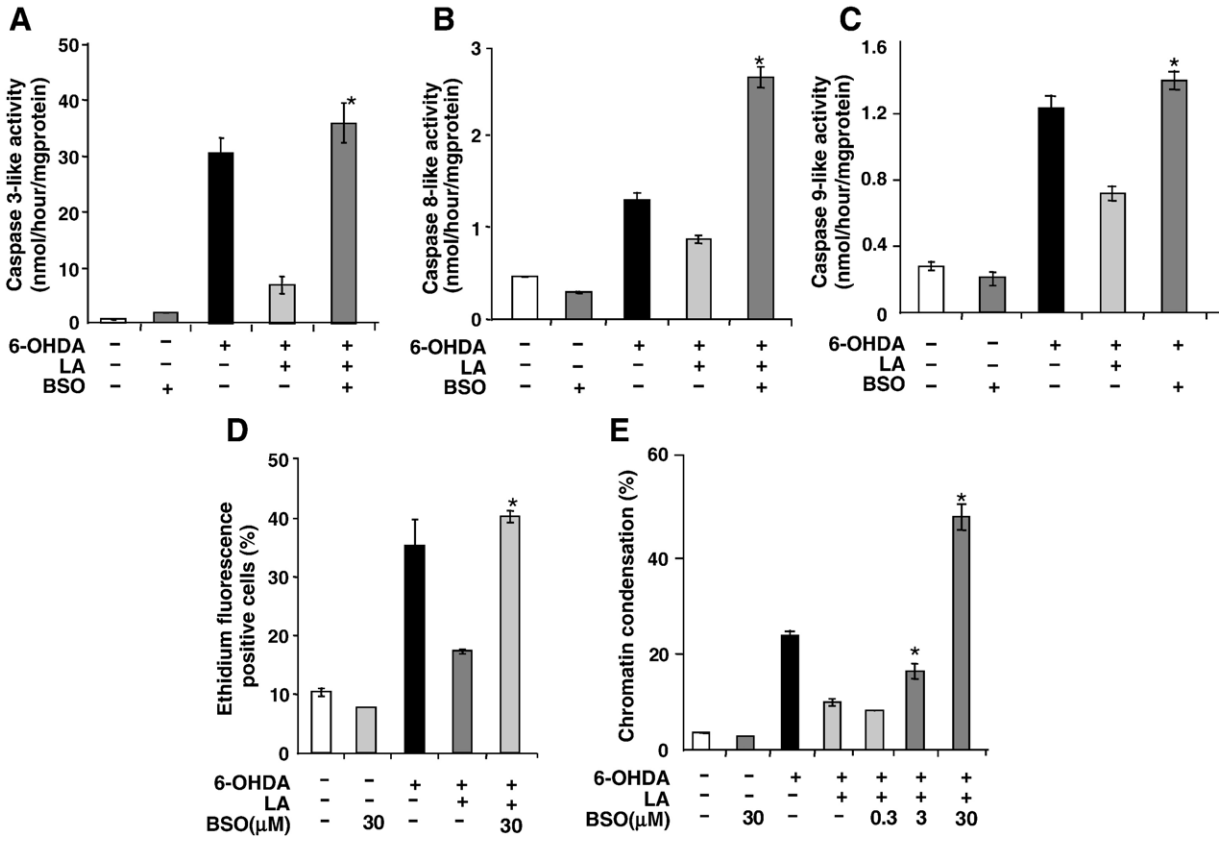

Fig. 7 – Effect of γ-glutamylcysteine synthetase inhibitor on the suppression of various 6-OHDA-induced apoptotic events by LA. PC12 cells were incubated for 24 h with 0.3–30 μM buthionine sulfoximine (BSO) in the presence or absence of 300 μM LA, and then treated with 6-OHDA. (A–C) Effect of BSO on the suppression of 6-OHDA-induced caspase-3 like (A), caspase-8 like (B) and caspase-9 like (C) protease activity by LA. (D) Effect of BSO on the suppression of 6-OHDA-induced ROS generation by LA. (E) Effect of BSO on the suppression of 6-OHDA-induced chromatin condensation by LA. Experimental conditions of (A–C), (D) and (E) were the same as described for Figs. 3, 1 and 4, respectively. The asterisk indicated that BSO significantly attenuated the LA-induced suppression ($p<0.05$). Data are the means +/– SD from three independent experiments.

depended on the pretreatment time with LA (Fig. 1C) and BSO completely attenuated the suppression of ROS generation (Fig. 7D). These results suggested that LA worked through the induction of GSH-elevating proteins, although LA's direct antioxidant activity could not be excluded.

We also observed that intracellular GSH reached a maximum at 12 h after LA treatment, but the peak times of ROS and apoptosis suppression were at 24 h after LA pretreatment (Figs. 1C, 2F and 5A). It is likely that GSH regeneration capacity is more important than GSH level itself. When 6-OHDA produces oxidants in the cell, GSH is likely to be oxidized and no longer works as an antioxidant. Under these conditions, a rapid GSH regeneration, either by GSSG reduction or new GSH synthesis, is important for the continuous cell protection. As the mRNA level for γ-GCS is substantially higher than basal level at 12 h, the enzyme protein synthesis appears to be enhanced for the following time and the enzyme activity might be increased at 24 h.

A previous study showed that NGF suppresses 6-OHDA-induced oxidative stress and apoptosis in PC12 cells by increasing the expression of HO-1 in a phosphatidylinositol 3-kinase-dependent manner (Salinas et al., 2003). In another report, LA increased the HO-1 protein level and the resistance of vascular smooth muscle cells to hydrogen-peroxide-induced cell death, and this protection of LA depended on HO activity (Cheng et al., 2006). In the present experiment, however, the LA-suppressed apoptosis did not depend on HO-1 activity despite increasing expression of HO-1 by LA (Fig. 4). The reason for this discrepancy is not clear at this time, but it may be due to the different experimental conditions, such as the cell culture conditions, or to a difference in the kind of ROS which is involved in cell death. Further studies are needed to answer this question.

It is believed that GSH synthesis is transcriptionally regulated by activating protein-1 (AP-1) and Nrf2. AP-1 regulates mRNA expression of rate-limiting proteins in GSH synthesis including xCT and γ-GCS heavy subunit (Rahman et al., 1998; Sato et al., 2000). It is reported that LA activated AP-1 and its upstream pathway ERK signaling pathway in vascular smooth muscle cells (Cheng et al., 2006). These findings suggested that AP-1 had an important role in the increase of GSH synthesis by LA. In this study, we showed that Nrf2 was significantly translocated into nuclei by LA treatment (Fig. 8). In addition, Nrf2 also regulates gene expression of γ-GCS_L and cystine/glutamate antiporter in some cells (Gail et al., 2004; Moinova and Mulcahy, 1998; Sasaki et al., 2002). These results suggested that Nrf2 might cooperate with AP-1 in promoting gene expression of GSH synthesis-related protein by LA.

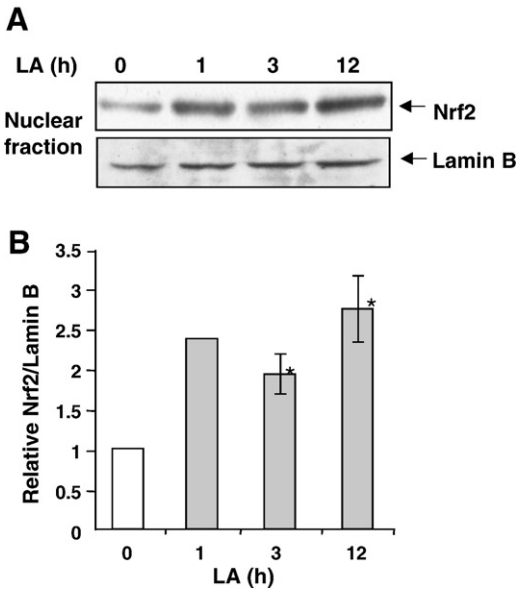

Fig. 8 – Effect of LA on the level of nuclear Nrf2 in PC12 cells. Experimental conditions were the same as described in Fig. 5. Cells were incubated with 300 μM LA for 0–12 h and the nuclear fraction extracted. Nrf2 were detected by Western blotting with Nrf2 specific antibodies. (A) Time-dependent increase in Nrf2 nuclear translocation. (B) The ratio of Nrf2/lamin B band densities is shown in panel A. The asterisk indicates that LA significantly increased nuclear Nrf2 ($p<0.05$). Data are the means +/– SD from three independent experiments.

Recently, the adaptive response induced by pro-oxidants including ROS has received increased attention. It has been reported that low levels of ROS and end product of lipid peroxidation can induce adaptive responses including GSH synthesis and Nrf2 nuclear translocation, and enhance tolerance against subsequent oxidative stress in culture cells (Chen et al., 2006; Seo et al., 2004). LA may also work as a pro-oxidant under some conditions (Moini et al., 2002; Aoyama et al., 2006). In our experiment, 1 mM LA alone had cytotoxicity in PC12 cells (data not shown) and 300 μM LA was hardly attainable physiologically. Thus, 300 μM LA might induce low levels of oxidative stress and enhance tolerance against subsequent oxidative stress. We also showed that LA suppressed 6-OHDA-induced oxidative stress through increasing GSH content and increased Nrf2 nuclear translocation in PC12 cells (Figs. 1, 5A and 8). In addition, it has been reported that LA significantly increased ROS and suppressed subsequent hydrogen-peroxide-induced oxidant stress in vascular smooth muscle cells (Cheng et al., 2006). These results did not exclude the possibility that induction of an adaptive response by LA might suppress 6-OHDA-induced oxidative stress in PC12 cells.

Since 6-OHDA generated the intracellular ROS in PC12 cells (Fig. 1), the contents of GSH and GSSG in 6-OHDA-treated cells were examined. Interestingly, 50 μM 6-OHDA-treatment for 12 h significantly increased the intracellular GSH content and did not increase GSSG content (data not shown). No change of GSSG content by 6-OHDA might be due to rapid reduction by glutathione reductase (Ochiai et al., 2004). In this study, LA suppressed apoptosis due to the enhancement of GSH synthesis in PC12 cells. These findings suggested that the 6-OHDA-increased GSH might be the response to avoid 6-OHDA-induced oxidative stress in non-apoptotic PC12 cells. Thus intracellular GSH in non-apoptotic adherent cells and apoptotic non-adherent cells was examined. In this case, intracellular GSH content in adherent cells was significantly higher than in non-adherent cells (data not shown). In this context, oxidative stress induced by lipid peroxidation products and oxysterols at sublethal concentrations significantly increased the cellular GSH (Chen et al., 2006). These results suggested that non-apoptotic cells treated by 6-OHDA might respond to the 6-OHDA-induced oxidative stress via induction of GSH. It is likely that LA might promote this cell response in the suppression of 6-OHDA-induced apoptosis by LA.

Although caspase-8 mediates the death receptor signal pathway in death ligand-induced apoptosis, 6-OHDA induced an increase of caspase-8 like activity in PC12 cells (Fig. 2B). In this context, the oxidative stress-induced p38 phosphorylation in the 6-OHDA-induced apoptosis pathway was linked to the activation of caspase-8 in neuronal culture cells (Choi et al., 2004). In addition, we have reported that 6-OHDA induced p38 phosphorylation, increase of caspase-8-like activity and tBid in PC12 cells (Fujita et al., 2006). Thus, presumably, caspase-8 is upstream of tBid and downstream of p38 phosphorylation (Fig. 9).

Taken together, we propose the following causal sequence of 6-OHDA-induced apoptosis of PC12 cells and its suppression by LA: intracellular generation of ROS by 6-OHDA is an initial event in 6-OHDA-induced apoptosis, and the ROS stimulates p38 phosphorylation, activating caspase-8, the cleavage of Bid, cytochrome c release, activating caspase-9, and activating caspase-3, thereby inducing chromatin condensation. LA stimulates the translocation of Nrf2 into nuclei, and thereby promotes the gene expression of GSH synthesis-related proteins such as γ-GCS_L and 4F2hc. The increased γ-GCS then increases intracellular GSH content, thereby suppressing the 6-OHDA-induced ROS and subsequent chromatin condensation independently from HO-1 expression (Fig. 9).

4. Experimental procedures

4.1. *Chemicals*

HE and CMFDA were obtained from Molecular Probes (OR, USA). 6-Hydroxydopamine, fetal bovine serum (FBS) and buthionine sulfoximine (BSO) were obtained from Sigma Chemical Co. (St. Louis, MO). Sn-MP was obtained from Frontier Scientific Inc. (Logan, UT). Sn-MP was dissolved in a solvent consisting of 40% propanediol, 10% ethanol, 153 mM arginine and H_2O. Fluorogenic tetrapeptide substrates, such as Ac-DEVD-MCA for caspase-3-like protease, Ac-IETD-MCA for caspase-8-like protease and Ac-LEHD-MCA for caspase-9-like protease were obtained from the Peptide Institute (Osaka, Japan). Polyclonal antibodies of HO-1 and Mn-SOD were from Stressgen Biotechnology Corp (BC, Canada). Anti-Cu/Zn-SOD antiserum was prepared in our laboratory. Polyclonal antibodies of Bax, Bcl-xL, lamin B and Nrf2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). LA was donated by Cargill Co. Ltd. (USA). BIOXYTECH GSH/GSSG-412 Assay Kit was obtained from OXIS international, Inc.

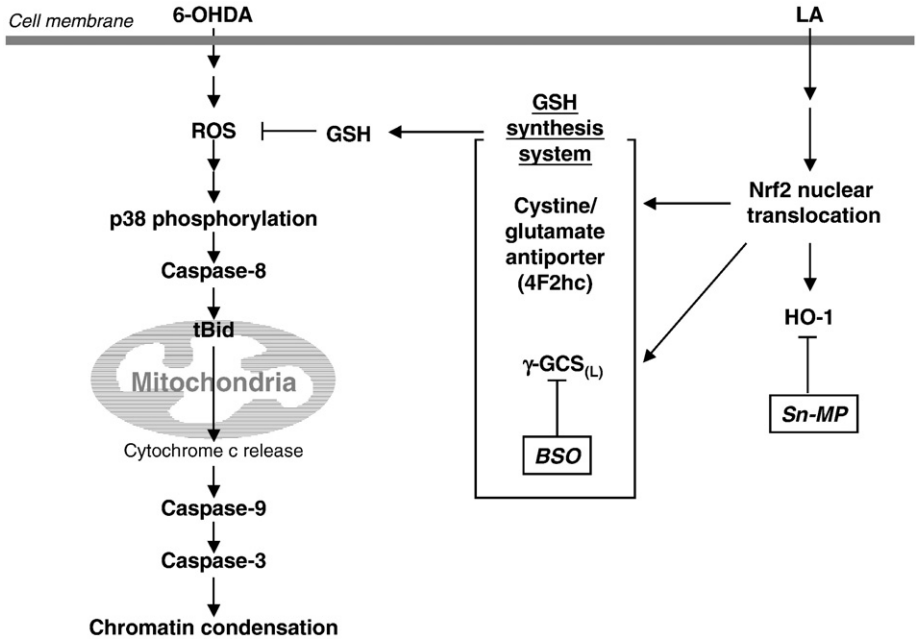

Fig. 9 – Schematic diagram summarizing the cross talk of 6-OHDA-activated apoptosis pathway and LA-activated glutathione synthesis pathway. 6-OHDA probably induces PC12 cell apoptosis by the following mechanisms: intracellular ROS production by 6-OHDA is an initial event, and then the ROS increases the activating caspase cascade and chromatin condensation. LA rapidly increases the nuclear Nrf2 levels and then enhances gene expression of antioxidant related enzymes such as HO-1, γ-GCS$_L$ and 4F2hc, thereby activating the glutathione synthesis system. LA-induced GSH suppresses ROS induced by 6-OHDA. HO-1 and γ-GCS$_L$ were heme oxygenase-1 and γ-glutamylcystein synthetase L subunit, respectively.

(Portland, OR) All other chemicals were of analytical grade and obtained from Nacalai Tesque (Kyoto, Japan).

4.2. *Cell culture*

The PC12 cell line was maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin on collagen type I-coated dishes as described in a previous paper (Furuno et al., 2001). Cells were grown in a humidified incubator at 37 °C under 5% CO_2/95% air and used for experiments during the exponential phase of growth.

4.3. *Assay for ROS generation*

Intracellular ROS generation was measured using ROS-sensitive fluorescent precursor, HE (Fujita et al., 2006; Yamada et al., 2003). Cells (2×10^5 cells/ml) were plated onto collagen-coated-24 well plates in 0.5 ml culture medium and incubated for 24 h before LA treatment. Cells were pretreated with or without 300 μM LA for the indicated times and incubated with 75 μM 6-OHDA for 30 min at 37 °C. Cells were washed with PBS and stained with 10 μM hydroethidine for 15 min at 37 °C in the dark. Then the cells were analyzed with light and fluorescence microscopy and a FACScan flow cytometer to determine the ROS generation.

4.4. *Assay for caspase activity*

Activities of caspases were determined as described previously (Fujita et al., 2005; Yabuki et al., 2000) in 20 mM HEPES buffer (pH 7.5) containing 0.1 M NaCl and 5 mM dithiothreitol at 37 °C using 10 μM of Ac-DEVD-MCA, Ac-IETD-MCA or Ac-LEHD-MCA as substrates for caspase-3, 8 and 9-like protease, respectively. Fluorescence of released AMC was measured using a fluorescence plate reader with excitation and emission wavelengths of 355 and 460 nm, respectively.

4.5. *Assay for chromatin condensation*

Cells (2×10^5 cells/ml) were plated onto collagen-coated-24 well plates in 0.5 ml culture medium and incubated for 24 h before LA treatment. Cells were pretreated with or without LA for the indicated times. After incubation with 6-OHDA for 12 h, cells were stained with Hoechst33342 and the chromatin-condensed cells were determined under fluorescence microscopy. Total cells (500–1000 cells) and chromatin-condensed cells were counted in the same field, and the percentage of chromatin condensation was calculated (Fujita et al., 2005).

4.6. *Western blot analysis*

Cells (2×10^5 cells/ml) were plated onto collagen-coated-100 mm dishes in 15 ml culture medium and incubated for 24 h before LA treatment. Cells were treated with or without LA for the indicated times. Cell lysates were prepared as described elsewhere (Fujita et al., 2005). Cells were dissolved in ice-cold lysis buffer [20 mM Tris–HCl (pH 7.5), 150 mM NaCl 1% Triton X-100, 1 mM EDTA, 1 mM EGTA] supplemented with protease inhibitors (0.1 mM PMSF, 100 μg/ml leupeptin, 5 μg/ml pepstatin A). Lysates were cleared by centrifugation.

Nuclear proteins were fractionated from PC12 cells (Wielandt et al., 2006). Briefly, cells were collected by centrifugation at

800 ×*g* for 5 min at 4 °C and then were resuspended in 400 μl of ice-cold buffer A (10 mM HEPES (pH 7.8), 10 mM KC1, 1.5 mM $MgCl_2$, 0.2 mM PMSF, and 0.5 mM DTT). After 15 min incubation on ice, NP-40 was added to a final concentration of 0.6%, and cells were vortexed and centrifuged for 1 min at 16,000 ×*g*. The nuclear pellet was extracted with 50 μl of ice-cold buffer B [l0 mM HEPES (pH 7.8), 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT, and 25% glycerol] for 30 min at 4 °C on a rocking platform, and debris were removed by centrifugation at 16,000 ×*g* for 20 min at 4 °C.

Lysates and nuclear proteins were added with the same volume of SDS sample buffer (125 mM Tris–HCl, pH 6.8, 4% SDS, 10% 2-mercaptoethanol, 20% glycerol, and 0.002% bromophenol blue) and boiled at 100 °C for 5 min. The samples (10–50 μg protein) were then subjected to SDS-polyacrylamide gel electrophoresis. Proteins in the gel were transferred onto a PVDF membrane, and then incubated with the primary antibody (1:5000 dilution for HO-1 and actin, 1:1000 dilution for others) and finally with horseradish peroxide-linked second antibody (1:2000 or 1:25,000 dilution) and analyzed using ECL plus kit (Amersham). Protein concentration was determined by the method of Bradford (Bradford, 1976) using bovine serum albumin as a standard.

4.7. *Assay for intracellular glutathione content*

Measurements of GSH were performed using the BIOXYTECH GSH/GSSG-412 Assay kit. Cells (2×10^5 cells/ml) were plated onto collagen-coated 60 mm dishes in 5 ml culture medium and incubated for 24 h before LA treatment. Cells were treated with or without LA and BSO for the indicated time. Cells were suspended in ice-cold 5% trichloroacetic acid (w/v) and lyzed by a vortex mixer. The supernatant was centrifuged for 10 min at 20,000 ×*g* and 4 °C. After removing insoluble materials by centrifugation, samples were assayed spectrophotometrically following the manufacturer's instructions. The protein contents of the samples were determined by the method of Bradford (1976).

The thiol-reactive fluorescent dye CMFDA was used for GSH determination. CMFDA forms a GSH adduct in a reaction catalyzed by glutathione-S-transferase. After conjugation with GSH, CMFDA is hydrolyzed to the fluorescent 5-chloromethylfluorescein by cellular esterase (Poot et al., 1991). Cells were treated with or without LA and BSO for the indicated times, incubated for an additional 30 min in a CFMDA (30 μM)-containing culture medium at 37 °C, and then analyzed with a FACScan flow cytometer to determine the GSH content.

4.8. *Semi-quantitative RT-PCR analysis for mRNA expression of GSH synthesis-related proteins*

To determine the relative expression level of γ-GCS transcripts, RT-PCR was performed as follows. Total RNA was isolated from PC12 cells using TRIzol reagent (Invitrogen) following the manufacturer's instructions. Oligo dT-primed cDNA was prepared from 3–5 μg of total RNA using Superscript III (Invitrogen). One-fiftieth of the cDNA obtained was used for each PCR. Primer sequences and product sizes were as follows: 5′-TAA TAC GAC TCA CTA TAG TGC GGC GCC TCA GTG ACG CTT TTT G-3′ (containing T7 promoter sequence) and 5′-GAT TTA GGT GAC ACT ATA GAA CCA AGT TAA TCT TGC CTC C-3′ (containing SP6 promoter sequence) for γ-GCS_L (456 bp); 5′-CCTGGCATTTG-GACGCTACAT-3′ and 5′-TCAGAATTGCTGTGAGCTTGC-3′ for xCT (182 bp); 5′-CTCCCAGGAAGATTTTAAAGACCTTCT-3′ and 5′-TTCATTTTGGTGGCTACAATGTCAG-3′ for 4F2hc (141 bp); and 5′-ATT TGG CAC CAC ACT TT TAC A-3′ and 5′-TCA CGC ACG ATT TCC CTC TCA G-3′ for actin (379 bp). The cDNA were amplified by 28 cycles (γ-GCS_L or actin) or 35 cycles (xCT or 4F2hc) with the following conditions: denaturing at 94 °C for 0.5 min, annealing at 52 °C (γ-GCS_L or actin) or 60 °C (xCT or 4F2hc) for 1 min, extension at 72 °C for 1 min and final elongation at 72 °C for 3 min. The amplified products were separated on 1.5% agarose gels and visualized by ethidium bromide staining. The intensity of the bands was quantified using an image analyzer (NIH Image Software). The identity of PCR products was verified by sequencing.

4.9. *Statistical analysis*

Results are expressed as means +/− SD. The significance of differences between experimental conditions was determined using the two-tailed Student *t*-test. A probability of $p<0.05$ was considered significant.

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