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Lig-8, a Highly Bioactive Lignophenol Derivative from Bamboo Lignin, Exhibits Multifaceted Neuroprotective Activity

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ABSTRACT

Lignin is a durable aromatic network polymer that is second only to cellulose in natural abundance. Lig-8, a lignophenol derivative from bamboo lignin, is a highly potent neuroprotectant. It protects human neuroblastoma cells (SH-SY5Y) from hydrogen peroxide (H_2O_2)–induced apoptosis by preventing caspase-3 activation via either caspase-8 or caspase-9. It exerts this antiapoptotic effect by protecting mitochondrial membrane permeability from damage by H_2O_2 or the peripheral benzodiazepine receptor ligand PK11195. Lig-8 has been also shown to scavenge the reactive oxygen or nitrogen species *in vitro*. Furthermore, lig-8 suppresses apoptosis induced by oxygen-glucose deprivation, tunicamycin (endoplasmic reticulum [ER]–stress inducer), or proteasome inhibitor in pheochromocytoma cells. In addition, *in vivo*, lig-8 reduced intravitreal *N*-methyl-D-aspartate–induced retinal damage (decreases in retinal ganglion cells and inner plexiform layer thickness) in mice. Lig-8 prevents neuronal damage partly by inhibiting excessive endoplasmic reticulum stress. In this article, we review the protective effects of lig-8 against apoptosis induced by various stimuli. Apoptosis is an active, energy-dependent process through which living cells initiate their own death. It can be induced by a variety of physiological and pharmacological stimuli. Apoptotic cell death is associated with neurodegenerative disorders such as Alzheimer, Parkinson, or Huntington disease as well as glaucoma. We believe that

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the elucidation of the mechanism of antiapoptotic action of lig-8 may help in finding new approaches to the treatment of neurodegenerative disorders.

INTRODUCTION

Lignin is a characteristic chemical and morphological compound forming an integral part of the cell wall of some cells (e.g., tracheids, xylary fibers, and sclereids of plants). After cellulose, lignin is the second most abundant organic substance in the plant world, making up 20–30% of the global plant biomass; it forms a durable three-dimensional aromatic network polymer. Lignin is highly resistant toward chemical and biological degradation and gives wood its mechanical strength (Martinez et al. 2005). Typical lignin derivatives that were industrially isolated, for example, in the production of pulp, have been hardly utilized. A successful medicinal application for them has not yet been found, partly because lignin molecules are heterogeneous and complex. To find ways of utilizing lignin, a new phase-separation system has been recently developed (Funaoka et al. 1989, 1996). The phase-separation system for the production of lignophenols, a functional lignin derivative from native wood, has been designed. Various lignophenol derivatives obtained by the phase-separation system were evaluated for their antiapoptotic effect. As shown in Table 1, 7 of the 11 lignophenol derivatives tested had neuroprotective activity in the H_2O_2-induced apoptosis. The lignocresol derivatives had more potent neuroprotective action as compared with other lignophenols. One of the lignocresol derivatives that were obtained from bamboo and chemically carboxymethylated (CM) was named lig-8.

The pharmacological effects of lignin have not been studied. We performed various screening tests, therefore, to elucidate the pharmacological actions of lig-8 and found that lig-8 has antiapoptotic action and protects endoplasmic reticulum (ER) from stress. These effects of lig-8 have been demonstrated *in vitro* and *in vivo*.

CHEMISTRY

Chemical Isolation of Lig-8 from Native Lignin

A phase-separation system for production of functional lignin derivatives from native lignins was devised (Funaoka et al. 1989, 1996). This system utilizes phenols and concentrated acid. The resulting lignin derivatives, lignophenols, have highly phenolic functions, high stability, and are less heterogeneous compared with those after the conventional lignin preparations. The phenolic properties of lignophenols can be modulated by using different phenols (Funaoka et al. 1995). Thus, it can be assumed that the lignophenols derived from native lignins have different phenolic functionalities, and exhibit various biological activities. Moreover, the functionality of lignophenols can be easily modulated by various modifications.

Native lignins with complicated three-dimensional structures were converted to lignophenols by the phase-separation system, the summary of which is shown in Fig. 1. Lignophenols retain the original interunit linkages formed by dehydrogenative polymerization during the biosynthesis of native lignin, and have high phenolic functionality. By using

TABLE 1. Properties of lignophenol derivatives and their effects on H_2O_2-induced apoptosis in SH-SY5Y cells

		Properties of lignophenol derivatives				
		Hydroxyl groups** (mol/C_9)		Combined carboxymethyl groups***	Weight-average molecular weight****	
No.	Lignophenol derivatives	Phenolic OH	Total OH	(mol/C_9)	(MW)	Antiapoptotic effect
1	Lignin alkali	–	–	–	28000	+
2	Lignopyrogallol (Japanese cedar)	3.61	4.27	–	3410	–
3	Lignocatechol (Japanese cedar)	2.78	3.46	–	4820	–
4	Lignoresorcinol (Japanese cedar)	2.66	3.37	–	4810	+
5	Lignopyrogallol (rice husk)	Not measured		–	1950	–
6	AT-lignopyrogallol (Japanese cedar)	3.11	3.63	–	2430	–
7	CM-lignocresol (rice husk)	0.62	1.53	0.60	3810	+
8	CM-lignocresol (bamboo)	0.46	1.31	0.66	5330	+++
9	CM-lignocresol (beech)	0.64	1.75	0.67	5460	+
10	CM- and AT-lignocresol* (beech)	0.63	1.77	0.75	1500	+
11	AT-lignocresol (beech)	1.21	2.18	–	1700	++

–, no effect; +, blockage 0–25%; ++, 25–50%; +++, 50–100%.
Abbreviations: AT = alkaline-treated; CM = carboxymethylated.
All lignophenol derivatives are water-soluble. *Carboxymethylated materials of the water-insoluble fraction of alkaline-treated lignocresol. **Hydroxyl groups of lignopolyphenols and CM derivatives were determined by ^{1}H NMR spectra for original lignopolyphenols in CDCl3-C5D5N (1:3, v/v) and their acetylated derivatives in CDCl3 (containing TMS as the internal reference) and nonaqueous potentiometric titration, respectively. ***Carboxymethyl groups were determined by nonaqueous potentiometric titration. ****Average molecular weights were calculated by gel permeation chromatograms (columns: Shodex KF602, KF603, and KF604; eluent: tetrahydrofuran; detector: UV at 280 nm) for acetylated derivatives, and alkali lignin was provided by technical data of Aldrich Chemical Company, Inc. Data from ref. Akao et al. 2004.

different phenols for the phase-separation system, the different kinds of lignophenols were produced. Lignopolyphenols, such as lignocatechol, lignoresorcinol, and lignopyrogallol, which have many phenolic hydroxyl groups, are highly hydrophilic. On the other hand, lignomonophenols combined with monohydric phenols such as cresol are water-insoluble. However, when water-insoluble lignophenols were CM and/or alkaline treated, they became highly hydrophilic and water-soluble. Especially, CM lignophenols showed a high binding

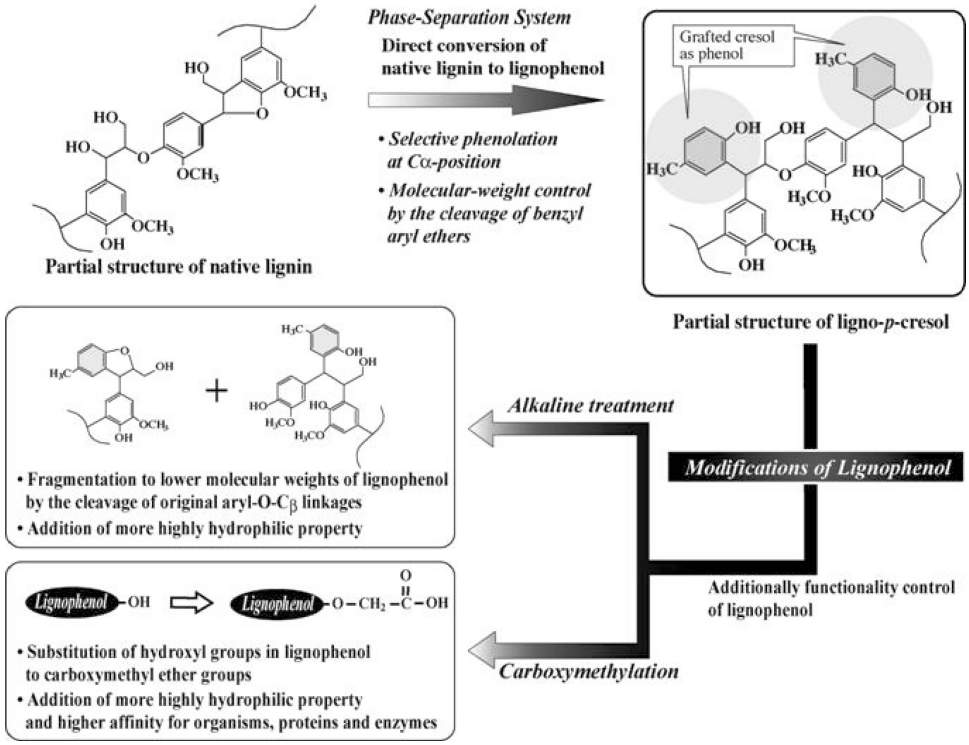

FIG. 1. Conversion of native lignin into lignophenol derivatives and control of their functionality. Data from Akao et al. (2004).

affinity for proteins because of the introduced carboxyl groups (Suzuki et al. 1989). The carboxymethyl groups were recognized by ^{1}H NMR (Table 1) and ionizing radiation (IR) spectroscopy (Fig. 2). CM lignocresol from bamboo was named lig-8 (Akao et al. 2004).

BIOLOGICAL ACTIVITY

Cytoprotective Effects of Lig-8 *In Vitro*

Lig-8 protected SH-SY5Y cells from hydrogen peroxide (H_2O_2)–induced cell death and displayed the most potent apoptosis-preventing effect at 20 and 30 μM (Fig. 3). However, at 10–50 μM, lig-8 had no effect on the growth of SH-SY5Y cells. H_2O_2-induced apoptosis in SH-SY5Y cells involves activation of caspase-8 and caspase-3. Lig-8 blocked the caspase-3 activation and concentration-dependently prevented the proteolysis of procaspase-3 by caspase-8 or caspase-9 (Akao et al. 2004).

In the last several years, it has become increasingly clear that mitochondria play a major rate-limiting role in the apoptosis of neuronal cells. The decision/effector phase of the apoptotic process converges on mitochondria, where permeabilization of mitochondrial membranes occurs as a result of the permeability transition pore complex (PTPC)

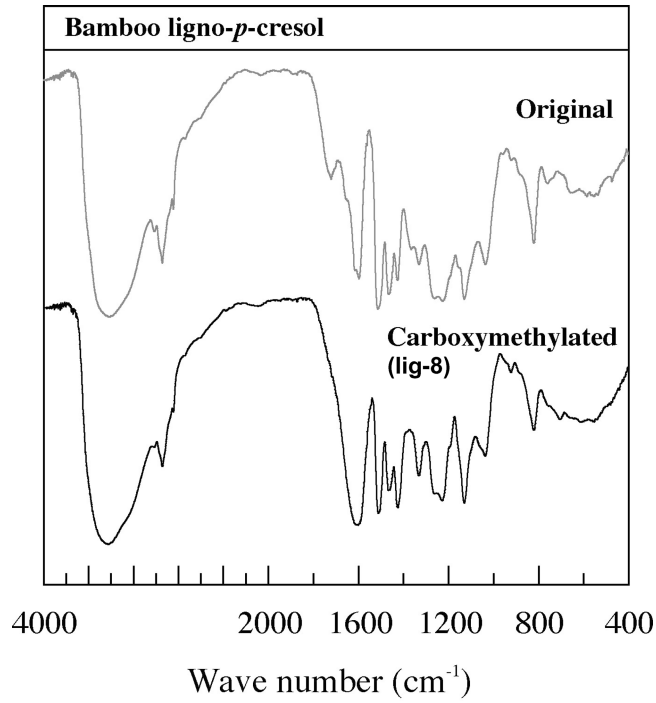

FIG. 2. Ionizing radiation (IR) spectra of original (preparation after phase separation) and carboxymethylated (CM) bamboo lignocresol derivative lig-8. Absorption bands around 1600–1710 and 1425 cm^{-1} in the IR spectra of CM lignocresols were markedly increased in intensity compared with those of original lignophenols after phase separation, owing to the overlapped absorptions of introduced carboxyl groups and aromatic nuclei in the original lignophenols. Data from Akao et al. (2004).

(Zoratti and Szabo 1995; Tsujimoto and Shimizu 2000). Lig-8 has protective effects on mitochondrial membrane permeability transition (PT) in the apoptosis. The release of cytochrome c from mitochondria and simultaneously the sequential activation of caspase-9 are suppressed by lig-8 (Akao et al. 2004). H_2O_2-induced apoptosis is thought to involve a mitochondrial pathway. Lig-8 was shown to ameliorate apoptosis by preventing depolarization of mitochondrial membrane PT. To further assess the effect of lig-8 on mitochondrial membrane PT, the decrease in mitochondrial membrane potential by 100 μM PK11195 [peripheral benzodiazepine receptor ligand, 1-(2-chloro-phenyl)-*N*-(1-methylpropyl)-3-isoquinolinecarboxamide] was prevented by lig-8 at 30 μM (Fig. 4). Moreover, the activation of caspase-9 by PK11195 was almost completely blocked by lig-8 at 30 μM. Thus, lig-8 prevented PK11195-induced cell death (mediated by mitochondrial membrane PT), as observed in H_2O_2-induced apoptosis (Akao et al. 2004).

Lig-8 also attenuated cell death induced by either H_2O_2, which generates reactive oxygen species (ROS), or by nitric oxide free radical donor, SIN-1 (Oh-hashi et al. 1999), which generates reactive nitrogen species (RNS) (Feelisch et al. 1989) in SH-SY5Y cells. Lig-8 exhibited ROS and RNS scavenging activity.

Lig-8 protected against cell damage induced by oxygen-glucose deprivation (OGD) stress in pheochromocytoma (PC12) cells (Fig. 5) (Ito et al. 2006). Lig-8 displayed potent

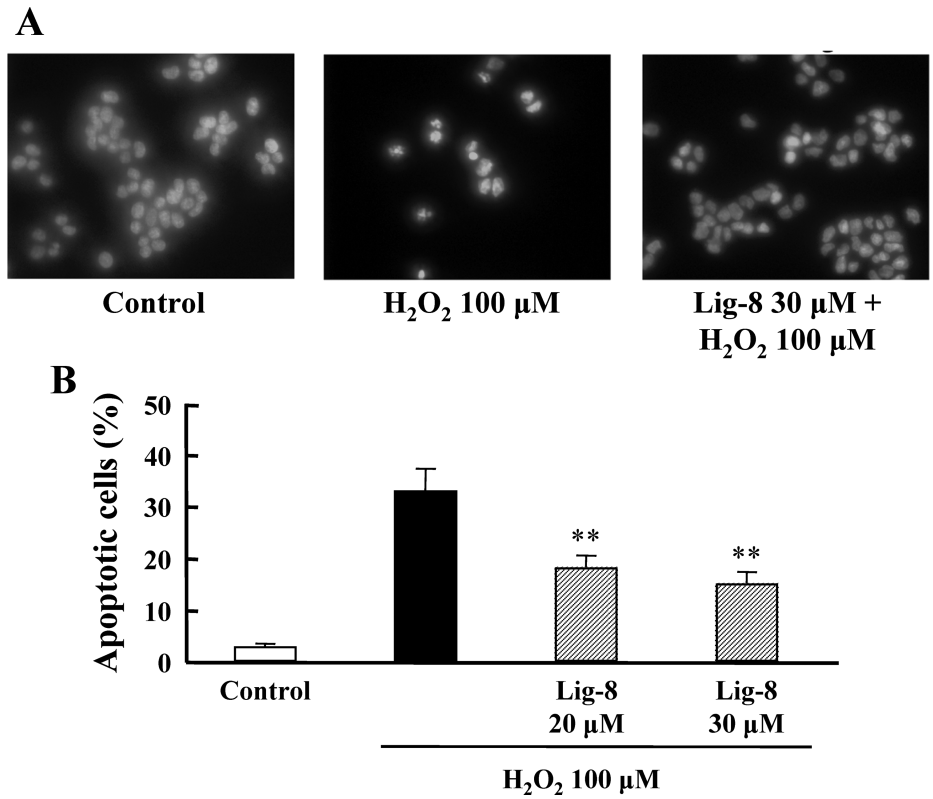

FIG. 3. H_2O_2-induced apoptosis and its blockade by lig-8 for 12 h in SH-SY5Y cells: (A) apoptotic cell death evaluated by DNA ladder formation in Hoechst 33342 staining; (B) apoptotic cell death evaluated by Hoechst 33342 staining. Results are mean ± SD of three independent experiments, $^{**}P < 0.01$ vs. H_2O_2 100 μM (Fisher PLSD test). Data from Akao et al. (2004).

neuroprotective activity against H_2O_2-induced apoptosis in SH-SY5Y by preventing caspase-3 activation via either caspase-8 or caspase-9. Accordingly, the neuroprotective effect of lig-8 against OGD-induced cell damage may at least partly involve caspase-3, caspase-8, and/or caspase-9. However, the cell death induced by OGD has been reported not to involve caspase-3 (Hillion et al. 2005). Hence, it is probable that caspase-3 is not involved in the protective effect of lig-8 against OGD stress. It has been recently reported that after cerebral ischemia, ER-stress pathways are activated, including an unfolded protein response (UPR) and protein synthesis inhibition (DeGracia and Montie 2004). If ER stress and UPR are prolonged, neurons were directed toward apoptosis. Thus, neuronal cell death is triggered by misfolded proteins or proteasome inhibition. OGD induces ER stress, resulting in an upregulation of glucose/oxygen-regulated protein 78 (GRP78/BiP). Lig-8 has also neuroprotective effect against tunicamycin (2 μg/mL)–induced cell death (Fig. 6), indicating that a decrease in the effect of ER stress may participate in the protective mechanisms activated by lig-8.

It has been reported recently that the ubiquitin-proteasome pathway is involved in the degradation of misfolded proteins under ER-stress conditions (Kawahara et al. 1997).

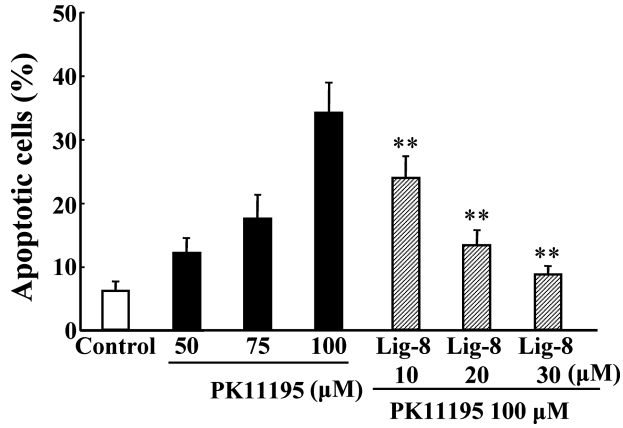

FIG. 4. Effect of lig-8 against PK11195-induced apoptosis evaluated by Hoechst 33342 nuclear staining. Results are mean ± SD of three independent experiments, $^{**}P < 0.01$ vs. the value of apoptotic cells (%) in 100 μM PK11195 treatment (Fisher PLSD test). Data from Akao et al. (2004).

During UPR, the accumulated unfolded protein is either correctly refolded or unsuccessfully refolded and subsequently degraded by the ubiquitin-proteasome pathway. When the amount of unfolded protein exceeds a certain threshold, damaged cells are committed to cell death, which is mediated by activation of the caspase-12, apoptosis signal-regulating kinase 1 (ASK-1), c-Jun N-terminal kinase (JNK), and DNA damage–inducible gene 153 (Gadd153)–signaling pathway (Yoneda et al. 2001; Imaizumi and Tohyama 2004; Oyadomari and Mori 2004). In fact, it has been reported that ER-stress–mediated cell death is

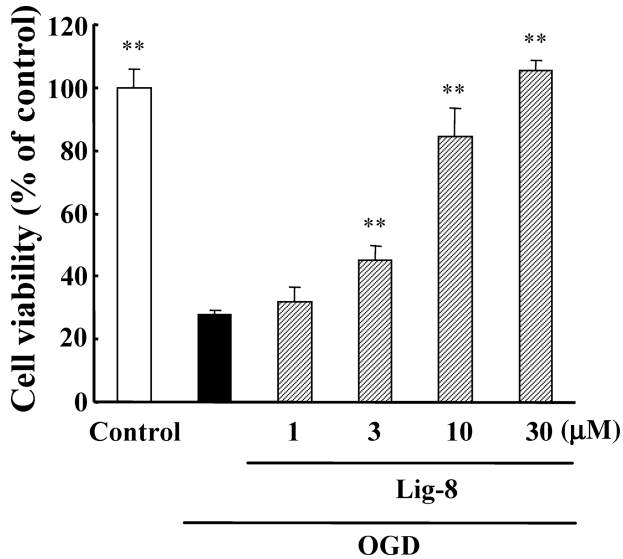

FIG. 5. Protective effect of lig-8 against oxygen-glucose deprivation (OGD)–induced cell death in PC12 cells. Lig-8 inhibited OGD-induced cell death. Results are mean ± S.E.M., $n = 4$; $^{**}P < 0.01$ vs. OGD treatment alone (Dunnett test). Data from Ito et al. (2006).

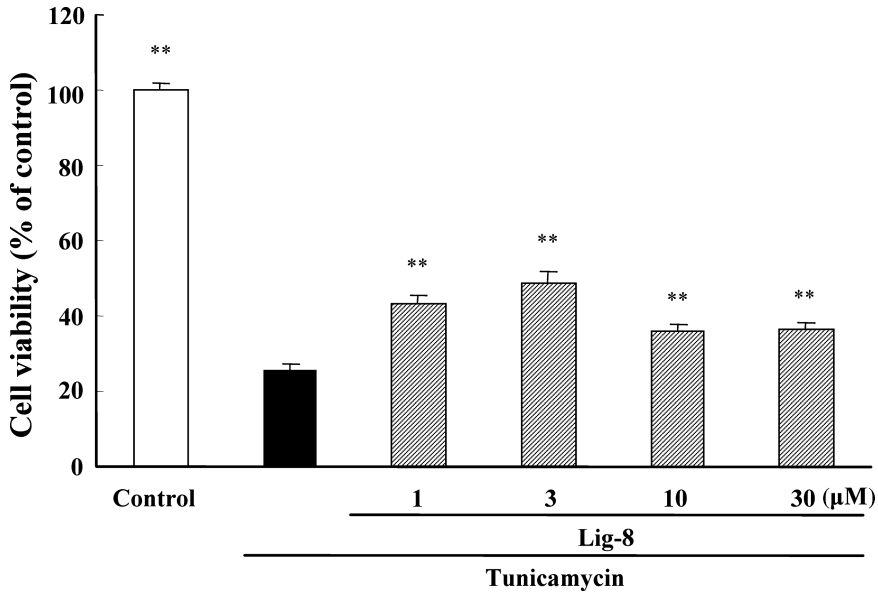

FIG. 6. Effect of lig-8 against tunicamycin-induced cell death in PC12 cells. Results are mean ± S.E.M., $n = 4$; $^{**}P < 0.01$ vs. tunicamycin treatment alone (Dunnett test). Data from Ito et al. (2006).

induced via an inhibition of proteasome activity (Hong et al. 2004). An inhibition of proteasome activity by means of proteasome inhibitor (PSI)–induced apoptotic cell death in SH-SY5Y cells, and lig-8 treatment significantly inhibited this apoptosis (Fig. 7). Lig-8 is capable of inhibiting ER-stress–induced cell death.

Protective Effects of Lig-8 on *in Vivo* Retinal Damage

Excessive activation of glutamate receptors by glutamate released from injured retinal ganglion cells (RGCs) is implicated in the glaucomatous RGC death process (Osborne et al. 1999). Glutamate is the principal excitatory neurotransmitter within the central nervous system (CNS), and it has been found to be increased in the vitreous body in glaucoma (Vorwerk et al. 1999; Martin et al. 2002; Yücel et al. 2006). Blockade of glutamate activity by modulation of its receptors—in particular, by modulation of *N*-methyl-D-aspartate (NMDA)–type glutamate receptors—has been advocated as an important strategy for neuroprotection in glaucoma. NMDA-receptor activation, and the downstream signal pathways, had an important involvement in retinal ischemic cell death (Sucher et al. 1997). ER-stress activation may play a pivotal role in the downstream signal pathways following activation of the NMDA receptor (Uehara et al. 2006). The animal model employed in the study (involving intravitreal injection of NMDA) displays glaucomatous symptoms in the retina. This model exhibits high sensitivity and stability, as well as good reproducibility, and is widely used for investigating the mechanisms underlying neuronal cell death in the retina (Fig. 8) (Yoneda et al. 2001). Lig-8 exerted protective effects on NMDA-induced retinal damage in mice (Fig. 9). Its neuroprotective action may be due partly to inhibition of the effects of excessive ER stress associated with reduced proteasome activity. We have demonstrated

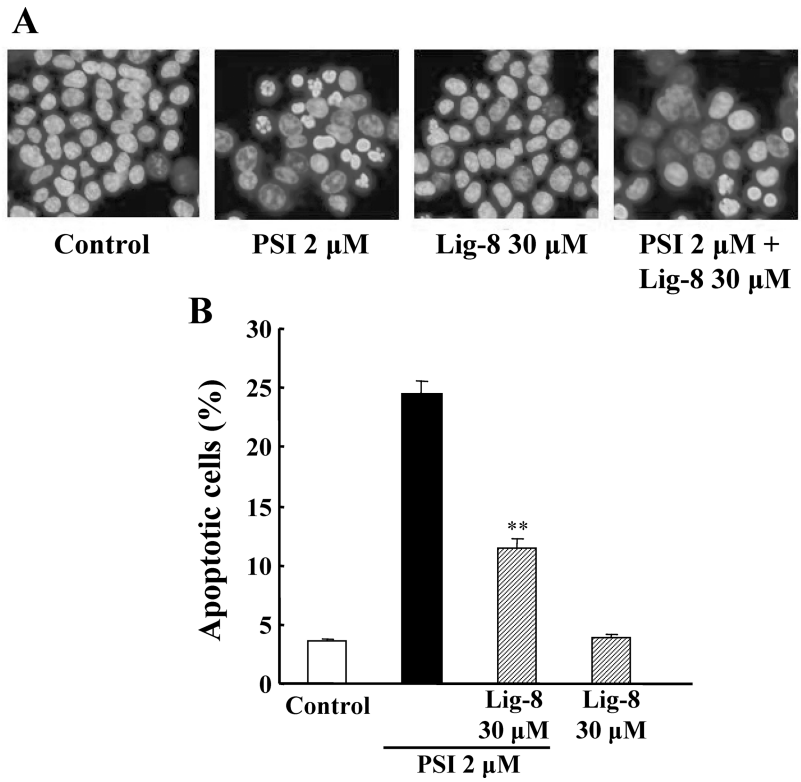

FIG. 7. Effect of lig-8 on proteasome inhibitor (PSI)–induced apoptotic cell death: (A) representative fluorescence microscopy of Hoechst 33342 staining after PSI treatment; (B) apoptotic cell death quantitatively evaluated by Hoechst 33342 staining. Results are the mean ± S.D. of three independent experiments. $^{**}P < 0.01$, PSI + lig-8 treatment vs. PSI treatment alone (Fisher PLSD test). Data from Ito et al. (2006).

that lig-8 intravitreously administered protects from neuronal death. We hope that lig-8 will exert its effects by oral administration as well.

SUMMARY

Using a newly developed phase-separation technique, lignin (a durable aromatic network polymer second to cellulose in abundance) was converted into highly bioactive lignophenol derivatives. Among the compounds examined, lig-8, a lignocresol derivative from bamboo, was found to protect SH-SY5Y cells from H_2O_2-induced apoptosis. Lig-8 also had potent neuroprotective effects against oxidative stress, OGD stress, tunicamycin stress, PSI stress, and retinal damage induced by NMDA. It has been determined that lig-8 exhibited protective effects by preventing activation of caspase-3 via either caspase-8 or caspase-9. Furthermore, lig-8 exerts the antiapoptotic effects by inhibiting dissipation of the mitochondrial membrane permeability transition. The neuroprotective effects of lig-8 may represent an anti–ER-stress mechanism owing to an improvement in proteasome activity that occurs during ER stress (Fig. 10). These findings suggest that lig-8 may be useful in the therapy of neurodegenerative disorders.

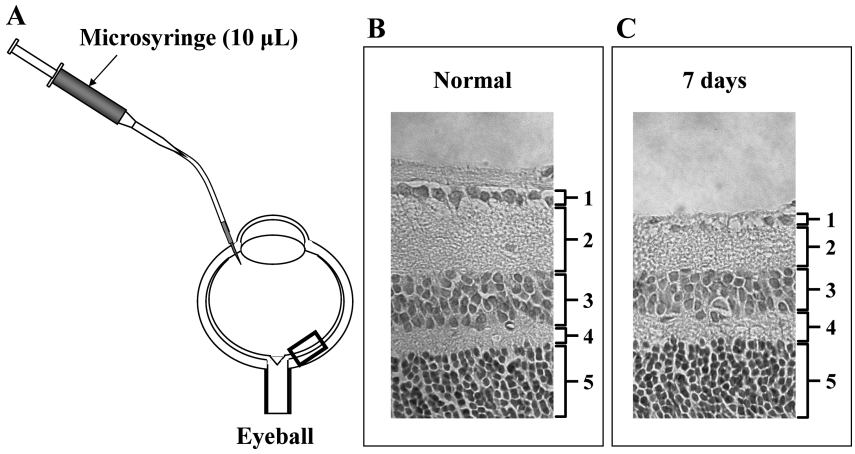

FIG. 8. Illustrations showing (A) model of retinal damage induced by *N*-methyl-D-aspartate (NMDA) injection. NMDA is injected into the vitreous body of the unilateral eye under isoflurane anesthesia; boxed area is shown diagrammatically in (B) and (C). Hematoxylin and eosin staining of sections obtained from control mice and at 7 days after NMDA injection. Representative microphotographs showing nontreated control retina (B) and retina at 7 days after NMDA injection (C). 1: ganglion cell layer (GCL), 2: inner plexiform layer (IPL), 3: inner nuclear layer (INL), 4: outer plexiform layer (OPL), and 5: outer nuclear layer (ONL).

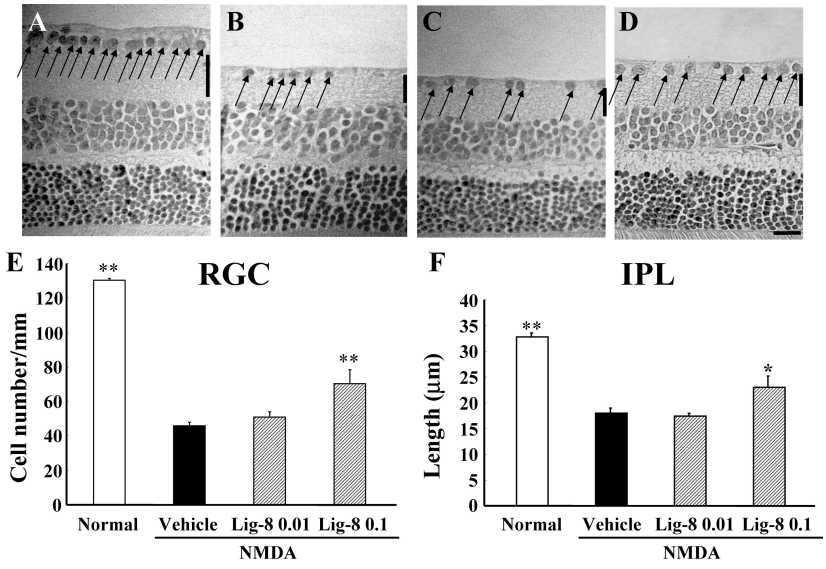

FIG. 9. Effect of lig-8 on *N*-methyl-D-aspartate (NMDA)–induced retinal damage in mice. Hematoxylin and eosin staining of sections obtained from mice at 7 days after the intravitreal injection of NMDA. Representative photographs showing nontreated normal retina (A), NMDA + vehicle–treated retina (B), NMDA + lig-8 (0.01 nmol/eye)–treated retina (C), and NMDA + lig-8 (0.1 nmol/eye)–treated retina (D). Vertical bars show thickness of inner plexiform layer (IPL). Horizontal bar represents 25 μm. Arrows show retina ganglion cells (RGC). Lig-8 protected against NMDA-induced retinal damage in mice. Each eye was enucleated together with its optic nerve at 7 days after treatment with either NMDA (20 nmol/eye) plus vehicle (vehicle-treated group) or NMDA (20 nmol/eye) plus lig-8 (0.01 nmol/eye or 0.1 nmol/eye) (lig-8–treated group). Number of RGCs (E) and thickness of IPL (F) were measured. Results are mean ± S.E.M. for 8–12 eyes. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. NMDA plus vehicle (Dunnett test). Data from Ito et al. (2006).

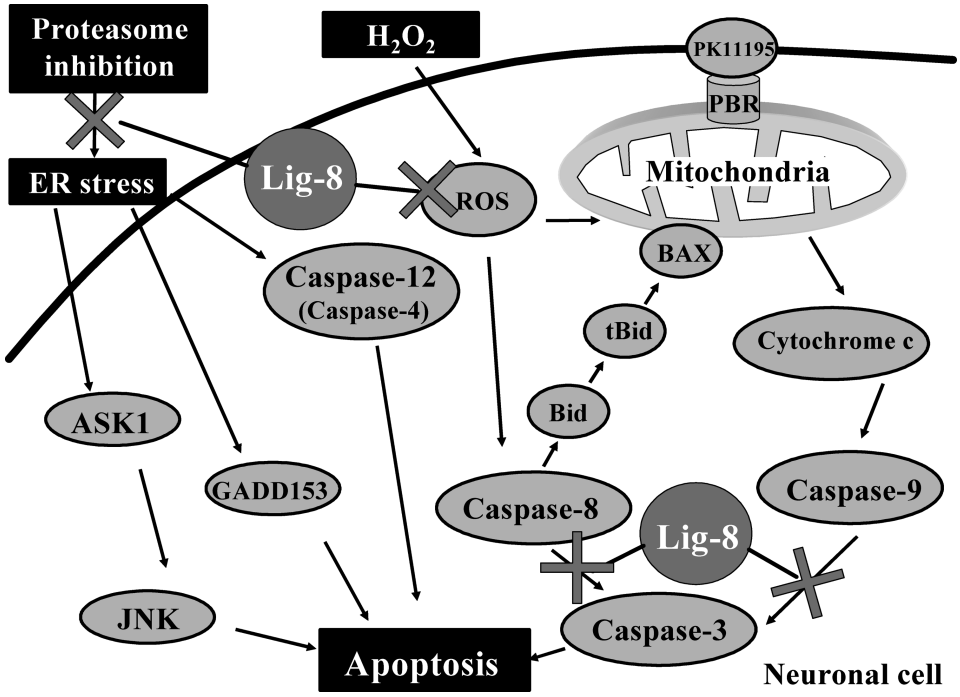

FIG. 10. Protective signal of lig-8 against oxidative stress and ER stress. ×: inhibitiory points of lig-8. Lig-8 exhibited the neuroprotective activity by preventing the caspase-3 activation via either caspase-8 or caspase-9, and by inhibiting a decrease in proteasome activity. Lig-8 was also shown in reactive oxygen species (ROS)–scavenging potency.

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