N-methyl-D-aspartate (NMDA) receptor-mediated excitotoxicity is one of the major causes for neuronal cell death during cerebral ischemic insult. Previously, we reported that the final product of lipid membrane peroxidation 4-hydroxy-2E-nonenal (HNE) synergistically increased NMDA receptor-mediated excitotoxicity (J Neurochem., 2006). In this study, we investigated the mechanism involved in the synergistic neuronal cell death induced by co-treatment with HNE and NMDA. Although neither HNE (1 μM) nor NMDA (2 μM) alone induced the death of cortical neurons, simultaneous treatment of neuronal cells with HNE and NMDA synergistically evoked the death of the cells. However, the synergistic effect on neuronal death was observed only in the presence of calcium. HNE neither increased the cytosolic calcium level ([Ca$^{2+}$]) nor altered the NMDA-induced intracellular calcium influx. However, HNE together with NMDA elevated the mitochondrial calcium level and depolarized the mitochondrial transmembrane potential. Furthermore, HNE evoked damage of isolated mitochondria at the cytosolic calcium level (200 nM), which is maximally induced by 2 μM NMDA. Consistently, ATP was depleted in neurons when treated with both HNE and NMDA together. Ciclopirox, a potent inhibitor of mitochondrial permeability transition pore opening (Br. J. Pharmacol., 2005), largely prevented the synergistic damage of mitochondria and death of cortical neurons. Therefore, although low concentrations of HNE and NMDA cannot individually induce neuronal cell death, they can evoke the neuronal cell death by synergistically accelerating mitochondrial dysfunction.

**Key words:** 4-hydroxy-2(E)-nonenal (HNE), NMDA, neuronal death, calcium, mitochondria
radicals-induced oxidative stress, mitochondrial dysfunction, inflammatory reaction, and others. In general, excess release of glutamate causes over-stimulation of its receptors, including NMDA and AMPA/kainate receptor subtypes, and sustained increase of intracellular calcium level. Intracellular calcium overload in turn induces oxidative stress and damages the cellular membrane and microorganelles including mitochondria [1]. Even though each of these deteriorating events is accepted as one of the key factors for neuronal death, the interplay among the events has also been suggested to be important for neuronal cell death [2].

Mitochondria play a crucial role in the regulation of intracellular calcium concentration ([Ca$^{2+}$]), oxidant level, and ATP synthesis. Thus, mitochondrial damage is recognized as a common neuropathogenesis in numerous brain diseases, including Alzheimer’s disease, Parkinson’s disease, and cerebral ischemia/reperfusion [3,4]. Prolonged oxidative stimuli cause mitochondrial dysfunction, including the decrease of mitochondrial transmembrane potential ($\Delta W_{m}$), overload of mitochondrial calcium, and opening of mitochondrial permeability transition pore (MPTP) [5,6].

Lipid peroxidation by oxidative stresses does much harm to neurons. Lipid peroxidation is an important mode of oxidative damage, particularly in the brain that is enriched with lipid molecules such as polyunsaturated fatty acids. A major deleterious outcome of lipid peroxidation is the generation of reactive aldehydes, and the most cytotoxic product among those is 4-hydroxy-2(E)-nonenal (HNE) [7]. HNE is normally present in human plasma below 0.1 μM, but it can reach up to 1 mM in response to oxidative insults [8]. In brain, accumulated HNE is known to be an important mediator of free radical damage in numerous neurodegenerative diseases [8, 9]. Moreover, HNE accumulation has been reported in the brain challenged by in vivo and in vitro cerebral ischemic-like insults [10, 11]. Thus, certain drugs reducing HNE levels in brain ameliorate the extent of cerebral ischemic injury and improves the neurological functions after ischemic insults [12-14]. Recently, plasma HNE has been recognized as a biomarker for ischemic stroke [15].

HNE has earlier been shown to increase the vulnerability of neurons to glutamate receptor-mediated excitotoxicity [9, 16]. We also reported that HNE induced synergistic death of neurons when treated together with NMDA [11]. However, the exact mechanism for the synergistic cytotoxicity by HNE and NMDA remains largely unclear. In the present study, we demonstrated for the first time that the rise of [Ca$^{2+}$]i by NMDA facilitates HNE-induced mitochondrial calcium overload and dysfunction, and eventually neuronal cell death.

**MATERIALS AND METHODS**

**Primary cortical neuron culture**

Primary cultures of rat cortical neurons were prepared from embryonic 16 days old fetal Sprague-Dawley rats. In brief, brain tissues free of meninges were dissociated by triturating through a Pasteur pipette, and cell suspension (cell number: 5×10^5 cells/ml) was added onto the culture plates pre-coated with poly-D-lysine and laminin. Cells were maintained in Dulbecco’s modified Eagle medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS) in humidified 95% air/5% CO$_2$ at 37°C. Three days later, cytosine arabinoside (5 μM) was added for 1 day to block non-neuronal cell division. Subsequently medium was replaced twice a week. Experiments were performed on 16-18 days cultures.

**Determination of HNE- and/or NMDA-induced cell death**

Cells were exposed to HNE (1 μM) and/or NMDA (2 μM) in nominally Mg$^{2+}$-free Hank’s balanced salt solution (HBSS) containing 1.5 mM CaCl$_2$, 1 mM glycine, and 10 mM glucose. For the calcium-free condition, CaCl$_2$ was not included in the HBSS. Six hours later, cell injury or death was assessed by the morphological examination using a phase-contrast microscopy and quantified by measuring the amount of lactate dehydrogenase (LDH) released into the bathing medium. LDH activity was measured using a diagnostic kit (Sigma Chemical Co., St. Louis, MO). Cell death was expressed as percentage of total LDH, which was measured in sister cultures frozen and thawed after the experiments.

**Measurement of [Ca$^{2+}$]i**

[Ca$^{2+}$], was measured fluorometrically according to the fura-2 method, as we described before (Lim et al., 2006). Cells were cultured on poly-D-lysine and laminin-coated coverslips (diameter=13 mm). They were then washed twice with HBSS, incubated with 1 μM fura-2AM for 30 min at 37°C, and then washed twice with HBSS. A coverslip with cells was diagonally placed inside the cuvette, and the bathing medium was stirred with a teflon-coated magnetic bar to allow adequate mixing of added drugs. The fluorescence was stimulated at 340 and 380 nm of incident light and collected at 510 nm. Constants were determined for each coverslip with 0.1% Triton-X100 for Rmax and 40 mM EGTA for R$_{min}$. The ratios of [Ca$^{2+}$], at 340 an 380 nm were calculated according to the formula: [Ca$^{2+}$]=K$_{d}$(R×R$_{min}$)/(R$_{max}$- R)×S$_{0}$/S$_{d}$, where R is the 340/380 wavelength ratio of the sample, R$_{min}$ is the ratio obtained with fura-2 in the absence of Ca$^{2+}$, and S$_{0}$/S$_{d}$ is the ratio of fluorescence values at 380 nm for free and Ca$^{2+}$-bound dye. A K$_{d}$ value of 224 nM was used in this study.

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Measurement of mitochondrial transmembrane potential ($\Delta \Psi_m$) in cells

Cells were loaded with JC-1 (1.0 μg/ml; Molecular Probes, Eugene, OR) for 20 min at 37°C. Depolarization of $\Delta \Psi_m$ was assessed by measuring fluorescence intensities at the excitation wavelength of 485 nm and dual emission wavelengths of 530 and 590 nm (for measuring JC-1 monomers and aggregates, respectively) using a fluorescence microplate reader (SpectraMax GeminiXS, Molecular Devices, CA). During measurements, cells were maintained at 37°C and protected from light. Fluorescence intensity was measured every 15 or 30 min. All fluorescent measurements were corrected by autofluorescence that was determined in cells not loaded with JC-1. Autofluorescence was constant throughout the experiment. In control experiments, no photobleaching was observed during fluorescence monitoring.

Measurement of mitochondrial calcium concentration ([Ca$^{2+}$]$_m$) in cells

In order to measure [Ca$^{2+}$]$_m$, cultured cortical neurons were incubated with the mitochondrial Ca$^{2+}$-sensitive fluorescent indicator, rhod-2 AM (5 μM, Molecular Probes), for 30 min at room temperature. Rhod-2 fluorescence was excited at 560 nm and emitted fluorescence was collected through a 590 nm long pass barrier filter using a fluorescence microplate reader (SpectraMax GeminiXS, Molecular Devices, CA). The rhod-2 fluorescence intensities were expressed as $F/F_0$ ($F_0$: fluorescence before drug addition, F: maximal fluorescence after drug addition).

Isolation of liver mitochondria

Mitochondria were isolated from livers of male Sprague-Dawley rats (220-280 g) (Charles River Breeding Lab), as we described before [17]. Rats were starved overnight before sacrifice. All steps of mitochondria isolation were carried out on ice and in a cold medium containing 0.25 M sucrose, 2 mM K$^+$-EDTA, and 3 mM HEPES, adjusted to pH 7.4 with KOH. Rat liver was washed, minced, and homogenized on ice using a ground-glass homogenizer. The homogenate was subjected to centrifugation at 600 g for 10 min, and the supernatant obtained to 8,000 g for 20 min. The subsequent mitochondrial pellet was washed in EDTA-free medium and used for experiments. Mitochondrial protein concentration was determined by the Bradford method using bovine serum albumin as the standard.

Measurement of $\Delta \Psi_m$ in isolated mitochondria

The $\Delta \Psi_m$ in isolated mitochondria was measured using JC-1 [17] or rhodamin 123 [18]. Rhodamine 123 has spectral and metabolic properties as a probe of transmembrane potential in isolated rat-liver mitochondria. Thus, mitochondria (0.5 mg protein) were suspended with 2 ml of recording buffer containing 150 mM sucrose, 5 mM MgCl$_2$, 5 mM succinate, 2.7 μM rotenone, 200 mM Ca$^{2+}$, 50 mM potassium phosphate, and 20 mM HEPES (pH 7.4). Before each experiment, mitochondria in the recording medium were incubated with each dye [0.2 μM rhodamine 123 (Molecular Probes) or JC-1 (1.0 μg/ml)], for 20 min in the dark in a 3 ml recording cuvette at 35°C. The emission signals at 590 and 530 nm elicited by the excitation at 485 nm for JC-1 and the emission signals at 529 nm elicited by excitation at 507 nm for rhodamin 123 were measured with a spectrofluorophotometer RF-5301PC (Shimadzu, Kyoto, Japan). The ratio of the signal at 590 nm over that at 530 nm (red/green ratio) of JC-1 or fluorescence intensity of rhodamin 123 was calculated to estimate $\Delta \Psi_m$. The recording chamber was magnetically stirred, and the measurements were carried out at 35°C.

Measurement of MPTP opening in isolated mitochondria

MPTP opening was assessed by measuring mitochondrial swelling under energized and de-energized conditions, as described previously [19]. Mitochondrial swelling was determined spectrophotometrically (Shimadzu UV-2401PC, Kyoto, Japan) by measuring absorbance changes at 540 nm. Mitochondria (1 mg protein) were incubated at 25°C under energized conditions in 2 ml of medium containing 13 mM mannitol, 70 mM sucrose, 3 mM HEPES (pH 7.4), 10 mM succinate, and 1 μM rotenone. When required, de-energized condition was achieved by incubating mitochondria in KSCN buffer (150 mM KSCN, 20 mM MOPS, 10 mM Tris, 0.5 μM rotenone, and 0.5 mM antimycin, pH 7.4).

Measurement of intracellular ATP levels

The level of intracellular ATP was measured using the method described previously (Choi et al., 2002). Briefly, cells were lysed with 10% trichloroacetic acid, sonicated for 1 min on ice, and 2 mM EDTA and 2 mg/ml bovine serum albumin (BSA) were added with. After centrifugation, the supernatant was collected and neutralized with 4 N to the lysate. The ATP content was determined using a luminescence detection kit (Molecular Probes, Eugene, OR).

Statistical analysis

Data are expressed as mean ± standard error of mean (S.E.M.) and analysed for statistical significance using repeated measures of ANOVA or two-way ANOVA by running SAS Window v.9.1 program. If needed, post-hoc Scheffe’s test was performed for multiple comparisons. A p value <0.05 was considered significant.
Table 1.

| Antagonists** | WAY-100635 | SB-224289, GR-127935 | Ketanserin, M100907 | Mesulergine, SB-200907 | Ondansetron, Tropisetron | GR-113808, SB-204070 | − SB-399885 | Amisulpiride, SB-269970 |
|----------------|-------------|----------------------|---------------------|------------------------|------------------------|------------------------|----------------|------------------------|

RESULTS

Neuronal cell death and [Ca\(^{2+}\)]\(_i\) by co-treatment with HNE and NMDA

In the present study, neither HNE (1 μM) nor NMDA (2 μM) alone injured neuronal cells (Fig. 1A). However, combined treatment of the cells with HNE and NMDA evoked the release of LDH (Fig. 1A) with apparent morphological deterioration (data not shown). Furthermore, it is of a great interest to note that the synergistic death was observed only in the presence of calcium (Fig. 1). HNE alone neither increased the cytosolic calcium level ([Ca\(^{2+}\)]\(_i\)) nor altered the NMDA-induced intracellular calcium influx (Fig. 2), which is consistent with our previous observation (Lim et al., 2006). Therefore, our previous and present results together suggest that NMDA-mediated [Ca\(^{2+}\)]\(_i\) rise interplays with HNE to augment the neuronal cell death.

Enhanced ∆Ψ\(_m\) depolarization and [Ca\(^{2+}\)]\(_m\) by co-treatment with HNE and NMDA

Mitochondrial dysfunction is one of the prominent features of oxidative stress-mediated cell death. Oxidative stress, including HNE, has been shown to depolarize the ∆Ψ\(_m\) and facilitate Ca\(^{2+}\)-evoked MPTP opening [20]. Similarly, NMDA receptor activation is also known to induce mitochondrial dysfunction [21]. As shown in Fig. 3A, however, neither 1 μM HNE nor 2 μM NMDA altered the ∆Ψ\(_m\) in neuronal cells (Fig. 3A). On the other hand, when the cells were incubated for 3 h with 1 μM HNE together with 2 μM

Fig. 1. Combined application of HNE and NMDA induces the synergistic death of cortical neurons. Cortical neurons were exposed to NMDA (2 μM), MK-801 (2 μM) and/or HNE (1 μM) in nominally Mg\(^{2+}\)-free Hank's balanced salt solution (HBSS) with (A) or without (B) 1.5 mM CaCl\(_2\). Supernatants were taken at 3 h for LDH assay. Data represent mean±S.E.M of five independent experiments. with control, ***p<0.001, compared with HNE plus NMDA-treated group.

Fig. 2. HNE does not affect the [Ca\(^{2+}\)]\(_i\) rise induced by NMDA in cortical neurons. Cells were loaded with 1 μM fura-2AM for 30 min at 37°C and they were then treated with 2 μM NMDA for 3 min with or without prior treatment with 1 μM HNE for 5 min. The fluorescence intensity was continuously measured during the treatment. Each bar indicates the peak [Ca\(^{2+}\)]\(_i\) evoked by each treatment. Data represent mean±S.E.M of three independent experiments.

Fig. 3. Combined application of HNE and NMDA induces ∆Ψ\(_m\) decrease and mitochondrial calcium accumulation. To measure ∆Ψ\(_m\), cells were loaded with JC-1 (1 μg/ml) for 20 min and then exposed to NMDA (2 μM) and/or HNE (1 μM) in nominally Mg\(^{2+}\)-free HBSS with (A) or without (B) 1.5 mM CaCl\(_2\). At indicated time points, the fluorescence intensities of JC-1 monomer (green) and aggregates (red) were measured and the ratio of those intensities was then calculated. (C) To measure [Ca\(^{2+}\)]\(_m\), cells were loaded with rhod-2 AM (5 μM) for 30 min and then exposed to NMDA (2 μM) and/or HNE (1 μM) in nominally Mg\(^{2+}\)-free HBSS with 1.5 mM CaCl\(_2\). At indicated time points, the fluorescence intensity of rhod-2 was measured. Data represent mean±S.E.M of five independent experiments.
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In isolated mitochondria, there was complete depolarization of $\Delta \Psi_m$ (Fig. 3A), which was comparable to the extent induced by 5 μM FCCP, a potent mitochondrial uncoupler (data not shown). Interestingly, the potentiated depolarization of $\Delta \Psi_m$ by HNE and NMDA occurred only in the presence of extracellular calcium (Fig. 3A, B). Accumulation of calcium in mitochondria is well associated with the depolarization and dysfunction of mitochondria. Our results clearly showed that co-treatment with HNE and NMDA increased $[Ca^{2+}]_m$ in cortical neurons (Fig. 3C). However, neither 1 μM HNE nor 2 μM NMDA alone showed any significant changes in $[Ca^{2+}]_m$ (Fig. 3C). The results imply that the synergistic cytotoxicity by HNE and NMDA may be related to the rise of $[Ca^{2+}]_m$ and mitochondrial dysfunction.

**Calcium-dependent effect of HNE on $\Delta \Psi_m$ decrease and MPTP opening**

Our previous and present data showed that 2 μM NMDA elevated the $[Ca^{2+}]_i$, up to 200 nM. Therefore, we tested in isolated mitochondria whether HNE (1 μM) could induce $\Delta \Psi_m$ depolarization and MPTP opening in the presence of 200 nM calcium. Neither 1 μM HNE nor 2 μM NMDA alone affected $\Delta \Psi_m$ in isolated mitochondria (data not shown). However, 1 μM HNE significantly depolarized $\Delta \Psi_m$ in the presence of 200 nM calcium in the test buffer (Fig. 4). Mitochondrial swelling experiments also showed that 1 μM HNE strongly promoted the MPTP opening induced by 200 nM CaCl$_2$ in both energized (Fig. 5A) and de-energized (Fig. 5B) conditions. These findings indicate that HNE facilitates the calcium-induced opening of MPTP. The HNE/Ca$^{2+}$-facilitated MPTP opening shown in Fig. 4 was completely blocked by two different MPTP blockers, cyclosporine A and ciclopirox (data not shown).

**Ciclopirox blocks HNE plus NMDA-induced ATP depletion and neuronal death**

We previously reported that ciclopirox protected adenocarcinoma cells from H$_2$O$_2$ toxicity via complete prevention of $\Delta \Psi_m$ decrease and MPTP opening [17]. Unlike cell membrane-impermeable cyclosporine A or bonkrekic acid, the cell membrane-permeable MPTP blocker ciclopirox rapidly and markedly reduced mitochondrial damage induced by H$_2$O$_2$ in cultured cells. In the present study, therefore, we further examined whether ciclopirox blocked the synergistic toxicity of HNE and NMDA on mitochondria, and found that combined treatment of HNE and NMDA strongly increased $[Ca^{2+}]_m$ (Fig. 6A) and synergistically decreased the intracellular ATP level (Fig. 6B). However, ciclopirox completely blocked the mitochondrial calcium overload, restored intracellular ATP levels and ameliorated the neuronal cell death caused by HNE and NMDA (Fig. 6).

**DISCUSSION**

Microdialysis study showed that the level of glutamate in cerebral ischemic lesion reached over 70 μM, whereas HNE is normally present in human plasma below 0.1 μM, but it can reach up to 1 mM in response to oxidative insults [8]. Therefore, the concentrations of HNE (1 μM) and NMDA (2 μM) used in this study should be pathologically relevant. At the concentrations used in this study, treatment with HNE or NMDA alone did not evoke neuronal cell injury/death, however, combined treatment with HNE and NMDA together markedly increased the neuronal cell...
Synergistic Toxicity of HNE and NMDA
dearth, implying that concerted play of NMDA and HNE could be responsible for the massive brain damage during the early period of ischemic insult.

One possible mechanism for neurotoxicity of HNE has been suggested as the consequence of intracellular calcium overload [9, 20, 21]. This possibility is supported by the previous findings that HNE induces the impairment of ion transporters, voltage-dependent Ca$^{2+}$ channels and glucose or glutamate transporters, and the phosphorylation of NMDA receptor subunit, resulting in intracellular calcium overload [20, 21]. However, the intracellular calcium overload was observed only at concentrations of HNE higher than that used in the present study: HNE significantly increased the [Ca$^{2+}$], at >10-fold higher concentrations in PC12 cells, hippocampal neurons, and spinal cord neurons [9, 20]. Furthermore, the [Ca$^{2+}$], rise by HNE may be dependent on the cell type. For example, HNE at very high concentrations (25 or 440 μM) failed to increase [Ca$^{2+}$], in human platelets [22], whereas 1 μM HNE increased [Ca$^{2+}$], in hepatocytes [23]. In our present and previous studies [11], 1 μM HNE did not induce any changes in [Ca$^{2+}$], in control or NMDA-treated neurons.

Even though NMDA-evoked [Ca$^{2+}$], rise was not changed by HNE, our present results showed that the [Ca$^{2+}$], enhanced by NMDA plays a crucial role in the neuronal cell death in the presence of HNE, because the synergistic cell death by cotreatment of HNE and NMDA was completely attenuated by a specific NMDA receptor antagonist MK-801 [11], or by removing extracellular calcium (the present study). A question arises, therefore, how HNE potentiates NMDA-induced neuronal death with no alteration of intracellular calcium level. One of key mechanisms for HNE cytotoxicity is mitochondrial failure via mitochondrial calcium overload [24, 25], and NMDA neurotoxicity has also been suggested to be caused by the mitochondrial calcium accumulation [26, 27]. At concentrations used in this study, however, neither HNE nor NMDA alone altered the [Ca$^{2+}$]m (the present results), but combined together, they evoked Ca$^{2+}$ accumulation in mitochondria. Previously, HNE has been reported to induce the opening of MPTP in isolated fetal brain mitochondria [28]. HNE enhances calcium-mediated MPTP opening via permeabilizing adenine nucleotide translocator that is a component of MPTP and has been suggested as the only target of MPTP and has been suggested as the only target of HNE [29, 30]. Furthermore, HNE inhibits efflux of Ca$^{2+}$ from mitochondria by inhibiting pyridine nucleotide hydrolysis [31]. In the present study, we found that 2 μM HNE evoked MPTP opening in isolated mitochondria at very low calcium level (200 nM) induced by 1 μM NMDA. This synergistic calcium accumulation in mitochondria and opening of MPTP may result in the failure of mitochondrial ATP generation, as shown in Fig. 6B of this study.

Much effort has been paid to develop drugs for the treatment of oxidative stress-mediated neurodegenerative diseases. Mitochondrial damage is a common phenomenon in the pathogenesis of numerous brain diseases, including Alzheimer’s disease, Parkinson’s disease, and cerebral ischemic stroke [3, 4, 32]. We previously demonstrated that ciclopirox markedly reduced peroxynitrite- or hydrogen peroxide-induced mitochondrial damage and cell death by inhibiting ∆Ψm decrease and MPTP opening [17, 33]. In the present study, we also observed that ciclopirox blocked the HNE plus NMDA-induced toxic effects on mitochondria. Therefore, further studies are needed in view of medicinal chemistry to develop new therapeutic drugs for

![Fig. 6. Ciclopirox (CPX) reduces the cytotoxicity enhanced by HNE and NMDA. (A) Mitochondrial Ca$^{2+}$ overload. Cells were loaded with rhod 2 AM (5 μM) for 30 min and then exposed to NMDA (2 μM) and/or HNE (1 μM). For evaluation of the effect of CPX, cells were treated with CPX 30 min before and throughout NMDA and HNE treatment. Two hours later, the fluorescence intensity of rhod-2 was measured. (B) ATP content. Cells were exposed to each condition as described in Fig. 1A. Three hours later, they were lysed, and intracellular ATP content was then measured. (C) LDH release. Cells were exposed to HNE plus NMDA for 6 h in the presence of various concentrations of CPX. Cell death was then assessed by measuring LDH activity. Data represent mean±S.E.M of five independent experiments. ’p<0.05, ’’p<0.01, ’’’p<0.001: compared with control. ”p<0.01, ###p<0.001, compared with HNE plus NMDA-treated group.
protection of mitochondrial damage and resulting cell death.

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