Identification of a lipid peroxidation product as a potential trigger of the p53 pathway

Takahiro Shibata1, Kumiko Iio1, Yoshichika Kawai2, Noriyuki Shibata3, Motoko Kawaguchi4, Sono Toi5, Makio Kobayashi3, Masahiko Kobayashi6, Kenichi Yamamoto6, and Koji Uchida1,7

1Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601, Japan, 2Graduate School of Nutrition and Biosciences, The University of Tokushima, Tokushima 770-8503, Tokushima, Japan, Departments of 3Pathology, 4Surgical Pathology, and 5Neurology, Neurological Institute, Tokyo Women's Medical University, Tokyo 162-8666, Japan, and 6Cancer Research Institute, Kanazawa University, Kanazawa 920-0934, Japan

Running title: A lipid peroxidation product that trigger the p53 pathway

The tumor suppressor and transcription factor p53 is a key modulator of cellular stress responses, and activation of p53 can trigger apoptosis in many cell types including neurons. We found that this nuclear protein was significantly phosphorylated when human neuroblastoma SH-SY5Y cells were exposed to in vitro oxidized polyunsaturated fatty acids. To identify an oxidized lipid that induces p53 phosphorylation, we conducted a screening of lipid peroxidation products in human neuroblastoma SH-SY5Y cells and identified 4-oxo-2-nonenal (ONE), a recently identified aldehyde originating from the peroxidation of ω6 polyunsaturated fatty acids, as a potential inducer of the p53 phosphorylation. We also found that ONE induced the phosphorylation of ataxia telangiectasia-mutated (ATM), which plays an essential role in transmitting DNA damage signals by the phosphorylation of p53. In addition, exposure of the cells to ONE resulted in an accumulation of ubiquitinated proteins and in a significant inhibition of proteasome activities, suggesting that ONE acted on the ubiquitin-proteasome pathway, a regulatory mechanism of p53 turnover. These and the observation that the ONE-induced p53 response was associated with the induction of apoptosis suggested that ONE activated the p53-dependent apoptosis mechanism via activation of the p53 signaling pathway and down-regulation of the p53 turnover. Finally, we observed that the ONE-2'-deoxyguanosine adduct, 7-(2-oxo-heptyl)-substituted 1,Λ2-etheno-2'-deoxyguanosine, was accumulated in the spinal cord motor neurons of patients with sporadic amyotrophic lateral sclerosis. These data may suggest the potential critical role for ONE in the induction of a neuronal apoptosis program during oxidative processes.

Lipid peroxidation in tissue and in tissue fractions represents a degradative process, which is the consequence of the production and the propagation of free radical reactions primarily involving membrane polyunsaturated fatty acids (PUFAs), and has been implicated in the pathogenesis of numerous diseases including atherosclerosis, diabetes, cancer, and rheumatoid arthritis, as well as in drug-associated toxicity, postischemic reoxygenation injury, and aging (1). The peroxidative breakdown of polyunsaturated fatty acids has also been implicated in the pathogenesis of many types of liver injury and especially in the hepatic damage induced by several toxic substances. There is increasing evidence that aldehydes endogenously generated during the process of lipid peroxidation are causally involved in most of the pathophysiological effects associated with oxidative stress in cells and tissues (2). Compared to free radicals, lipid peroxidation-derived aldehydes are generally stable and can diffuse within or even escape from the cell and attack targets far from the site of the original free radical-initiated event, therefore, suggesting that they are...
not only end products and the remnants of lipid peroxidation processes, but also may act as mediators for the primary free radicals that initiated lipid peroxidation.

Many types of mammalian cells undergo apoptosis during normal development or in response to a variety of stimuli, including DNA damage, growth factor deprivation, and the abnormal expression of oncogenes or tumor suppressor genes (3-5). Apoptosis induced by these agents appears to be mediated by a common set of downstream elements that act as regulators and effectors of apoptotic cell death. In many cases, apoptosis requires the p53 tumor suppressor protein (6). p53 encodes a transcription factor (7, 8), which is activated following DNA damage (9) and may play a role in apoptosis (10, 11). The induction of p53 mRNA has been associated with neuronal damage following excitotoxic or ischemic brain injuries (12). Mice deficient in p53 were reported to be resistant to stroke-induced neuronal damage (13), and kainate-induced hippocampal cell death (14). In addition to its role in apoptotic cell death, p53 may also participate in regulating the cell cycle (15).

p53 is the most frequently altered tumor suppressor gene in a wide spectrum of human cancer (16) with a mutation frequency of up to 50% (17). In contrast to many cancers (18), p53 is not mutated in human neuroblastoma, thus, several neuroblastoma cell lines, commonly used as a model to study the differentiation and the development of neural cells in vitro (19), can also be used in the study of the modulation of p53 family members after treatment with cytotoxic or cytostatic agents. In the present study, based on an observation that the in vitro oxidized polyunsaturated fatty acids significantly induced phosphorylation of p53 in human neuroblastoma SH-SY5Y cells, we evaluated the effect of oxidized fatty acid metabolites on p53 phosphorylation and identified 4-oxo-2-nonenal (ONE), one of the major lipid peroxidation products, as an inducer of p53 phosphorylation in neuronal cells. To fully understand the signaling mechanism that triggers this response, the cellular events that may lead to the activation of p53 were investigated. Moreover, we investigated the presence of this aldehyde in the tissue from patients with sporadic amyotrophic lateral sclerosis. This study demonstrates that the ONE is a potential inducer of the p53 pathway that involves ataxia telangiectasia-mutated (ATM) and proteasome. The identification of ONE as a trigger of p53 signaling may provide new clues to the apoptotic mechanism in neurodegenerative diseases.

**EXPERIMENTAL PROCEDURES**

**Material.** 4-Oxo-2-nonenal (ONE) was synthesized by the oxidation of 4-hydroxy-2-nonenal (HNE) dimethyl acetal with pyridinium dichlorochromate followed by HCl hydrolysis. Other aldehydes were purchased from the Cayman Chemical Co. (Ann Arbor, MI). The antibodies against p53 and ubiquitin were obtained from the Santa Cruz Biotechnology Co. (Santa Cruz, CA). The antibodies against phosphorylated p53 at Ser-15 and poly(ADP-ribose) polymerase (PARP) were purchased from the Cell Signalling Technology, Inc. (Beverly, MA). The anti-ATM monoclonal antibody and anti-phospho-ATM (Ser-1981) monoclonal antibody were obtained from the Upstate Biotechnology Co. (Charlottesville, VA) and the Rockland Immunochemicals Co. (Philadelphia, PA), respectively. The monoclonal antibody (mAb 6A3) against the ONE-2'-deoxyguanosine adduct, 7-(2-oxo-heptyl)-substituted 1,N2-etheno-2'-deoxyguanosine, was used for immunohistochemical detection of the ONE-modified DNA (20). 13S-Hydroperoxy-9Z,11E-octadecadienoic acid (13-HPODE) was enzymatically synthesized from linoleate by soybean lipoxygenase as described previously (20).

**Cell culture and cell viability.** Human neuroblastoma SH-SY5Y cells were grown in Cosmedium-001 (Cosmo-Bio, Tokyo, Japan) containing 5% FBS. The cells were seeded in plates coated with polylysine and cultured at 37 °C.

**Cell viability.** Cell viability was quantified by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, cells incubated with ONE were treated with 10 μl of MTT solution (5 mg/ml) for 4 h. The cells were then lysed with 0.04 N HCl in isopropanol, and the absorbance was read at 570 nm.
Autoxidation of polyunsaturated fatty acids (PUFAs). The autoxidation of the PUFAs, such as linoleate and arachidonate, was performed by incubating the emulsified PUFA (200 μg/ml) with Fe²⁺ (40 μM) and ascorbate (1 mM) in 30 mM sodium phosphate buffer (pH 7.4) at 37 °C.

Lipid peroxidation assay. The degree of lipid peroxidation of the PUFAs was determined by measurement of the 2-thiobarbituric acid-reactive substances (TBARS). The reaction mixture (10 μl) was treated with 20 μl of 8.1% (w/v) SDS and 150 μl of 0.08% 2-thiobarbituric acid and 70 μl of H₂O and then boiled for 60 min. After cooling, the sample was centrifuged (11,000 x g, 3 min), and the absorbance of the supernatant solution was measured at 532 nm. Malondialdehyde bis(dimethylacetal), which yields malondialdehyde (MDA) by acid treatment, was used as the standard.

Formation of ONE from 13S-hydroperoxy-9Z,11E-octadecadienoic acid (13-HPODE). 13-HPODE (5 mM) was incubated in 50 mM sodium phosphate buffer (pH 7.4) at 37 °C. ONE generated during the autoxidation of 13-HPODE was derivatized with a semicarbazide as previously described (21). The semicarbazone derivative of ONE was extracted with chloroform, evaporated, and dissolved in ethanol. Ten microliters of the aliquot was injected and analyzed on a Develosil ODS-HG-5 column (Nomura Chemicals, Aichi, Japan; 4.6 x 250 mm) with a stepwise gradient of water/acetic acid (100/0.01, by vol.) (solvent A) - acetonitrile (solvent B) (time = 0-5 min (A100%); 5-50min (→B100%); 50-55min (B100%); 60min (→ A100%) at a flow rate of 0.8 ml/min. The elution profiles were monitored by absorbance at 300 nm.

Nuclear staining assay. To determine the apoptotic nuclei, cells treated with 10 μM ONE for 4 h were fixed in 4% paraformaldehyde solution and stained with a fluorescent DNA-binding dye, Hoechst 33258, and observed using a fluorescence microscope (Olympus Optical Co., Ltd., Tokyo).

Analysis of DNA fragmentation. For analysis of the DNA fragmentation by agarose gel electrophoresis, cellular DNA was extracted from whole cells treated with 10 μM ONE for 24 h. After treatment with ONE, the cells were harvested, washed once with ice-cold PBS, and resuspended in 100 μl of lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM EDTA, 0.5% Triton X-100), and the samples then left on ice for 10 min. After centrifugation at 13,000 rpm for 5 min, the supernatant was transferred to another Eppendorf tube, and sequential extractions were carried out. RNase was added to the samples at the final concentration of 0.5 mg/ml, and the mixture was incubated at 37 °C for 1 hr, then proteinase K was added to the mixture at the final concentration of 0.5 mg/ml, and the mixture was incubated at 50°C for 30 min. Electrophoresis was performed on a 2.0% agarose gel, and DNA was visualized by ethidium bromide staining.

Western blot analysis. The homogenates prepared from the cells were treated with SDS-sample buffer, then immediately boiled for 5 minutes. The protein concentrations were determined using the BCA protein assay kit (Pierce, Rockford, IL). The proteins were separated by SDS-polyacrylamide gel electrophoresis in the presence of 2-mercaptoethanol and electro-transferred onto a nitrocellulose membrane (Hybond ECL) (Amersham Biosciences, Piscataway, NJ). To detect the immunoreactive proteins, we used horseradish peroxidase-conjugated anti-rabbit or mouse or goat IgG and ECL blotting reagents (Amersham Biosciences).

RT-PCR. Total RNA was isolated from the cells using TRIZOL reagent (Invitrogen Co., Carlsbad, CA) according to the manufacturer's protocol and spectrophotometrically quantified. The total RNAs (5 μg) were reverse transcribed into cDNA and used for the RT-PCR analysis. GAPDH was used as an internal standard. The PCR products were separated in a 1% agarose gel, and the positive signals were quantified by densitometry analysis after staining with ethidium bromide. The following primer pairs (Genset) were used:

GADD45: F: 5’-TGCCATTATCTCAAGGTGA-3’
R: 5’-GAAGCTGCTTATTCGCAGTA-3’
Fas: F: 5’-TTGCTGGAGTCATGACACTAAGTC-3’
R: 5’-AACATCCTTGGAGGCAGAATCA-3’
MDM2:F: 5’-GGATCTTGATGCTGGTGTAAG-3’
R: 5’-GCTACTAGAAGTTGATGGCTG-3’
Bax: F: 5’-GGTTTCATCCAGGATCGAGC-3’
R: 5’-GAGGAAGTCCAATGTCCAGC-3’
Bcl-2: F: 5’-AACTCCCAATACYGGCTC-3’
R: 5’-GCCACACAGCCAACGTGCAT-3’

Localization of p53. For the immunocytochemistry, cells were fixed overnight in PBS containing 2% paraformaldehyde and 0.2% picric acid at 4 °C. After the membranes were permeabilized by exposing the fixed cells to PBS containing 0.3% Triton X-100, the cells were sequentially incubated in PBS solutions containing blocking serum (5% normal goat serum) and immunostained with the anti-p53 antibody. The cells were then incubated for 1 h in the presence of fluorescein isothiocyanate-labeled goat anti-rabbit, rinsed with PBS containing 0.3% Triton X-100, and covered with anti-fade solution (1 mg/ml p-phenylenediamine, 90% glycerol). Images of the cellular immunofluorescence were acquired using a confocal laser microscope (Zeiss) with a 40x objective (488-nm excitation and 518-nm emission).

Proteasome activity. The proteasome activity was also evaluated using SH-SY5Y cells stably transfected with the Proteasome Sensor Vector™ (BD Biosciences Clontech, Palo Alto, CA). The ZsProSensor™ protein accumulated through the inhibition of the proteasome, resulting in a strong green emission signal, was measured by a flow cytometer (Epics XL, Beckman coulter).

RNA interference using siRNA. The siRNA for human ATM was generated by Santa Cruz Biotechnology. RL34 cells were seeded at 80% density the day before transfection. The cells were transfected with Lipofectamine2000™ transfection reagent; 2.5 μl of siRNA stock (20 μM) and 5 μl of Lipofectamine2000™ were each diluted with 250 μl of Opti-MEM (Invitrogen). After 5 min at room temperature, they were combined and incubated for 20 min. The reaction mixtures were overlaid on the cell culture for 6 h. The medium was then changed to a fresh one containing 5% FBS.

Immunohistochemical detection of ONE-2'-deoxyguanosine adduct in sporadic ALS patients. The presence of the ONE-2'-deoxyguanosine adduct in vivo on spinal cords obtained at autopsy from five sporadic ALS patients [age: 52-79 (61.40 ± 8.81) yr; sex: four males and six females] and five age-matched control individuals [sex: six males and four females; age: 52-75 (62.40 ± 7.62) years] without neurological disorders, diabetes mellitus, or hyperlipidemia was examined. Each autopsy was performed after the family members granted informed consent in accordance with the Ethical Guideline of Tokyo Women’s Medical University and the Helsinki Declaration of 1983. The spinal cord specimens were processed for preparing formalin (10%-)-fixed, paraffin-embedded materials stored at room temperature and OCT (Sakura Fine Technical, Tokyo, Japan) compound-embedded, frozen materials stored at –80°C.

Multiple 6-μm-thick sections were cut out from each spinal cord, and used for hematoxylin-eosin staining and immunohistochemical staining. The paraffin-embedded sections were deparaffinized, and some of the frozen sections were postfixed in 100% acetone. These sections were rehydrated, quenched for 10 min at 4°C with 3% hydrogen peroxide for inhibiting endogenous peroxidase activity, rinsed in PBS containing 0.3% Triton X-100, and covered with anti-fade solution (1 mg/ml p-phenylenediamine, 90% glycerol). Images of the cellular immunofluorescence were acquired using a confocal laser microscope (Zeiss) with a 40x objective (488-nm excitation and 518-nm emission).

RESULTS
Induction of p53 phosphorylation by oxidized PUFAs. To investigate whether lipid peroxidation is associated with the activation of p53, we evaluated the effect of the oxidized lipids on p53 phosphorylation in human neuroblastoma SH-SY5Y cells. To this end, the cells were exposed to the oxidized lipids prepared upon incubation of the PUFAs (linoleate and arachidonate) with 40 \( \mu \text{M} \) Fe\(^{2+} \) and 1 mM ascorbate in 30 mM sodium phosphate buffer (pH 7.4). As shown in Fig. 1A, the autoxidation of linoleate with an Fe\(^{2+} \)/ascorbate-mediated free radical generating system was associated with the formation of TBARS. In parallel with the increase in the TBARS values, the p53 phosphorylation was significantly induced in the cells exposed to the oxidized linoleate. Arachidonate was more rapidly oxidized with the Fe\(^{2+} \)/ascorbate compared to linoleate, and the p53 phosphorylation reached a plateau within 1 h before the TBARS values reached a maximum (Fig. 1B). These data indicate that the oxidized PUFAs represent the sources of a p53 phosphorylation inducer.

Identification of ONE as a selective inducer of p53 phosphorylation. In view of the fact that the generation of lipid hydroperoxides and reactive aldehydes is a phenomenon common in lipid peroxidation, we hypothesized that some of these reactive species might be involved in the p53 phosphorylation. To test this hypothesis, the effect of authentic lipid hydroperoxides and reactive aldehydes on the p53 phosphorylation in SH-SY5Y cells was studied. Remarkably, among the tested oxidized lipids, the p53 phosphorylation was induced only by ONE (Fig. 2). The hydroxy and hydroperoxy fatty acids and other short-chain aldehydes had no significant effects on the p53 phosphorylation. Of interest, HNE, an analog of ONE, was ineffective in the concentration range up to 20 \( \mu \text{M} \) (Fig. 3). These results suggest that the oxidized PUFAs represent the sources of a p53 phosphorylation inducer.

ONE is a lipid peroxidation product, which has been recently established to be formed by the free radical-initiated degradation of \( \omega \)-polyunsaturated fatty acids (22, 23). To evaluate the effectiveness of lipid peroxidation as the origin of ONE, we examined the generation of ONE during the in vitro peroxidation of 13-HPODE following derivatization with semicarbazide (Fig. 4A). A time-course analysis of the ONE generation during the autoxidation of 13-HPODE, indeed, revealed that significant amounts of ONE were generated during the peroxidation (Fig. 4B). This and the observation that the yield of ONE was almost comparable to that of HNE (data not shown) proved that ONE is one of the major products during lipid peroxidation.

ONE-induced activation of the p53 pathway.

(i) Activation of p53. To confirm that the p53 accumulated in response to the treatment with ONE was functional, we assayed the subcellular localization of p53 and expression of the p53 target genes in cells treated with ONE. In parallel with the enhanced p53 phosphorylation, ONE induced p53 in a time-dependent manner (Fig. 5A). The dose-dependent effects of ONE were also analyzed at the same time of maximum induction (4 h), showing that even 5 \( \mu \text{M} \) of ONE produced an inducibility. Accompanied by the enhanced phosphorylation of p53, ONE enhanced the nuclear accumulation of p53 (Fig. 5, panels B and C).

(ii) Involvement of ATM in the ONE-induced p53 phosphorylation. In neurons, several kinases have been identified that detect DNA damage and initiate signaling pathways through p53 phosphorylation at the serine or threonine residues. It has been shown that ATM, among them, plays an essential role in transmitting DNA damage signals through phosphorylation of p53 (24, 25). The autophosphorylation of ATM at Ser-1981 activates the kinase and is largely responsible for phosphorylating p53 at Ser-15 in response to DNA damage (26, 27). Hence, experiments were carried out to assess the possibility that the ONE-induced p53 response is mediated via the ATM function. As shown in Fig. 6A, ONE, but not HNE, induced ATM phosphorylation in SH-SY5Y cells. The pretreatment and continued presence of an ATM inhibitor (caffeine or wortmannin) partially reduced the ONE-induced phosphorylation of p53 (Fig. 6B), suggesting that ATM is involved in the p53 response to ONE. To provide evidence that there is a link between ATM and p53 in response to ONE, we wanted to know whether blockage of ATM signaling inhibited the ONE-induced p53 phosphorylation. To this end, we utilized RNAi to block the expression of ATM in the SH-SY5Y cells. As shown in Fig. 6C, the SH-SY5Y cells expressing the ATM siRNA partially reduced the ATM levels. The reduced expression of the ATM...
was accompanied by reduced p53 phosphorylation in response to ONE. Thus, it appears that ONE activates p53 via ATM.

(iii) Involvement of proteasome in the ONE-induced p53 accumulation. Because the rapid demise of p53 is known to be largely achieved through the proteasome-dependent protein degradation pathway (28), it was hypothesized that ONE might act on this mechanism. Indeed, the treatment of cells with ONE resulted in the enhanced accumulation of ubiquitinated proteins (Fig. 7A). It was also observed that ONE partially inhibited the proteasome activities (Fig. 7, panels B and C).

These and the observations that ONE induced accumulation of p53 and ubiquitinated proteins in rat primary cerebral hemisphere neurons (Supplementary Data Fig. S1) and rat primary cortical neurons (Supplementary Data Fig. S2) suggest that the modulation of proteasome may also be involved in the mechanism responsible for the accumulation of p53 and ubiquitinated proteins in the cells exposed to ONE.

Induction of apoptosis by ONE. The tumor suppressor gene product, p53, has been reported to mediate apoptosis in many experimental systems. It is believed that this activity is responsible for the tumor suppressive function of p53 (29-31). Hence, we characterized whether ONE-induced cell death included apoptosis. As shown in Fig. 8A, the exposure of SH-SY5Y cells to ONE led to a dose- and time-dependent decrease in the number of viable cells. When human neuroblastoma SH-SY5Y cells were exposed to 10 μM ONE for 4 h, fragmented nuclei were found in cells exhibiting typical morphological features of apoptosis (Fig. 8B). In addition, the gel electrophoresis of DNA from SH-SY5Y cells exposed to ONE also displayed nucleosomal DNA fragmentation (Fig. 8C). These and the observation (Fig. 8D) that ONE, but not HNE, led a proteolytic cleavage of PARP, a hallmark of apoptosis, resulting in the accumulation of the 85 kDa fragment and decreasing of the 116 kDa protein, indicate that ONE induces the apoptotic cell death of SH-SY5Y cells.

On the other hand, the RT-PCR analysis demonstrated the up-regulation of p53-responsive genes, such as *Fas* and *gadd45*, in the cells exposed to ONE, while induction of the pro-apoptotic Bcl-2 family members, such as Bax and Bcl-2, was scarcely observed (Fig. 8E), suggesting that p53, which is thought to monitor the integrity of the cellular genome and responds to DNA damage by inducing cell cycle arrest and/or apoptosis, may be activated by ONE. The precise mechanism by which p53 modulates cell viability has not yet been determined. However, the prime candidates for its regulation may be Fas and its ligand (FasL), which are known to be one of the downstream mediators in the p53-dependent apoptotic pathway (32).

**Immunohistochemical detection of ONE-2'-deoxyguanosine adduct in sporadic ALS patients.** To determine the role for ONE in neuronal degeneration, we investigated the immunohistochemical localization of an ONE adduct in the control and ALS spinal cords, using a monoclonal antibody (mAb 6A3) that specifically recognizes the ONE-2'-deoxyguanosine adduct, 7-(2-oxo-heptyl)-substituted 1,N2-etheno-2'-deoxyguanosine (Fig. 9A) (20). The spinal cords of the sporadic ALS patients demonstrated the characteristic histopathological features of the classical form of this disease, such as degeneration of the pyramidal tracts, chromatolysis, atrophy and loss of the lower motor neurons, appearance of spheroids in the anterior horns, and reactive astrocytosis and microgliosis in these affected areas. Many of the surviving motor neurons bore lipofuscin, Bunina bodies, skein-like inclusions, and round hyaline inclusions. The spinal cords of the control subjects exhibited no significant morphological alterations. In the control subjects, the ONE-2'-deoxyguanosine immunoreactivity was diffuse and found in the cytoplasm of only a few motor neurons (Fig. 9, panels a, d, g). By contrast, in the ALS patients, the immunoreactivity was significant and localized to the nucleus and cytoplasm of the atrophic motor neurons and the surrounding reactive glial cells in the anterior horns and pyramidal tracts (Fig. 9, panels b, e, h). Bunina bodies, skein-like inclusions, round hyaline inclusions, and spheroids were negatively immunostained by mAb 6A3 (data not shown). No immunoreaction product deposits were detectable in the sections processed with omission of the primary antibodies (Fig. 9, panels c, f, i). These immunohistochemical results in the paraffin-embedded sections were similar to those in the frozen sections (data not shown).
DISCUSSION

It has long been thought that HNE is the major lipid hydroperoxide-derived bifunctional electrophile that reacts with DNA and proteins. However, it is now recognized that ONE is more reactive than HNE toward the DNA-bases 2'-deoxyguanosine (20, 22), 2'-deoxyadenosine (33,34), and 2'-deoxycytidine (35-37), generating a variety of bulky DNA adducts, such as 2-oxo-heptyl-substituted etheno-type adducts. In the present study, we identified ONE as a potential inducer of the p53 response in the human neuroblastoma cells. Other lipid peroxidation products, possessing an analogous functionality to that of ONE, were inactive upon induction of the p53 phosphorylation (Fig. 2). The inability of HNE to induce p53 phosphorylation is particularly interesting. Regardless of the previous observation that nonbulky functional groups on the 4-position of 2-nonenal only modestly influence the cytotoxic response elicited in the human colorectal carcinoma cell line RKO (38), HNE was ineffective not only on p53 phosphorylation, but also on ATM phosphorylation (Fig. 6), accumulation of ubiquitinated proteins (Fig. 7), and PARP cleavage (Fig. 8) in the SH-SY5Y cells. This seems to agree with the latest finding that ONE is both more neurotoxic to the neuroblastoma cells and more reactive in modifying and cross-linking proteins than HNE (39). The mechanism by which ONE can mediate activation of p53 in SH-SY5Y cells has not been fully established; however, these findings suggest that ONE leads to the accumulation of protein and/or DNA damage followed by apoptotic signaling that requires p53 in neuronal cells. Further studies will be needed to understand the remarkable distinction between HNE and ONE in terms of the p53-dependent neuronal apoptosis.

It is believed that p53 protein turnover is regulated by multiple mechanisms including regulation of the p53 protein degradation and covalent modifications of p53, particularly protein phosphorylation. p53 activation is dependent on the protein kinases, such as ATM. It is known that ATM transmits the signal of DNA damage induced by oxidative stress. For instance, oncogenic insults promote the accumulation of reactive oxygen species, resulting in DNA damage and apoptosis by a p53-dependent pathway (40-42). More recently, ATM has been shown to play an essential role in transmitting DNA damage signals, through phosphorylation of p53 (Ser-15) (24, 25). We showed that ONE significantly induced the phosphorylation of ATM (Fig. 6A). In addition, we used siRNA targeting ATM and the ATM inhibitor caffeine to investigate the involvement of ATM in the p53 response induced by ONE and demonstrated that the inhibition of ATM signalling resulted in the reduction of ATM (Ser-1981) and p53 (Ser-15) (Fig. 6, panels B and C). Our results thus suggest that apoptosis induced by ONE in SH-SY5Y cells is mediated by the ATM/p53 pathway (Scheme 1). Additional pathways regulating the p53-mediated cell death after DNA damage may exist. One possible additional effector is the ATM-related protein (ATR), which can also signal to p53 (43); however, this has not been addressed in neurons.

It is generally accepted that the accumulation of active p53 in response to stress also mainly occurs through the post-translational mechanisms (28). Pivotal is the increase in the protein half-life of p53. p53 is usually a very labile protein, turning over with a half-life sometimes as short as a few minutes. In response to DNA damage and other types of stress, p53 is markedly stabilized. It has been suggested that a rapid increase in the p53 concentration without a need for de novo transcription is particularly advantageous in cells with severely damaged genomes. Meanwhile, an elevation in the p53 protein can also be achieved by the increased translation of the p53 mRNA, involving relief of a translational repression mechanism operating through the 3'-untranslated region of this mRNA (44). In the present study, we found that ONE caused significant reduction of the proteasome activity (Fig. 7). This result suggests that ONE affected the regulatory mechanism of the p53 protein turnover via the ubiquitin-proteasome system. Proteasome is responsible for the majority of cellular proteolysis in eukaryotic cells and may contribute to controlling the intracellular levels of a variety of short-lived proteins (45-47).

Substrates of proteasome include a number of cell regulatory molecules, such as cyclins, the Myc oncogene protein, and p53; the regulated degradation of these molecules has been linked to the control of cell proliferation and cell cycle progression (45-47). Thus, it is likely that the ONE-induced disruption of the proteasome pathway results in the buildup of pro-apoptotic and detrimental proteins, such as p53 and ubiquitinated proteins. This may, at least in part,
be the mechanism underlying the ONE-induced apoptosis of the SH-SY5Y cells (Scheme I).

West et al. (38) have recently compared the apoptotic responses induced by a series of toxic α,β-unsaturated aldehydes, including ONE, in a human colorectal cancer cell line and have shown that the apoptotic response induced by ONE and other related aldehydes involves the activation of caspases, proteolysis of downstream caspase targets, and nucleosomal DNA fragmentation. Meanwhile, the present study has suggested the involvement of the p53 signaling pathway in neuronal cell death induced by ONE. The p53 protein is a tumor suppressor protein that transmits signals arising from various forms of cellular stress, including DNA damage and hypoxia, to genes and factors that induce cell cycle arrest and apoptosis (31). Not only is p53 induced by a variety of apoptotic stimuli, but the overexpression of p53 has also been demonstrated to induce apoptosis in a variety of cell types (6, 48). Based on the enhanced nuclear accumulation of p53 (Fig. 5) and the gene expression of p53-responsive genes (Fig. 8E), p53 might be a key molecule in the apoptosis induced by ONE. Moreover, the observation that ONE significantly induced the gene expression of Fas (Fig. 8E) suggests the involvement of the Fas/FasL signaling pathway, which is known to be one of the downstream mediators in the p53-dependent apoptotic pathway (32). The p53- and Fas-associated apoptoses have been suggested to be a common mechanism of cell loss in several neurodegenerative diseases (49).

The monoclonal antibody 6A3 used for the immunohistochemical detection of the ONE-2'-deoxyguanosine adduct has been raised using the chemically synthesized hapten–protein conjugate as the immunogen (20). Characterization of the antibody revealed that mAb 6A3 is specifically bound to the oxo-heptyl-2'-deoxyguanosine moiety, but not to various aldehyde-modified DNA containing exocyclic DNA adducts (20). The development of specific antibodies against the ONE-2'-deoxyguanosine adduct has made it possible for us to obtain evidence for the occurrence of ONE in vitro and in vivo. Using mAb 6A3, we have recently observed an intensive ONE-2'-deoxyguanosine staining in the liver section of rats fed a choline-deficient, and L-amino acid-defined diet (20), an experimental model for endogenous rat liver carcinogenesis associated with oxidative stress. In addition, Kamada et al. (50) have detected the ONE-2'-deoxyguanosine immunoreactivity in the human cancer cell line HCT8 exposed to H$_2$O$_2$ (50). In the present study, we demonstrated for the first time that the ONE-2'-deoxyguanosine adduct determinants accumulated in the spinal cord of ALS patients but not control subjects (Fig. 9). The immunoreactivity was localized to the nucleus and cytoplasm of both motor neurons and reactive glia, suggesting that ONE attack mitochondrial DNA as well as nuclear DNA. It is of interest that motor neurons were degenerated, while reactive glia were viable. This may reflect a difference in tolerance for ONE between motor neurons and glial cells. A previous study demonstrated increased expression of the proapoptotic, p53-induced Bax and reduced expression of the antiapoptotic, p53-inhibited Bel-2 in ALS spinal cord (51). These observations suggest that the enhanced lipid peroxidation, through its pivotal role in oxidative stress, followed by the activation of p53 signalling pathway, may be involved in the ALS neurodegenerative process.

In summary, this report demonstrates that the potential DNA alkylating agent ONE strongly activates p53 in SH-SY5Y neuroblastoma cells. We have shown that ONE is a specific activator of ATM which may phosphorylate p53. The ONE-mediated activation of p53 may eventually lead to the up-regulation of many p53-dependent genes. Moreover, we observed that the level of the ONE adduct increased in the tissue cells from patients with sporadic ALS. These data represent a further demonstration of a link between oxidative stress/lipid peroxidation and neuronal cell loss in neurodegenerative disorders. We are currently investigating the further upstream cell signalling induced by ONE. Our future challenge is to define a target molecule that triggers signal transduction pathways leading to p53 activation and to define the biological significance of the activation of this stress signalling pathways mediated by ONE. Studies focusing on these biochemical steps would extend our understanding of the regulation of stress signalling cascades stimulated by various reactive products generated during lipid peroxidation.

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Footnote:

Abbreviations: ALS, amyotrophic lateral sclerosis; ATM, ataxia telangiectasia-mutated; FasL, Fas ligand; HNE, 4-hydroxy-2-nonenal; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ONE, 4-oxo-2-nonenal; PUFAs, polyunsaturated fatty acids; RNAi, RNA interference; siRNA, short interfering RNA; TBARS, 2-thiobarbituric acid-reactive substances.

To whom correspondence should be addressed: Koji Uchida, Ph.D., Laboratory of Food and Biodynamics, Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601, Japan. Tel: 81-52-789-4127, Fax: 81-52-789-5741. E-mail: uchidak@agr.nagoya-u.ac.jp
LEGENDS FOR FIGURES

Fig. 1. Induction of p53 phosphorylation in SH-SY5Y cells treated with in vitro oxidized PUFAs. The oxidized PUFAs were prepared by incubating linoleate (A) or arachidonate (B) (200 μg/ml) with Fe²⁺ (40 μM) and ascorbate (1 mM) in 30 mM sodium phosphate buffer (pH 7.4). At the end of the incubation period, the reaction mixtures were collected and stored at -80 °C. The mixture (1 ml) was immediately extracted with 3 x 1 ml of ethyl acetate, and the solvent was evaporated under nitrogen. The residue was reconstituted in 200 μl of DMSO and subjected to the lipid peroxidation assay (TBARS assay) and to the incubation with SH-SY5Y cells for induction of the p53 phosphorylation.

Fig. 2. Identification of ONE as a selective inducer of p53 phosphorylation. SH-SY5Y cells were treated for 4 h with 10 μM of the indicated compounds and induction of the p53 phosphorylation was examined by an immunoblot analysis. Abbreviations: 9-HPODE, 9S-hydroperoxy-10E,12Z-octadecadienoic acid; 13-HPODE, 13S-hydroperoxy-9Z,11E-octadecadienoic acid; 9-HODE, 9R-hydroxy-10E,12Z-octadecadienoic acid; 13-HODE, (+)-13-hydroxy-9Z,11E-octadecadienoic acid; 9-oxo-ODE, 9-oxo-10E,12Z-octadecadienoic acid; 13-oxo-ODE, 13-oxo-9Z,11E-octadecadienoic acid; ACR, acrolein; MDA, malondialdehyde; CRA, crotonaldehyde; HNE, 4-hydroxy-2-nonenal; ONE, 4-oxo-2-nonenal.

Fig. 3. ONE, but not HNE, induces p53 phosphorylation. (A) Chemical structures of ONE and HNE. (B) Inability of HNE on induction of p53 phosphorylation in SH-SY5Y cells.

Fig. 4. Formation of ONE during lipid peroxidation. (A) Derivatization of ONE with semicarbazide. (B) Time-dependent formation of ONE during autoxidation of 13-HPODE. 13-HPODE (5 mM) was incubated in 50 mM sodium phosphate buffer (pH 7.4) at 37 °C. ONE generated during autoxidation of 13-HPODE was derivatized with semicarbazide, and the semicarbazone derivative of ONE was analyzed by reversed phase HPLC.

Fig. 5. Activation of p53 by ONE. (A) Time-dependent induction of p53 phosphorylation and increase in the p53 protein levels in SH-SY5Y cells exposed to 10 μM ONE. (B) Immunocytochemical detection of p53 in SH-SY5Y cells exposed to 10 μM ONE. The cells were fixed in 2% paraformaldehyde and 0.2% picric acid then immunostained with the anti-p53 antibody. Images of the cellular immunofluorescence were acquired using a confocal laser scanning microscope. (C) Immunoblot analysis of nuclear p53 and phosphorylated p53 in SH-SY5Y cells exposed to 10 μM ONE for 4 h.

Fig. 6. Involvement of ATM in the ONE-induced p53 phosphorylation. (A) ONE, but not HNE, induces ATM phosphorylation. (B) Effect of pharmacological inhibitors (caffeine and wortmannin) of ATM on p53 phosphorylation in SH-SY5Y cells exposed to 10 μM ONE for 4 h. The data are representative of one of three experiments with similar results. (C) Effects of specific siRNA of ATM on p53 phosphorylation in SH-SY5Y cells treated with 10 μM ONE for 4 h.

Fig. 7. Involvement of proteasome in the ONE-induced p53 accumulation. (A) Accumulation of ubiquitinated proteins. SH-SY5Y cells were exposed to the indicated aldehyde (10 μM) for 4 h. The proteins were separated by 7.5-15% gradient SDS-polyacrylamide gel electrophoresis in the presence of 2-mercaptoethanol and electro-transferred onto a nitrocellulose membrane (Hybond ECL) (Amersham Biosciences, Piscataway, NJ). To detect the ubiquitinated proteins, we used anti-ubiquitin antibody. (B) Inhibition of proteasome. The proteasome activity was evaluated using SH-SY5Y cells stably transfected with the Proteasome Sensor Vector™. (C) Time-dependent inhibition of proteasome by ONE. SH-SY5Y cells were exposed to 10 μM ONE and the proteasome activity was evaluated using SH-SY5Y cells stably transfected with the Proteasome Sensor Vector™.
Fig. 8. **Induction of apoptosis by ONE.** (A) Cytotoxicity of ONE. The cells were exposed to 0 – 25 μM ONE for 4 h. Cell viability was measured by the MTT assay. In the MTT assay, data are expressed as % of control culture conditions. **Symbols:** ■, 0.5 μM ONE; ◆, 1 μM ONE; ▲, 10 μM ONE; ●, 25 μM ONE. (B) Nuclei condensation in SH-SY5Y cells exposed to 10 μM ONE. SH-SY5Y cells were fixed with paraformaldehyde, stained with Hoechst 33258, and examined by fluorescence microscopy. **Upper,** nuclear staining. **Lower,** phase contrast. (C) DNA fragmentation in SH-SY5Y cells exposed to 10 μM ONE or 10 μM HNE for 4 h. Nucleosomal DNA fragmentation was visualized by agarose gel electrophoresis. (D) PARP cleavage in SH-SY5Y cells exposed to 10 μM ONE. The cleavage of PARP was analyzed by Western blotting. **Upper,** time-dependent cleavage of PARP. **Lower,** Inability of HNE on induction of PARP cleavage. (E) RT-PCR analysis of p53-responsive gene expression in SH-SY5Y cells exposed to 10 μM ONE for different time intervals.

Fig. 9. **Immunohistochemical detection of ONE-2′-deoxyguanosine adduct in sporadic ALS patients.** (A) Chemical structure of ONE-2′-deoxyguanosine adduct, 7-(2-oxo-heptyl)-substituted 1,N2-etheno-2′-deoxyguanosine. (B) Immunohistochemistry with mAb 6A3 in paraffin-embedded sections of spinal cords from a sporadic ALS patient and an age-matched control subject. **Magnification:** x 10 (panels a, d, g) and x 400 (panels b, c, e, f, h, i). ONE-2′-deoxyguanosine adduct immunoreactivity was diffuse or undetectable in the control spinal cord (panels a, b, c). By contrast, it was intense in the nucleus and cytoplasm of motor neurons in the anterior horn, and/or reactive astrocytes and activated microglia in the anterior horns and pyramidal tract in the ALS spinal cord (panels d, e, f). No immunoreaction product deposits were detectable on negative reaction control sections consecutive to those in middle row (panels g, h, i). In panel f, arrows and arrowheads indicate reactive astrocytes and activated microglia, respectively.

**Scheme I.** A mechanism proposed for the activation of p53 cascade by ONE.
Fig. 1.
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Fig. 2.
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Fig. 3. Shibata et al.
Fig. 4.
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Fig. 5.
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Fig. 6.
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Fig. 7.
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Fig. 8.
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Fig. 9.
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Apoptosis is

Caffeine
Wortmannin

p53

ATM

MDM2

Proteasome

ONE

DNA damage

p53

Apoptosis

Scheme 1
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Supplementary Data

Fig. S1. The phosphorylation of p53 and accumulation of ubiquitinated proteins in rat primary cerebral hemisphere neurons treated with ONE.

Rat primary cerebral hemisphere neurons (Sumitomo Bakelite Co., LTD., Tokyo, Japan) were plated onto a polylysine coated culture plate and cultured in Sumitomo nerve-cell culture system/culture medium for 3 days. The cells were treated with 20 μM ONE for the indicated periods.
Fig. S2. The phosphorylation of p53 and accumulation of ubiquitinated proteins in rat primary cortical neurons treated with ONE. Rat primary cortical neurons (Sumitomo Bakelite Co., LTD., Tokyo, Japan) were plated onto a polylysine coated culture plate and cultured in Sumitomo nerve-cell culture system/culture medium for 3 days. The cells were treated with 20 μM ONE for the indicated periods.