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Promoting Effects of Polyunsaturated Fatty Acids on Chromosomal Giant DNA Fragmentation Associated with Cell Death Induced by Glutathione Depletion

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Glutamate and buthionine sulfoximine (BSO) both reduce intracellular glutathione (GSH) concentration but by different mechanisms, and thereby induce cell death in C6 rat glioma cells. The effects of lipid peroxidation on chromosomal DNA damage during the GSH depletion-induced cell death were assessed. Polyunsaturated fatty acids (PUFA), such as arachidonic acid (AA), γ-linolenic acid and linoleic acid enhanced lipid peroxidation, induced a loss of membrane integrity and consequently promoted 1–2 Mbp giant DNA fragmentation under both glutamate- and BSO-induced GSH-depletion. Treated C6 cells had 3′-OH termini in their DNA which were recognized by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) analysis. Antioxidants capable of scavenging reactive oxygen species and lipid radicals and iron or copper scavengers inhibited both lipid peroxidation and 1–2 Mbp giant DNA fragmentation, consequently protecting against cell death under GSH depletion. These results suggest that GSH depletion induces lipid peroxidation and leads to 1–2 Mbp giant DNA fragmentation; and that PUFAs can promote giant DNA fragmentation and 3′-OH termini in chromosomal DNA enhancing lipid peroxidation of C6 cells.

Keywords: Cell death; Glioma cells; Glutathione depletion; Giant DNA fragmentation; Lipid peroxidation; Polyunsaturated fatty acid

Abbreviations: AA, arachidonic acid; BSO, L-buthionine-(S, R)-sulfoximine; DDC, sodium *N*–*N*-diethyldithio-carbamate; Def, deferoxamine; DMPO, 5,5′-dimethyl-1-pyrroline-*N*-oxide; DPPD, *N*–*N*′-diphenyl-1,4-phenylene diamine; HMW, high molecular weight; GSH, reduced glutathione; L˙, lipid radical; LO˙, lipid alkoxyl radical; LOO˙, lipid peroxyl radical; LOOH, lipid hydroperoxide; MDA, malon dialdehyde; NAC, *N*-acetylcysteine; PFGE, pulsed-field gel electrophoresis; PI, propidium iodide; PUFA and LH, polyunsaturated fatty acid; ROS, reactive oxygen species; TBARS, TBA reactive substances; TBA, 2-thiobarbituric acid; α-Toc, α-tocopherol; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling

INTRODUCTION

Some agents producing reactive oxygen species (ROS) can induce cell death, causing lipid peroxidation of the cell membrane, protein modification and DNA damage such as single and double strand breakage of chromosomal DNA.[1] Chromosomal DNA fragments larger than 1 Mbp and 50 kbp–1 Mbp in size are called giant DNA and high molecular weight (HMW) DNA fragments, respectively. The 1–2 Mbp giant DNA and 100–800 kbp HMW fragmentations reflect features of high-order chromatin structures such as miniband (1–2 Mb) consisting of 18 loops (50–100 kb) of DNA.[2] Cell death associated with giant DNA fragmentation producing 1–2 Mbp fragments followed by 100–800 kbp HMW DNA fragments has been observed in T-24 human bladder cells treated with H_2O_2, X-rays or bleomycin; all of which can produce ROS in cells.[3] γ-Ray and ultraviolet also produced 1–2 Mbp giant DNA fragments in T-24 cells.[4] It has been reported that 50–300 kbp HMW DNA fragments were responsible for the initial degradation of chromosomal DNA in apoptosis in thymocytes caused by dexamethasone,[5,6] in mouse L-929 cells caused by tumor necrosis factor (TNF),[7] in several

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human epithelial cells caused by serum deprivation[8] and in HeLa nuclei treated with apoptosis-inducing factor (AIF).[9] However, little has been reported about the implications of ROS on DNA damage, the character of not only the 1–2 Mbp giant DNA fragments but also HMW DNA fragments, or about their significance or roles in ROS instigated cell death.

Glutamate and L-buthionine-(S, R)-sulfoximine (BSO) can reduce GSH content by inhibiting cystine transport of the cell membrane and GSH synthesis by γ-glutamyl cysteine synthetase, respectively, leading to oxidative stress sensitive conditions.[10] Under GSH-depleted conditions, polyunsaturated fatty acids (PUFA) produce alkoxyl and peroxyl radicals that play a crucial role in lipid peroxidation through a free radical chain reaction.

In this study, we use the different GSH reducing systems of glutamate and BSO for the induction of intracellular GSH depletion, and thereby examine the effects of PUFAs on chromosomal DNA damage such as 1–2 Mbp giant DNA fragmentation and DNA cleavage with 3′-OH termini during cellular GSH, and we clarify the mode of action of PUFAs in GSH depletion-induced chromosomal DNA damage such as DNA cleavage with giant DNA fragmentation

MATERIALS AND METHODS

Chemicals

BSO, DDC, deferoxamine mesylate (Def), DMPO, DPPD, NAC, propidium iodide (PI), sodium glutamate monohydrate (glutamate) and α-tocopherol (α-Toc) were purchased from Sigma Chemical Company (St. Louis). 4-Sulfamoyl-7-fluoro-2,1,3-benzoxadiazole (ABD-F) and proteinase K were purchased from Wako Chemical, Osaka, Japan. Arachidonic acid (AA), γ-linolenic acid (γ-linolenic A), linoleic acid (linoleic A) and oleic acid (oleic A) were purchased from Biomol Research Lab., U.S.A. Dulbecco's modified Eagle medium (DMEM) and fetal calf serum (FCS) was purchased from Gibco BRL and Life Technology, Rockville, U.S.A., respectively. α-Toc and DPPD were dissolved in dimethyl sulfoxide. AA, γ-linolenic A, linoleic A and oleic A were dissolved in ethanol. Glutamate and BSO were dissolved in PBS and other reagents were dissolved in distilled water.

Cell Culture

C6 rat glioma cells were grown in DMEM supplemented with 5% FCS containing 25 mM sodium bicarbonate, 100 units/ml of penicillin G and 60 μg/ml of kanamycin (DF-5). C6 cells were seeded into 60 mm plastic culture dishes and grown in 5 ml of DF-5 at 37°C in a humidified atmosphere containing 5% CO_2.

Cell Death Assay

As a cell death monitor, ^{51}Cr release assay was used and carried out according to a method described previously.[11] Briefly, C6 cells (2×10^6) were labeled with $Na_2^{51}CrO_4$ at a final concentration of 10 μCi/10^6 cells in 500 μl of DF-5, for 60 min at 37°C. After washing several times with chilled Hank's balanced salt solution (HBSS, pH 7.2), the labeled cells were used for the cytotoxic experiments. ^{51}Cr-labeled cells were treated with glutamate or BSO under the various conditions specified for the times indicated at 37°C in humidified air containing 5% CO_2. The specific ^{51}Cr release that indicates cytolysis was calculated using a formula described previously.[11]

Determination of Intracellular GSH

GSH levels were quantified by high performance liquid chromatography (HPLC) using the sulfhydryl group specific fluorigenic agent, ABD-F for thiol, as described by Toyooka *et al.*[12] The C6 glioma cell pellet was suspended in 50 μl of 5% trichloroacetic acid solution containing 5 mM EDTA, and the resultant suspension was allowed to stand for 10 min on ice. The acid extract was centrifuged at 12,000*g* for 10 min and 10 μl of the supernatant was mixed with 190 μl of ABD-F solution (4.6 mM ABD-F, 100 mM sodium borate, pH 9.3, 1 mM EDTA). The mixture was incubated at 60°C for 5 min and was extracted twice with 400 μl of ethyl acetate after being chilled. The GSH in the mixture was analyzed as follows: a Shimadzu solvent delivery system (model LC-9A HPLC, Kyoto) was used for the HPLC analysis. Separations were performed in a linear 4%–15% (w/w) acetonitrile gradient in 150 mM H_3PO_4 over 10 min in a Shimadzu Shimpack CLC-ODS column (4.6 mm × 15 cm) at a flow rate of 1.0 ml/min at room temperature. Fluorescence detection of the ABD-F derivative of GSH was carried out using a fluorescence HPLC monitor, a Shimadzu Model RF-535 at an excitation wavelength of 380 nm and an emission wavelength of 510 nm.

Determination of Lipid Peroxides

TBARS produced by lipid peroxidation of cell components were estimated as a MDA formation according to the method described by Buege and Aust.[13] C6 cells (0.5–1 mg protein corresponds to about 5×10^6 cells) were harvested and suspended in 500 μl of PBS, and 1 ml of TBA reagent (0.375% TBA, 15% trichloroacetic acid, 0.25 N HCl) was

added to the cell suspension. The mixture of the cell suspension and TBA reagent was heated in boiling water for 20 min, chilled quickly to room temperature and centrifuged at 1,500*g* for 10 min. The supernatant was measured at 535 nm with a photometer. The amount of TBARS was calculated according to the molar absorption coefficient of MDA, $\varepsilon = 1.56 \times 10^5\,M^{-1}\,cm^{-1}$ at 535 nm.

Determination of Cell Membrane Integrity with PI

To assess the membrane integrity of C6 cells, propidium iodide was used. The cells were harvested, washed in PBS, resuspended in 500 μl of DMEM (FCS-free medium) containing 15 μM PI and incubated for 15 min at 37°C. The cells treated with PI were washed in PBS several times, resuspended in 100 μl of cell lysis buffer and the amount of PI in the cell suspension was measured with a fluorimeter at 485 nm for excitation and 590 nm for emission wavelength using a Fluoro Scan Ascent FL analysis system.

Protein Determination

The amount of protein was determined using the Bio-Rad protein assay system (Bio-Rad Lab., Richmond, CA) and bovine serum albumin as a standard according to the method described by Bradford.[14]

Pulsed-field Gel Electrophoresis (PFGE)

PFGE experiments were carried out according to a method described previously.[15] Briefly, the harvested cells (4×10^6, 50 μl) were embedded in a 0.5% agarose block by mixing the cells with 50 μl of PBS containing 1% low melting point agarose. The agarose block was incubated with 250 μl of a mixture of proteinase K (1 mg/ml), *N*-lauroyl sarcosine sodium (1%, w/v) and 0.5 M EDTA (pH 9.2) for 48 h at 50°C. After the incubation, a quarter of the agarose block was loaded into a 1% separating agarose gel ($15 \times 15 \times 4.4\,cm^3$) in TBE (89 mM *Tris*-boric acid, 2 mM EDTA, pH 8.0) for PFGE. A Pulsaphor system of Pharmacia, Uppsala was used as the PFGE apparatus. PFGE was performed with TBE at 14°C for 28 h (constant 150 V, pulse time 90 s for 20 h and 120 s for 8 h). The gel was stained with ethidium bromide in TBE, visualized on a UV transilluminator at 302 nm with Polaroid 667 film, and the DNA bands measured densitometorically using the analytical software of the gel documentation system of Epi-Light UV FA1100 (Aishin Cosumosu, Japan). Chromosomal DNA from *Saccharomyces cerevisiae* (Bio-Rad Lab., Richmond, CA) was used as the DNA size marker.

In Situ Labeling of Free 3′-hydroxyl Ends on DNA (TUNEL)

Fragmented DNA in cells was labeled using a modified TUNEL method according to the Nippon Gene analyzing kit (Wako Chemicals, Osaka) based on the method described by Gavrieli *et al*.[16] Briefly, cells (1×10^5) grown in a chamber slide with four square wells (Iwaki, Japan) were fixed in 4% buffered formaldehyde for 30 min and the nuclei of the cell preparation on the slide permeabilized by incubation with 0.1% sodium citrate solution containing 0.1% Triton X-100 for 2 min on ice. After washing twice with PBS, the slides were covered with TdT (0.05 U/μl) and biotinylated dUTP in the terminal deoxynucleotidyl transferase (TdT) buffer consisting of 30 mM *Tris*-HCl, pH 7.2, 140 mM sodium cacodylate, 4 mM $MgCl_2$ and 0.1 mM dithiothreitol, and incubated for 10 min at 37°C. Incubation was terminated by transferring the slides to PBS and by washing three times at 5 min intervals. Slides were incubated with a horseradish peroxidase-streptavidin-conjugated antibody (1:100 dilution in PBS) for 10 min at 37°C, followed by incubation with 3, 3′-diaminobenzidine and its enhancer for 5 min at room temperature. Cells on the slides were washed in PBS and dehydrated in a series of 70%, 90% and 100% ethanol, and then cells were directly visualized by microscopic observation and photographs taken. Visualized cells were judged by classifying the staining into three degrees. For the positive cells, the number of the medium or more stained cells was recorded in a minimum of five fields at ×400 magnification. Two observers examined each preparation, and the results were averaged.

Statistical Analysis

The results from the experiments were analyzed using Student's *t*-test. Difference were considered significant when $P < 0.05$.

RESULTS

GSH Depletion, Lipid Peroxidation and Cell Death Induced by Glutamate and BSO in C6 Cells

GSH levels, lipid peroxidation and cell death of C6 cells treated with glutamate and BSO were examined (Fig. 1). GSH content in cells treated with glutamate was 1.0 nmol/mg protein, and corresponded to approximately 7% of the initial level at 9 h. The $T_{1/2}$ (half-life time) of GSH depletion was estimated to be about 3 h after exposure to glutamate (Fig. 1A). The GSH content in C6 cells also decreased to approximately 12% at 12 h and 2% of the control at 24 h after exposure to 10 mM BSO (Fig. 1A). The lipid peroxidation of C6 cells was estimated by TBARS

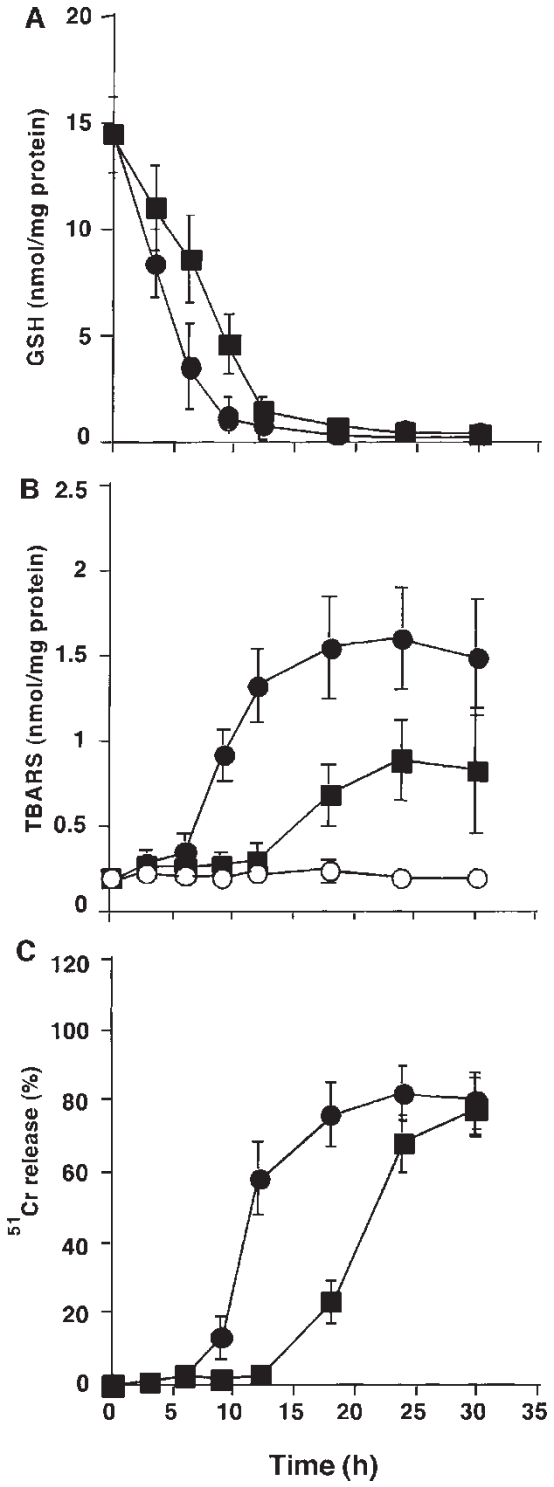


FIGURE 1 GSH depletion, lipid peroxidation and cell death induced by glutamate and BSO. C6 cells (2×10^6) were treated with 10 mM glutamate (●) or 10 mM L-buthionine-(S, R)-sulfoximine (BSO, ■) or without (○) for various periods. After incubation for the indicated periods, glutathione (GSH) levels (A) and 2-thiobarbituric acid reactive substances (TBARS) formed (B) in the cells and ^{51}Cr activity released in the medium (C) were determined. Values are the mean ± SD of four independent experiments.

formation in the cells; TBARS formed in C6 cells treated with glutamate were approximately 1.35 nmol/mg protein at 12 h and approximately 1.6 nmol/mg protein at 24 h; whereas, TBARS formation induced by BSO was delayed and at a lower level compared with that following glutamate treatment (Fig. 1B). The cytotoxic effects of glutamate and BSO on C6 cells were estimated by a ^{51}Cr release assay (Fig. 1C). ^{51}Cr release from the ^{51}Cr labeled C6 cells was begun at 9 h after exposure to 10 mM glutamate, reaching approximately 58% and 80% after 12 and 24 h, respectively. ^{51}Cr release from the cells was also observed after exposure to 10 mM BSO, and the release reached approximately 24% and 66% at 18 and 24 h, respectively (Fig. 1C).

PUFAs Enhanced Lipid Peroxidation and Cell Death under GSH Depletion

The effect of PUFAs such as AA ($C_{20:4}$), γ-linolenic A ($C_{18:3}$), linoleic A ($C_{18:2}$) and oleic A ($C_{18:1}$) on lipid peroxidation and C6 cell death under glutamate- or BSO-induced GSH depletion was examined by TBARS determination and ^{51}Cr release cytotoxic assay (Fig. 2). AA, γ-linolenic A and linoleic A enhanced TBARS formation in C6 cells treated with glutamate for 9 h. In contrast, oleic A suppressed both ^{51}Cr release and TBARS formation caused by both glutamate and BSO. AA, γ-linolenic A and linoleic A did not significantly affect either the glutamate or BSO-induced GSH reductions (Fig. 2A). The PUFAs used here did not on their own have any effect on ^{51}Cr release, GSH-depletion or TBARS formation in the absence of glutamate or BSO in C6 cells (data not shown). The effects of antioxidants including radical scavengers such as NAC, DMPO, α-Toc and DPPD, and iron/copper chelators such as Def and DDC were examined in respect to the GSH levels, the lipid peroxidation and the cell death in C6 cells caused by the treatment with glutamate or BSO in the presence of AA (Fig. 2). NAC, DMPO, α-Toc, DPPD, Def and DDC significantly suppressed lipid peroxidation and cell death caused by both glutamate and BSO (Fig. 2B and C).

PUFAs Enhanced Membrane Integrity Loss Induced by Glutamate and BSO

PI is a widely used as an indicator of membrane integrity.[17] In an attempt to quantify the cytoplasmic membrane integrity damaged by lipid peroxidation, we monitored the uptake of PI by incubating cells with PI after treatment with various reagents (Fig. 3). C6 cells treated with glutamate and BSO took up PI at about three- ($p < 0.05$) and two-fold ($p < 0.01$), respectively, compared with controls. AA, γ-linolenic A, and linoleic A, but not oleic A, enhanced the PI uptake of the C6 cells treated with glutamate. The effect of PUFAs on PI uptake by C6 cells treated with BSO was similar to that in the case of glutamate. Antioxidants, oxygen radical scavengers and iron/copper chelators suppressed

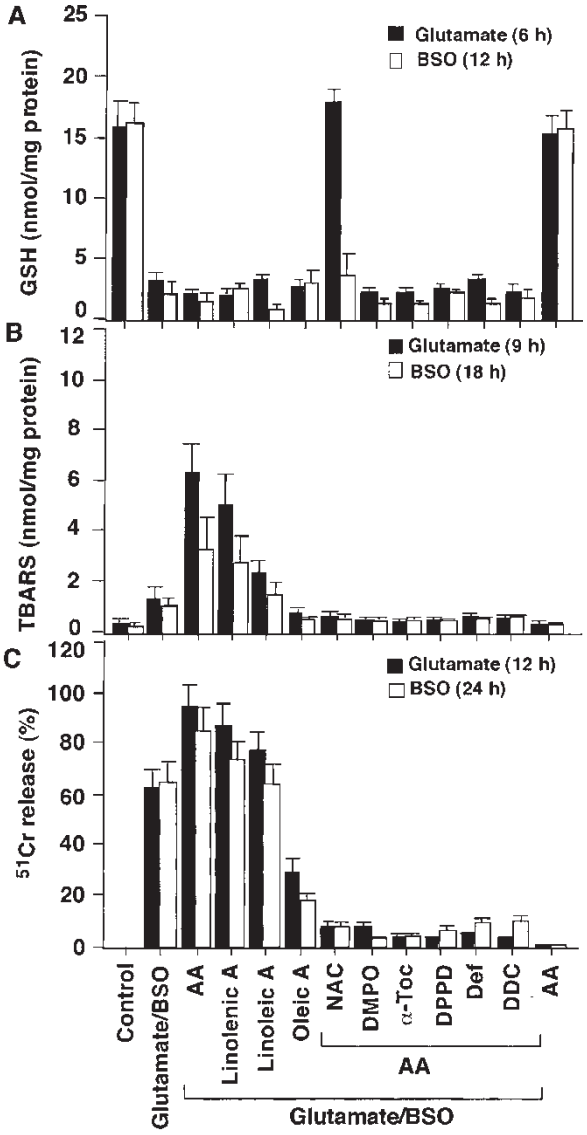


FIGURE 2 Effect of PUFAs, antioxidants, ROS scavengers and metal chelators on GSH depletion, the lipid peroxidation and cell death caused by glutamate and BSO. C6 cells (2×10^6) were treated with 10 mM glutamate (closed bar) for 6 h (A), 9 h (B), or 12 h (C) or with 10 mM BSO (open bar) for 12 h (A), 18 h (B) or 24 h (C) in the presence of 50 μM polyunsaturated fatty acids (PUFAs) together with 1 mM *N*-acetylcysteine (NAC), 10 mM 5,5′-dimethyl-1-pyrroline-*N*-oxide (DMPO), 100 μM α-tocopherol (α-Toc), 10 μM *N*–*N*′-diphenyl-1,4-phenylene diamine (DPPD), 10 μM deferoxamine (Def) or 10 μM sodium *N*–*N*-diethyldithiocarbamate (DDC). After incubation for the indicated periods, GSH levels (A) and TBARS formed (B) in the cells and ^{51}Cr activity released in the medium (C) were determined. Values are the mean ± SD of four independent experiments.

the enhancement of PI uptake induced by both glutamate and BSO, even if AA was present.

PUFAs Promoted Giant DNA Fragmentation Induced by Glutamate and BSO

The effects of antioxidants and oxygen, lipid radical scavengers and iron/copper chelators on chromosomal DNA fragmentation in C6 cells during glutamate- and BSO-induced cell death were examined (Fig. 4). Small amounts of 1–2 Mbp giant DNA fragments was observed in the chromosomal DNA of C6 cells treated with glutamate for 9 h. Giant DNA fragments were increased by further exposing the C6 cells to glutamate up to 12 h, which was further enhanced by AA, γ-linolenic A and linoleic A (Fig. 4A). The enhancement of 1–2 Mbp giant DNA fragmentation by these PUFAs was dependent on their species. Glutamate-induced giant DNA fragmentation was increased approximately 3.8-fold by addition of arachidonic A in a dose dependent manner, 2.5-fold by γ-linolenic A and 1.4-fold by linoleic A (Fig. 4B and C). In contrast, oleic A suppressed both glutamate- and BSO-induced giant DNA fragmentation. NAC, DMPO, α-Toc, DPPD, Def and DDC all inhibited the formation of 1–2 Mbp giant DNA fragments by both glutamate and BSO in the presence of AA (Fig. 4C and D).

PUFAs Promoted DNA Fragmentation with 3′-OH Termini Induced by Glutamate and BSO

DNA fragmentation patterns during the cell death under GSH depletion induced by glutamate or BSO in the presence or absence of AA, which is the most effective as an enhancer in PUFAs used on the cell death, were examined by TUNEL assay. TdT labeling was confined to the population of cells after treatment with glutamate alone or glutamate together with AA. DNA strand break labeling of 3′-OH termini (positive on TUNEL) was observed in most of the C6 cells treated with both glutamate for 12 h and glutamate together with AA for 24 h, but not in the presence of α-Toc, which almost rescued the cells from glutamate-induced cell death presenting negative staining on TUNEL, similar to controls (Fig. 5). In BSO-treated C6 cells, TUNEL analysis presented similar results to those of glutamate-treated cells in DNA features, even in the presence of AA (Fig. 6A and B). α-Toc suppressed the DNA damage estimated by TUNEL in BSO plus AA treated cells ($p < 0.01$). NAC, DMPO, α-Toc, DPPD, Def and DDC all standard reduced the ratio of TUNEL-positive cells caused by both glutamate and BSO in the presence of AA.

DISCUSSION

Glutamate inhibits cysteine transport resulting in a reduction of both intracellular GSH and cysteine levels,[18] whereas BSO reduces the GSH level, but not the cysteine level that is generally involved in the redox state controlling system. Therefore, the findings from this study suggest that oxidative stress in BSO-treated C6 cells could be milder than that

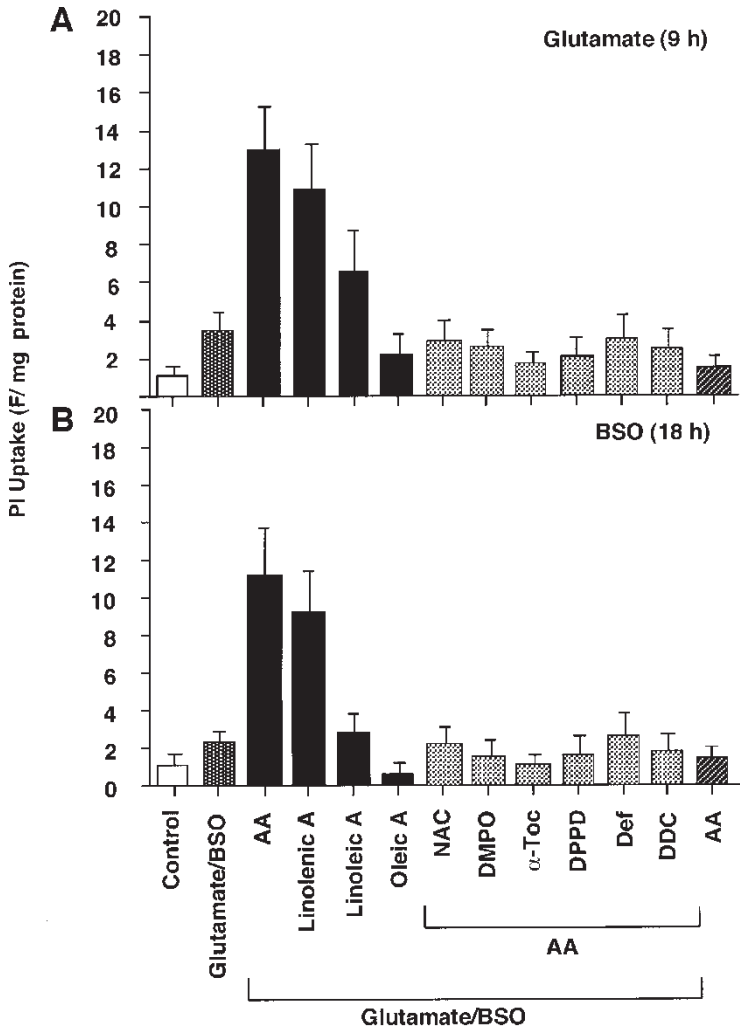


FIGURE 3 Effect of PUFAs, antioxidants, ROS scavengers and metal chelators on PI uptake by C6 cells treated with glutamate and BSO. C6 cells were treated with 10 mM glutamate for 9 h (A) or 10 mM BSO for 18 h (B) in the presence or absence of 50 μM PUFAs together with 1 mM NAC, 10 mM DMPO, 100 μM α-Toc, 10 μM DPPD, 10 μM Def or 10 μM DDC. PI uptake by cells was expressed as the fluorescence intensity, F/mg protein of whole cells. Values are the mean ± SD of three independent experiments.

in glutamate-treated cells (Fig. 1). 1–2 Mbp giant and 100–800 kbp HMW DNA fragmentation in both glutamate- and BSO-treated C6 cells was increased by the addition of PUFAs (Fig. 4). However, all of the antioxidants, ROS scavengers and metal chelaters used in this study suppressed not only 1–2 Mbp giant DNA fragmentation but also lipid peroxidation and cell death without affecting GSH depletion, even if the presence of PUFA (Fig. 2A and C). NAC is converted by deacetylation to cysteine that is a precursor of GSH synthesis in cells.[19] DMPO can scavenge hydroxyl radicals ($^{\cdot}OH$), superoxide anions (O_2^{-}), lipid radicals ($L^{\cdot}$) and lipid alkoxyl radicals ($LO^{\cdot}$).[20,21] α-Toc, which can scavenge $L^{\cdot}$, $LO^{\cdot}$ and $LOO^{\cdot}$ within membranes, has been classified as a chain-breaking antioxidant.[22] DPPD can scavenge $L^{\cdot}$ and $LOO^{\cdot}$.[23,24] Def and DDC can chelate Fe^{3+}/Fe^{2+} and Cu^{+} in several enzymes and other iron or copper proteins, respectively.[25] Therefore, it is likely that the 1–2 Mbp giant DNA fragmentation is enhanced by ROS or ROS-mediated lipid peroxidation. PUFAs are easily oxidized by $^{\cdot}OH$ radicals and also by enzymatic metabolisms by lipoxygenases, and thereby induce lipid peroxidation of cells without diminishing lipid hydroperoxides generated under GSH depletion and abundant oxygen.[26] PUFAs are particularly vulnerable to free radical attack, because the carbon double bond in PUFAs within membranes allows the easy removal of hydrogen atoms by ROS such as $^{\cdot}OH$ radicals.[26] Under aerobic conditions, lipid peroxidation continues as carbon double bonds with doubly allylic hydrogen combine with O_2 to form additional organic $LOO^{\cdot}$ (Scheme 1). The oxidizability of PUFAs may be linearly dependent on the number of doubly allylic positions at 37°C.[27,28] Oleic acid did not affect GSH-depletion but suppressed rather than enhanced both TBARS formation and ^{51}Cr release caused by both glutamate and BSO (Fig. 2). Oleic acid might act as an antioxidant

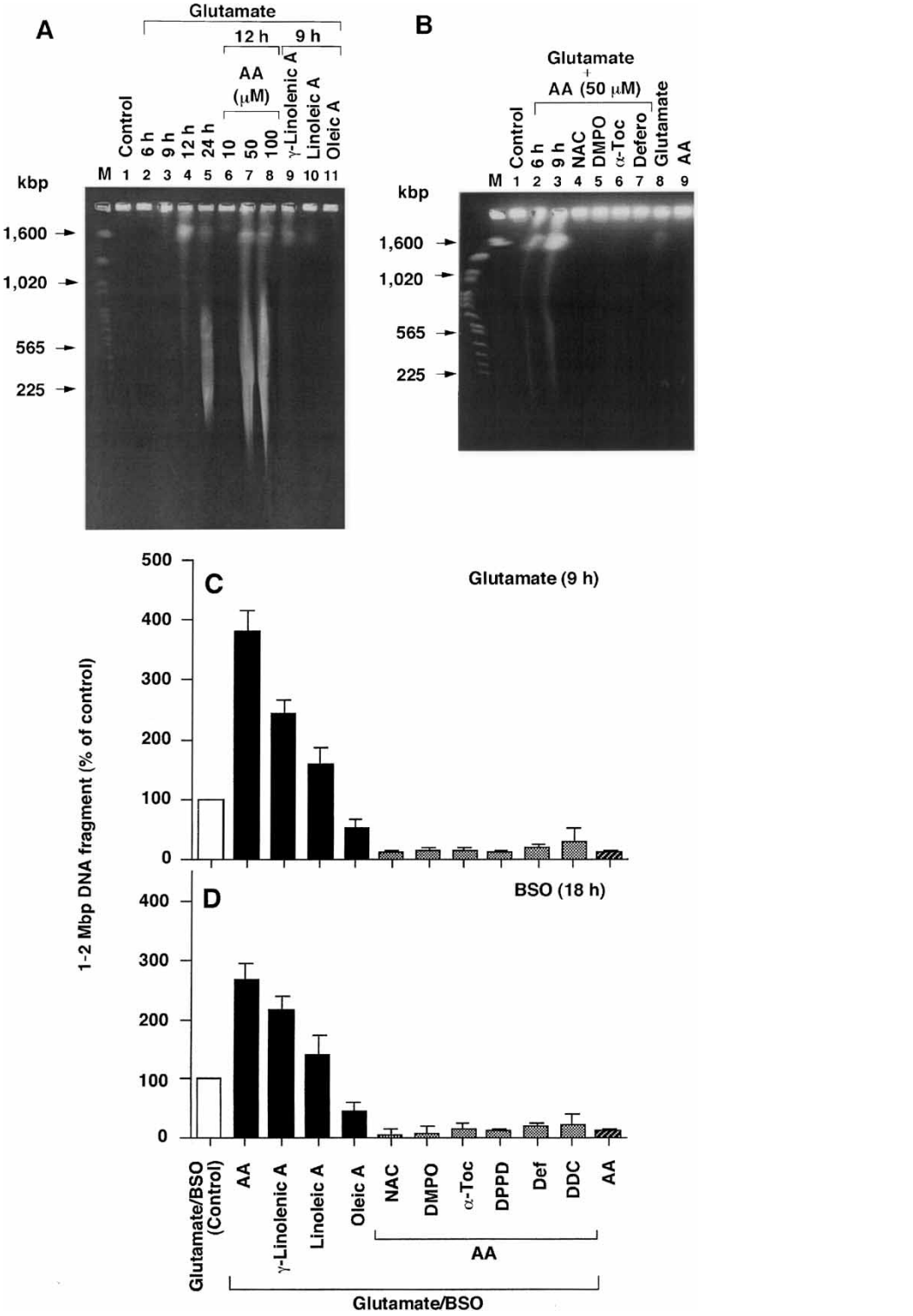


FIGURE 4 Effect of PUFAs, antioxidants, ROS scavengers and metal chelators on DNA fragmentation of C6 cells induced by glutamate and BSO. In panel A, each lane indicates the control (lane 1), samples from cells treated with 10 mM glutamate alone for 6 h (lane 2), 9 h (lane 3), 12 h (lane 4) and 24 h (lane 5), 10 mM glutamate for 12 h in the presence of 10 μM arachidonic acid (AA, lane 6), 50 μM AA (lane 7), 100 μM AA (lane 8), and for 9 h in the presence of 50 μM γ-linolenic acid (lane 9), 50 μM linoleic A (lane 10) and 50 μM oleic A (lane 11). In panel B, each lane indicates samples from cells treated with 10 mM glutamate for 9 h (lane 8) in the presence of 50 μM AA for 6 h (lane 2) and 9 h (lane 3), 1 mM NAC for 9 h (lane 4), 10 mM DMPO for 9 h (lane 5), 100 μM α-Toc for 9 h (lane 6), 10 μM Def for 9 h (lane 7), or treated with 50 μM AA alone for 9 h (lane 9). *Saccharomyces cerevisiae* chromosomal DNA (from Bio-Rad, lane M in both A and B) was used as giant-sized DNA markers. In panel C and D, the amount of 1–2 Mbp giant DNA fragments was determined according to the method described in "Materials and Methods Section" and expressed as a percentage of the control that indicates the amount of 1–2 Mbp DNA fragments in glutamate- or BSO-treated samples for 9 h or 18 h. The values are the mean ± SD of three independent experiments.

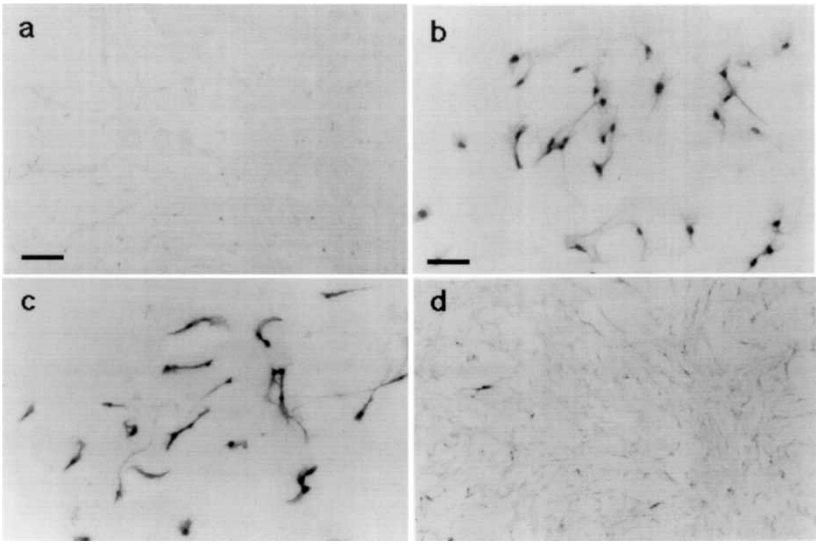


FIGURE 5 Effect of AA on DNA features of C6 cells treated with glutamate. C6 cells were treated with 10 mM glutamate for 12 h. Each picture shows the control (a), glutamate (b), glutamate and 50 μM AA (c), and glutamate, 50 μM AA and 100 μM α-Toc (d). The cells were labeled and visualized according to the TUNEL method. The pictures show different magnitudes between (a) and (b), in which the bars indicate 100 μm (a) and 25 μm (b). Pictures (c) and (d) are the same magnitude as (b) and (a), respectively.

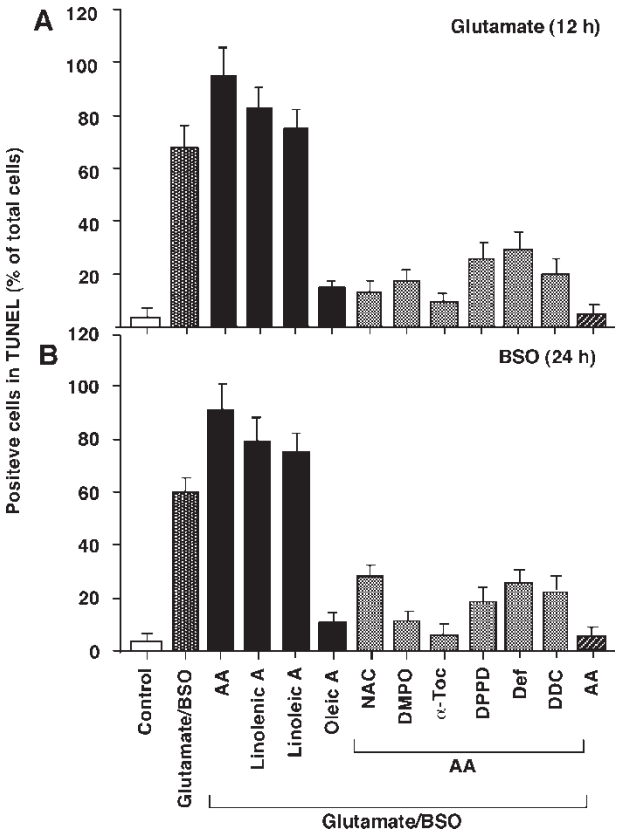
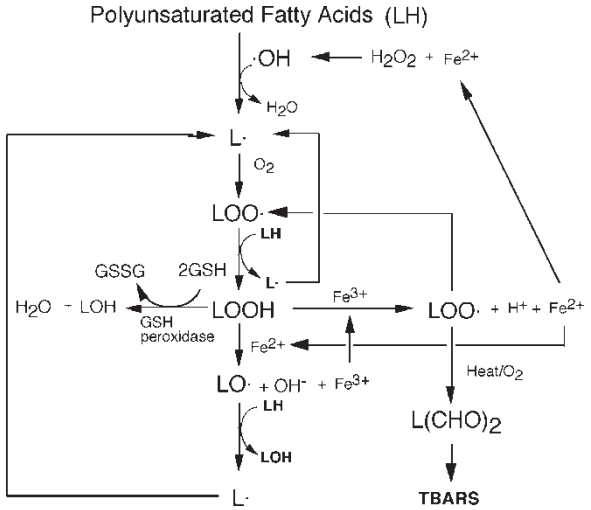


FIGURE 6 Effect of PUFAs, antioxidants, ROS scavengers and metal chelators on DNA features of C6 cells treated with glutamate and BSO. C6 cells were treated with 10 mM glutamate for 12 h (A) or 10 mM BSO for 24 h (B) in the presence of 50 μM PUFAs together with 1 mM NAC, 10 mM DMPO, 100 μM α-Toc, 10 μM DPPD, 10 μM Def, or 10 μM DDC. TUNEL positive cells were counted and expressed as a percentage of total cells. The values are the mean ± SD of three independent experiments.

in GSH depletion-induced cell death. Indeed, there are few reports on the effect of oleic acid; though both oleic acid and saturated fatty acids decrease the level of cellular ROS generated by the mitochondrial respiratory chain.[29] Olecic acid has an antioxidant action exerting a direct vascular atheroprotective effect[30] and preventing oxidative modification of lipoprotein.[31] Lipid free radicals such as L$^{\cdot}$, LO$^{\cdot}$ and LOO$^{\cdot}$ are most likely produced during lipid peroxidation through a chain reaction and amplified in the presence of iron or copper, not only in intracells but also in cell-cultured medium. The results here suggest that lipid peroxidation might induce



SCHEME 1 Lipid peroxidation by radical chain reaction under GSH depletion in C6 cells.

a loss of membrane integrity and enhance the fluidity of the membrane structure; so that thereafter chromosomal DNA might be more easily attacked by $^{\cdot}OH$ radicals or other types of ROS producing 1–2 Mbp giant DNA fragments.

TUNEL analysis showed positive results in both glutamate- and BSO-induced cell deaths, indicating that nuclear DNA from C6 cells treated with both glutamate and BSO has DNA fragments with 3′-OH termini, which might be in giant DNA fragments. The chromosomal DNA fragments with free 3′-OH termini caused by GSH depletion were enhanced by PUFAs in C6 cells (Fig. 6); thus, PUFAs could promote 1–2 Mbp giant DNA fragmentation with 3′-OH termini causing rapid necrotic cell death through lipid peroxidation. This suggestion is supported by a report in which *N*-methyl-D-aspartate and kainite induced TUNEL-positive internucleosomal DNA cleavage associated with both apoptotic and necrotic cell death in neonatal rat brain.[32] Activations of some phospholipases during necrosis, particularly cytosolic Ca^{2+}-dependent PLA_2, have been demonstrated.[33] PLA_2 is specific to the substrate with AA at the sn-2 position and its translocation to cell membranes. Lysophosphatidic acid, which is produced from phospholipids by removing the AA of PLA_2, also induces both apoptosis and necrosis in hippocampal neurons,[34] however, by unknown mechanisms. Thus, not only lysophosphatidic acid but also other unsaturated fatty acids should not be ignored in accessing necrotic cell death associated with lipid peroxidation.

A possible mechanism for 1–2 Mbp giant DNA fragmentation under GSH depletion is therefore lipid peroxidation-derived free radicals proceeding not only to plasma membranes but also to nuclear membranes close to the chromosomes. The loss of membrane integrity in the cell may thereby create circumstances suitable for oxygen radicals such as $^{\cdot}OH$ radicals to attack chromatin DNA. $LO^{\cdot}$ or $LOO^{\cdot}$ radicals may also cleave DNA strands leading to giant DNA fragmentation in chromatin. $LO^{\cdot}$ radicals are produced from lipid hydroperoxides (LOOH) by Fenton's reaction in the presence of iron or copper. Inoue[35] has reported that 13-hydroperoxy-octadecadienoic acid (LOOH), a metabolite of linoleic A (LH) and one of the lipid hydroperoxides, cleaves double stranded DNA at the position of guanosine nucleotides in pBR322; but neither linoleic A nor 13-hydroxyoctadecadienoic acid (LOH) was effective in such cleavage. The intracellular metal ions such as iron and copper might play an important role in the generation of hydroxyl radicals from H_2O_2 or O_2^- in or around chromatin.[36,37] Hydroxyl radicals can cause single strand DNA breaks associated with double strand DNA breaks.[4] Under the circumstance of membrane integrity loss induced by lipid peroxidation, ROS such as $^{\cdot}OH$ radicals might attack and directly cleave chromosomal DNA into single strand forms, consequently leading to double strand DNA breaks. Hydroxyl radicals caused single strand breaks with nicked forms leading to double strand breaks predominantly in the 5′-G-G-3′ or 5′-G-G-G-3′ sequences of pZ189.[38] Although the location(s) of the specific DNA target site(s) attacked by ROS in this cell system is still unclear, our study suggests that target sites in the chromosomal DNA occur at a relatively regular distance of 1–2 Mbp. In respect to the mechanism of giant DNA fragmentation, ROS-mediated action is more possible and preferable to lipid radical-mediated action for providing 3′-OH-termini in single or double strand breakages. Lipid hydroperoxides made from PUFAs induce a loss of membrane integrity and thereafter may alter the environment sufficiently to make it easier for ROS such as $^{\cdot}OH$ radicals to be around chromosomes.

This study using an exogenously added PUFAs experiment system suggests a possible mechanism as a model for conversion of apoptosis to necrosis under GSH depletion induced by various cell death triggers through the promotion of lipid peroxidation and giant DNA fragmentation. Therefore, some antioxidants, radical scavengers inhibiting peroxidation, might be useful for the suppression of glial cell death caused by oxidative stress.

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