Free Rad. Res., Vol. 35, pp. 281–299
Reprints available directly from the publisher
Photocopying permitted by license only

Enhancement of Hyperthermia-induced Apoptosis by a Free Radical Initiator, 2,2′-Azobis (2-amidinopropane) Dihydrochloride, in Human Histiocytic Lymphoma U937 Cells

FU-JUN LI[a,c], TAKASHI KONDO[a,*], QING-LI ZHAO[a], KIYOSHI TANABE[a], RYOHEI OGAWA[a], MIN LI[b] and YOKO ARAI[b]

[a]*Department of Radiological Sciences;* [b]*Department of Public Health, Faculty of Medicine, Toyama Medical and Pharmaceutical University, Toyama, 930-0194, Japan;* [c]*Department of Industrial Hygiene and Occupational Disease, School of Public Health, China Medical University, Shenyang, 110001, China*

Accepted by Prof. N. Taniguchi

(Received 14 November 2000; In revised form 18 January 2001)

To elucidate the mechanism how a free radical initiator, 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH), induces cell death at hyperthermic temperatures, apoptosis in a human histiocytic lymphoma cell line, U937, was investigated. Free radical formation deriving from the thermal decomposition of AAPH was examined by spin trapping with 5,5-dimethyl-1-pyrroline-N-oxide (DMPO). An assay for DNA fragmentation, observation of nuclear morphological changes, and flow cytometry for phosphatidylserine (PS) externalization were used to detect apoptosis and revealed enhancement of 44.0 °C hyperthermia-induced apoptosis by free radicals due to AAPH. However, free radicals alone derived from AAPH did not induce apoptosis. Hyperthermia induced the production of lipid peroxidation (LPO), an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) and enhanced expression of the type 1 inositol 1,4,5-trisphosphate receptor (IP_3R1). The effects of hyperthermia on LPO and $[Ca^{2+}]i$ were enhanced markedly by the combination with AAPH. A significant decrease in Bcl-2 expression, increase in Bax expression, a loss of mitochondrial membrane potential ($\Delta\Psi m$) and a marked increase in cytochrome c expression were found only in cells treated with hyperthermia and AAPH. Although an intracellular Ca^{2+} ion chelator, BAPTA-AM, completely inhibited DNA fragmentation, water-soluble vitamine E, Trolox, only partially suppressed DNA fragmentation and the increase in $[Ca^{2+}]i$. In contrast, LPO was inhibited completely by Trolox, but no inhibition by BAPTA-AM was found. These results suggest that apoptosis induced by hyperthermia alone is due to the increase in $[Ca^{2+}]i$ arising from increased expression of IP_3R1 and LPO. Additional increase in $[Ca^{2+}]i$ due to increased LPO and the activation of mitochondria-caspase dependent pathway play a major role in the enhancement of apoptosis by the combination with hyperthermia and AAPH.

Keywords: Apoptosis, 2,2′-azobis (2-amidinopropane) dihydrochloride, hyperthermia, lipid peroxidation, mitochondrial membrane potential, inositol 1,4,5-trisphosphate receptor, intracellular Ca^{2+}

*Corresponding author. Tel.: +81-76-434-7265. Fax: +81-76-434-5190. E-mail: kondot@ms.toyama-mpu.ac.jp.

Abbreviations: AAPH, 2,2′-azobis (2-amidinopropane) dihydrochloride; DMPO, 5,5-dimethyl-1-pyrroline-N-oxide; EPR, electron paramagnetic resonance; PS, phosphatidylserine; Annexin V/FITC, fluorescein isothiocyanate-labled annexin V; PI, propidium iodide; LPO, lipid peroxidation; MDA, malondialdehyde; $[Ca^{2+}]i$, intracellular Ca^{2+} ion concentration; IP_3R, Inositol 1,4,5-trisphosphate receptor; $\Delta\Psi m$, mitochondrial membrane potential; $DiOC_6(3)$, 3,3′-dihexyloxacarbocyanine iodide; BAPTA-AM, (acetoxymethyl)-1,2-bis-(o-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; Z-DEVD-FMK, benzyloxycarbonyl-Asp(OCH_3)-Glu(OCH_3)-Val-Asp(OCH_3)-fluoromethylketone

INTRODUCTION

A water-soluble azo compound, 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH), has been used as a temperature-dependent free radical generator. Upon activation due to hyperthermia, AAPH decomposes to yield two alkyl radicals and a subsequent reaction with oxygen can yield alkoxyl and/or peroxyl radicals.[1,2] Enhancement of hyperthermia-induced cytotoxicity with AAPH in Chinese hamster V79 cells has been reported.[2] However, the detailed mechanisms responsible for the lethal effect of exposure to hyperthermia are not yet fully understood.

Apoptosis induced by the diazo radical initiator, 2,2′-azobis (2,4-dimethylvaleronitrile) (AMVN) in PC12 rat pheochromocytoma cells,[3] and in HL60 human leukemia cells,[4] and apoptosis induced by oxidation of human low-density lipoprotein (LDL) due to AAPH in mature human monocyte-derived macrophages[5] have been reported. These results suggested that free radicals derived from azo compounds and their modification may play a role in the incidence and/or abrogation of apoptosis.

Therefore, considering the characteristics of thermally generating radicals from AAPH, whether AAPH enhance hyperthermia-induced cytotoxicity via the mechanism of apoptosis warrants further investigation.

Activation of Ca^{2+}-dependent cell death induced by free radicals in the unicellular organism *Trypanosoma brucei brucei*[6] and in Rat-1 fibroblasts[7] have been shown. Recently, we have reported that a human histiocytic lymphoma cell line, U937, contained Ca^{2+}/Mg^{2+}-dependent endonucleases.[8] Therefore, U937 cells appear to be a suitable cell line for examining the Ca^{2+}-dependent pathway of apoptosis induced by free radicals.[9] The significant inhibitory effect of peroxyl radicals derived from AAPH on Ca^{2+}-ATPase in plasma membranes (PMCA) has been reported.[10] Since PMCA plays a critical role in maintaining intracellular calcium homeostasis, changes in $[Ca^{2+}]i$ appear to be the intermediate link in the induction of apoptosis caused by AAPH.

One attractive target for oxidative signaling in apoptosis is lipid peroxidation (LPO), partially because polyunsaturated membrane phospholipids are extremely sensitive to oxidant attack. Indeed, a number of studies have shown significant relationships between LPO due to oxidative stress and apoptosis.[4] LPO has also been reported to cause elevation of $[Ca^{2+}]i$.[11] Previous studies demonstrated the production of LPO after treatment with AAPH.[1,12] It is therefore of interest to establish whether LPO can induce apoptosis directly or with the assistance of $[Ca^{2+}]i$.

In addition to LPO, there has also been recent interest in alterations to the mitochondrial membrane. Although characteristic changes in nuclear morphology and biochemistry define apoptosis, it has recently become clear that cytoplasmic, rather than nuclear, structures regulate the effector stage of apoptosis.[13] Thus, it has been established that mitochondrial alterations, especially changes in the mitochondrial membrane potential ($\Delta\Psi m$), have a major functional impact on apoptosis.

We therefore addressed whether AAPH or the combination of AAPH and hyperthermia induced apoptosis, including its mechanism. Here, the present results show that a hydrophilic azo compound, AAPH enhanced the apoptosis induced by hyperthermia without inducing apo-

ptosis by AAPH alone. Results with an antioxidant, Trolox, and an intracellular Ca^{2+} chelator, BAPTA-AM, indicated the increase in $[Ca^{2+}]i$ is the key event in the apoptosis induced by hyperthermia with AAPH, though LPO plays a role in this apoptosis. LPO, loss of $\Delta\Psi m$ and enhanced expression of the type 1 1,4,5-trisphosphate receptor (IP_3R1) appear to be causes of the increase in $[Ca^{2+}]i$.

MATERIALS AND METHODS

Chemicals and Reagents

5,5-Dimethyl-1-pyrroline-N-oxide (DMPO) was purchased from Labotec Ltd. (Tokyo, Japan). BAPTA-AM and Fura-2/AM were obtained from Dojindo Laboratory (Kumamoto, Japan). An annexin V/FITC (fluorescein isothiocyanate-labeled annexin V) kit was obtained from Immunotech, (Marseille, France). Trolox and caspase-3 inhibitor II (Z-DEVD-FMK) were from Sigma Chemical Co. Ltd. (St. Louis, MO) and Calbiochem-Novabiochem Corp. (San Diego, CA), respectively. Monoclonal or plyclonal antibodies to Bcl-2, Bax, IP3R1, cytochrome c and other proteins were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA). AAPH and other reagents were obtained from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan).

Detection of Free Radicals Induced by AAPH

Electron paramagnetic resonance (EPR) with spin-trapping was employed to detect thermally generated radicals from AAPH in 50 mM phosphate buffer solution (PBS, pH 7.4). DMPO was used as a spin trap. One ml of PBS solution containing AAPH and a spin trap in a test tube was prepared at ice temperature and the reaction was initiated by immersing the test tube in a water bath at the desired temperature. The incubation temperature was controlled within 0.1 °C. For EPR measurement, aliquots of the reaction mixture were sampled at different temperatures or incubation times. The EPR measurements were made using a RFR-30 Radical Analyzer System (Radical Research Co., Tokyo, Japan) operating at a X-band frequency of 9.4 GHz.

Cell Culture and Hyperthermic Treatment

U937 cells, a human histiocytic lymphoma cell line, obtained from the Japanese Cancer Research Resources Bank, Tokyo, Japan were grown in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum (Life Technologies, Rockville, MD). Cells were maintained in a humidified incubator maintained at 37.0 °C with 5% CO_2 and 95% air. For experiments, cells were seeded at 3×10^6 cells/3 ml in a plastic tube and exposed to a desired temperature from 37.0 °C to 46.0 °C by immersing the tubes in a precision controlled water bath. The temperature of the solution was monitored with a digital thermometer (#7563, Yokogawa, Tokyo, Japan). Preheated culture media containing various concentrations of AAPH were added to cell pellets in the plastic tubes just before heating.

Assessment of Apoptosis

The cells were incubated for various lengths after AAPH treatment at the desired temperatures for 10 min. The percentage of DNA fragmentation was determined by the method of Sellins and Cohen.[14] Cell morphology was examined with Giemsa staining. For detection of phosphatidylserine (PS) externalization U937 cells were stained simultaneously with Annexin V/FITC and propidium iodide (PI) and assessed with a Beckman–Coulter EPICS XL™ flow cytometer.

Determination of LPO

The products of LPO reacting with 2-thiobarbituric acid (TBARS), malondialdehyde (MDA),

was measured spectrophotometrically at an absorbance of 535 nm, as described by Buege and Aust.[15] MDA levels both in the cells and medium were measured after various incubation times.

Measurement of $\Delta\Psi m$

To measure $\Delta\Psi m$ after different treatments, cells were incubated at 37.0 °C for 15 min in the presence of 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6(3)$) (40 nM) and then analyzed with a flow cytometer.[13]

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

Cells were harvested after incubation for 6 h. About 10^6 cells were loaded with 5 μM Fura-2/AM for 30 min at room temperature. After washing with a HEPES-buffered Ringer solution (HR, pH 7.4; NaCl, 118 mM; KCl, 4.7 mM; $MgCl_2$, 1.13 mM; Na_2HPO_4, 1.0 mM; glucose, 5.5 mM and HEPES, 10 mM), cells were transferred onto a glass bottom dish coated with Cell-Tak (Collaborative Research, Bedford, MA). Digital imaging of Fura-2 fluorescence was done with a digital image processor (Argus 50CA, Hamamatsu Photonics, Japan) and the fluorescence ratio (340 nm/380 nm) at 510 nm was converted to $[Ca^{2+}]i$, as described previously.[16]

Western Blotting for Bcl-2, Bax, IP_3R Proteins and Cytochrome c

Cells were collected and washed in ice-cold PBS and then lysed in RIPA buffer (1 M Tris-HCl, 5 M NaCl, 1% NP-40, 1% sodium deoxycholate, 0.05% SDS) for 15 min. Protein concentration was determined with a Bio-Rad protein assay kit. An aliquot (30 μg/lane) of total protein was separated by SDS 10–20% polyacrylamide gel electrophoresis and transferred to PVDF membranes. After blocking buffer for 2 h at room temperature, membrane were incubated with the primary antibody, either mouse monoclonal IgG1 Bcl-2 (100), rabbit polyclonal IgG Bax (N-20), goat polyclonal antibody IP_3R (1, 2, and 3), or rabbit polyclonal IgG cytochrome c (H-104). As the internal standard, β-actin was used. After being washed in PBS, membranes were incubated with a HRP-linked anti-mouse IgG for Bcl-2, anti-rabbit IgG for Bax and anti-goat IgG for IP_3R (1, 2, and 3) for 40 min followed by chromogenic visualization with BLAST 4CN Plus (NEN Life Science Products, Boston, MA).

For preparation of cytosolic extract, cells were harvested by centrifugation at 200 × g for 5 min at 4 °C, washed twice with PBS, and resuspended in 500 μl of ice-cold buffer (20 mM Hepes-KOH, pH 7.0, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 250 mM sucrose, and protease inhibitors). After incubation on ice for 20 min, cells were disrupted by Dounce homogenization. Nuclei were removed by centrifugation at 1,000 × g for 10 min at 4 °C. Supernatants were then further centrifuged at 100,000 × g for 1 h and the resulting supernatants were stored at −80 °C.

RESULTS

Free Radical Formation Induced by AAPH

The EPR spectrum of an air-saturated aqueous solution of 10 mM AAPH containing 50 mM of DMPO in PBS (50 mM, pH 7.4) after heating for 10 min at 44.0 °C is shown in Figure 1A. The EPR spectrum indicated by 'a' was analyzed as a primary nitrogen triplet ($a_N = 1.46$ mT), further split by two secondary protons ($a_H^{\beta} = 1.52$ mT). This spectrum is consistent with the spin adduct of alkoxyl or peroxyl radicals formed by the reaction of oxygen and alkyl radicals due to the thermal decomposition of reaction AAPH, as reported previously.[2,16] When an air-saturated aqueous solution of AAPH was exposed to a temperature of 80.0 °C, the EPR spectrum displayed in Figure 1B was obtained. The EPR spectrum indicated by 'b' was analyzed as

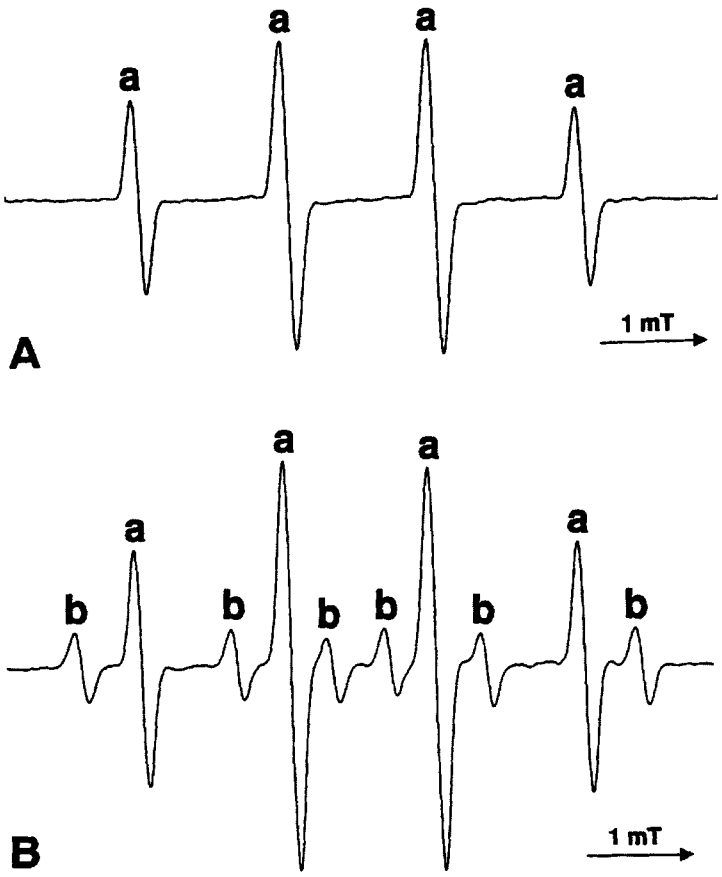

FIGURE 1 EPR spectra of the spin adducts from incubation of AAPH (10 mM) and DMPO (50 mM) for 8 min at 44.0 °C (A) or 80.0 °C (B) in a phosphate buffer (50 mM, pH 7.4). The hyperfine coupling constants were $a_N = 1.46$ mT, $a_H^\beta = 1.52$ mT in the spectrum labeled 'a', and $a_N = 1.54$ mT, $a_H^\beta = 2.54$ mT in the spectrum labeled 'b'. Instrumental settings of EPR: field, 335.5 mT; microwave power, 4.0 mW; sweep width, 5.0 mT; modulation width, 0.1 mT; sweep time, 1.0 min; time constant, 0.1 sec.

a primary nitrogen triplet ($a_N = 1.54$ mT), further split by two secondary protons ($a_H^\beta = 2.54$ mT). This spectrum is consistent with the spin adduct of the alkyl radical formed by the thermal decomposition of AAPH. A decrease in the EPR spectrum labeled 'a' and an increase in that labeled 'b' was observed in an argon-saturated aqueous solution of AAPH after heating (data not shown).

In order to explore the characteristics of thermally generating radicals from AAPH, EPR signal intensities were measured at different temperatures, graded concentrations of AAPH and incubation times. Figure 2A shows that free radical production increased with increasing the incubation time and temperature. Figure 2B shows that the production also increased with AAPH concentration and reached a plateau at a concentration of 50 mM. Here, the results show that free radical production due to the thermal decomposition of AAPH is temperature and concentration-dependent.

Apoptosis Induced by Hypertehrmia and the Combination with AAPH

The effects of different temperatures and AAPH concentrations on DNA fragmentation are shown in Figure 3. With increases in temperature, the percentage of DNA fragmentation

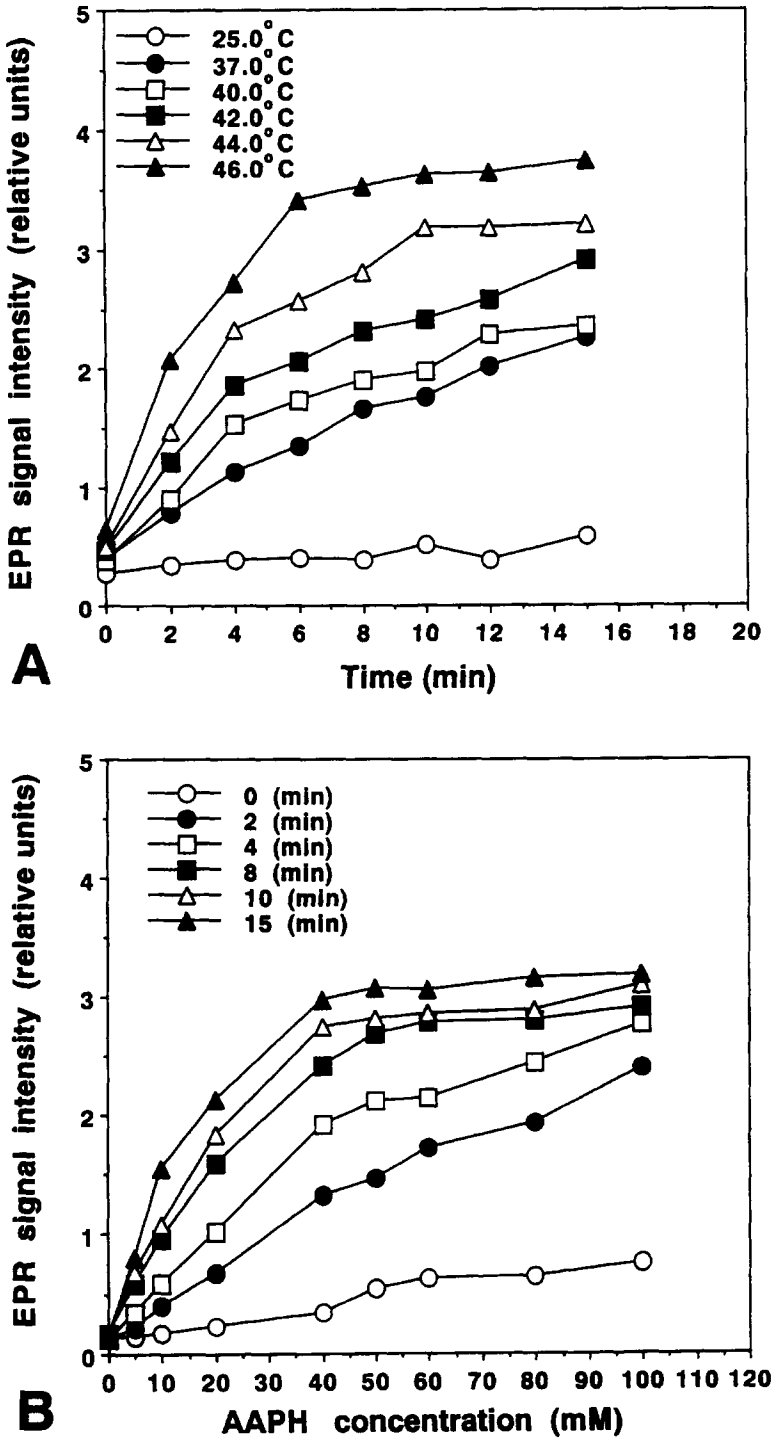

FIGURE 2 The relationships between EPR signal intensity of DMPO-spin adducts derived from AAPH and incubation time (A) or AAPH concentration (B). (A) Aerobic solutions of AAPH (50 mM) containing DMPO (40 mM) were treated at different temperatures for different incubation times; (B) An aerobic solution was treated at 44.0 °C with different AAPH concentrations for different incubation times. The DMPO concentration was 40 mM. The data shown in figure are representative of triplicate experiments.

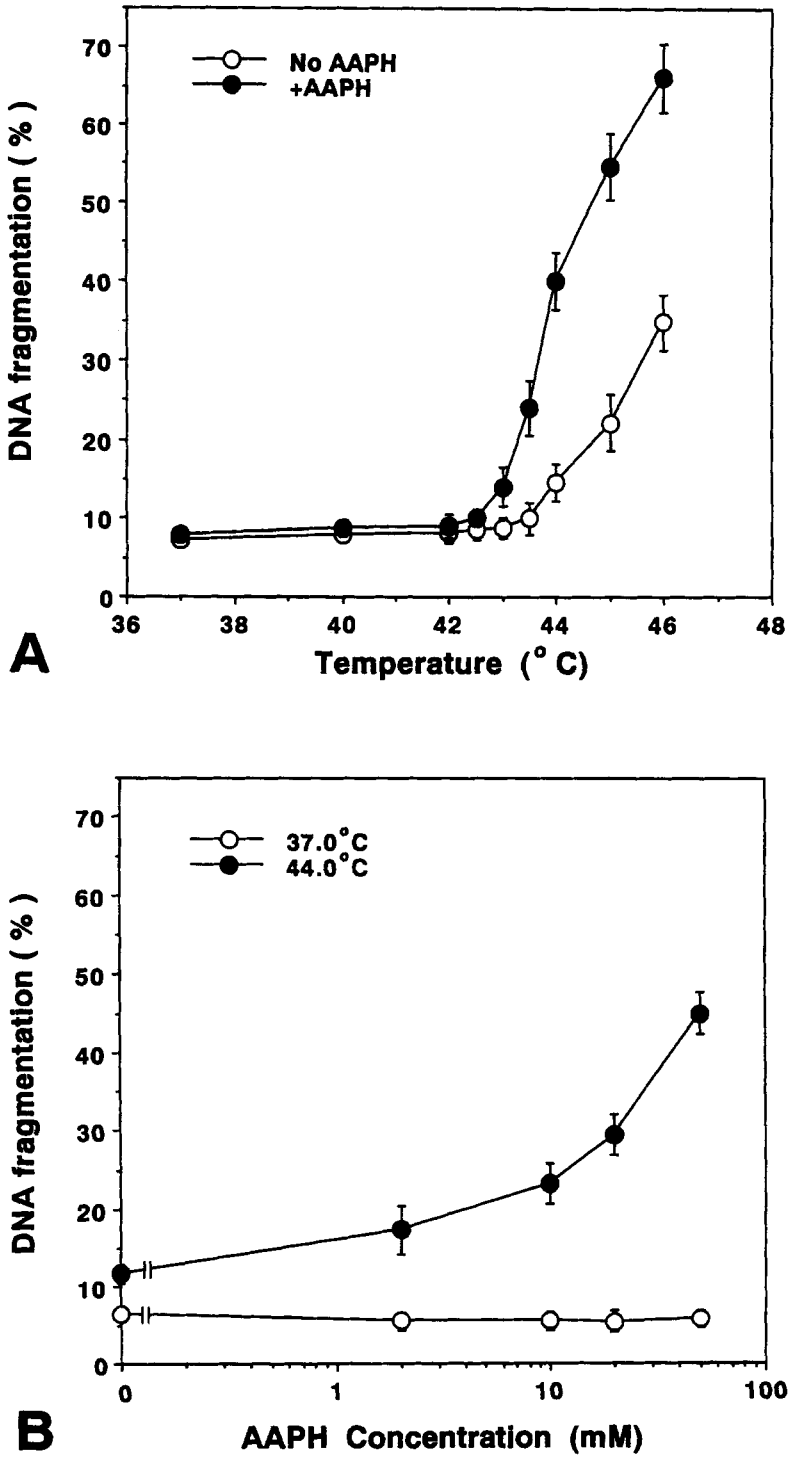

FIGURE 3 The effects of hyperthermia combined with AAPH on DNA fragmentation. DNA fragmentation was quantified after incubation for 6 h, with a diphenylamine reagent. (A) Percentage of DNA fragmentation at different temperatures in the presence or absence of 50 mM AAPH; (B) Percentage of DNA fragmentation after treatment of hyperthermia combined with different AAPH concentrations. Bars in figure represent means ± SD ($n = 3$).

increased gradually in the presence or absence of 50 mM of AAPH. Since a significant increase in DNA fragmentation was observed at 44.0 °C, this temperature was selected for the following hyperthermia experiments. AAPH enhanced the DNA fragmentation induced by hyperthermia in a concentration-dependent manner. In contrast, AAPH alone at 37.0 °C did not induce significant DNA fragmentation. Simultaneously, cells were stained with Giemsa for quantification of morphological apoptosis, including nuclear condensation and nuclear fragmentation. The results of morphological apoptosis coincided with those of DNA fragmentation.

Previous studies showed that PS externalization is a specific marker for apoptosis and may serve for the recognition, and subsequent removal, of apoptotic cells by phagocytosis.[3] Figure 4 shows early apoptosis and secondary necrosis of U937 cells when they were treated for 10 min at 44.0 °C and analysed with a flow cytometer after incubation for 6 h. Hyperthermia alone, or the combined treatment with AAPH, produced significant accumulation of U937 cells in the bottom right quadrant (Annexin V/FITC-positive and PI-negative cells are categorized as early apoptosis). In particular, the group treated with hyperthermia combined with AAPH, as shown in Figure 4D, showed early apoptosis. Simultaneously, the top right quadrant cells, called Annexin V/FITC and PI double-positive cells, also increased significantly. These represent the late phase of apoptosis and are known as secondary necrosis. The overall percentage of early apoptosis was $1.5 \pm 0.5\%$ in control cells, $2.4 \pm 0.4\%$ in cells treated with AAPH alone, $3.1 \pm 1.0\%$ in cells treated with hyperthermia alone, and $15.1 \pm 2.5\%$ in cells treated with hyperthermia and AAPH, respectively (mean $\pm$ SD, $n = 3$). The percentage of secondary necrosis was $6.4 \pm 2.1\%$ in control cells, $8.2 \pm 2.0\%$ in cells treated with AAPH alone, $12.2 \pm 5.6\%$ in cells treated with hyperthermia alone, and $37.1 \pm 9.3\%$ in cells treated with hyperthermia and AAPH, respectively (mean $\pm$ SD, $n = 3$).

Since the roles of Bcl-2 and Bax in apoptosis have been reported,[18] both apoptosis-associated proteins were studied regarding their effects on cells treated with hyperthermia and the combination with AAPH (Figure 5). The results revealed AAPH or hyperthermia alone did not change the expression of Bcl-2 or Bax. In contrast, after treatment with hyperthermia combined with AAPH, Bcl-2 expression began to decrease after 3 h incubation and Bax protein expression significantly increased after 6 h incubation.

Kinetics of Early apoptosis, DNA Fragmentation, LPO and Loss of $\Delta\Psi m$

Figure 6A shows the induction of early apoptosis stained with Annexin V/FITC positively and with PI negatively (cells with PS externalization) at various incubation times. After incubation for 1 h, the percentage of Annexin V/FITC-positive/PI-negative cells in the group treated with hyperthermia combined with AAPH increased 7–10 fold greater compared with the control cells (37.0 °C). The percentage reached 21.3% after 3 h and then decreased after 6 h of culture, indicating that some cells became secondary necrosis (Annexin V/FITC/PI double-positive cells). The kinetic analyses of DNA fragmentation after different treatments is shown in Figure 6B. The percentage of DNA fragmentation in cells treated with hyperthermia combined with AAPH began to increase after 1 h, then increased with incubation time. Hyperthermia induced early apoptosis and DNA fragmentation to a much lesser extent.

LPO is a free radical mediated chain reaction occurring in unsaturated fatty acids of membrane phospholipids which form lipid hydroperoxides. These are further degraded into mixtures of secondary products, which have been evaluated by the TBA assay to estimate their oxidative damage in biological systems.[12] Because the addition of water-soluble AAPH generates free radicals in the aqueous phase, we measured the

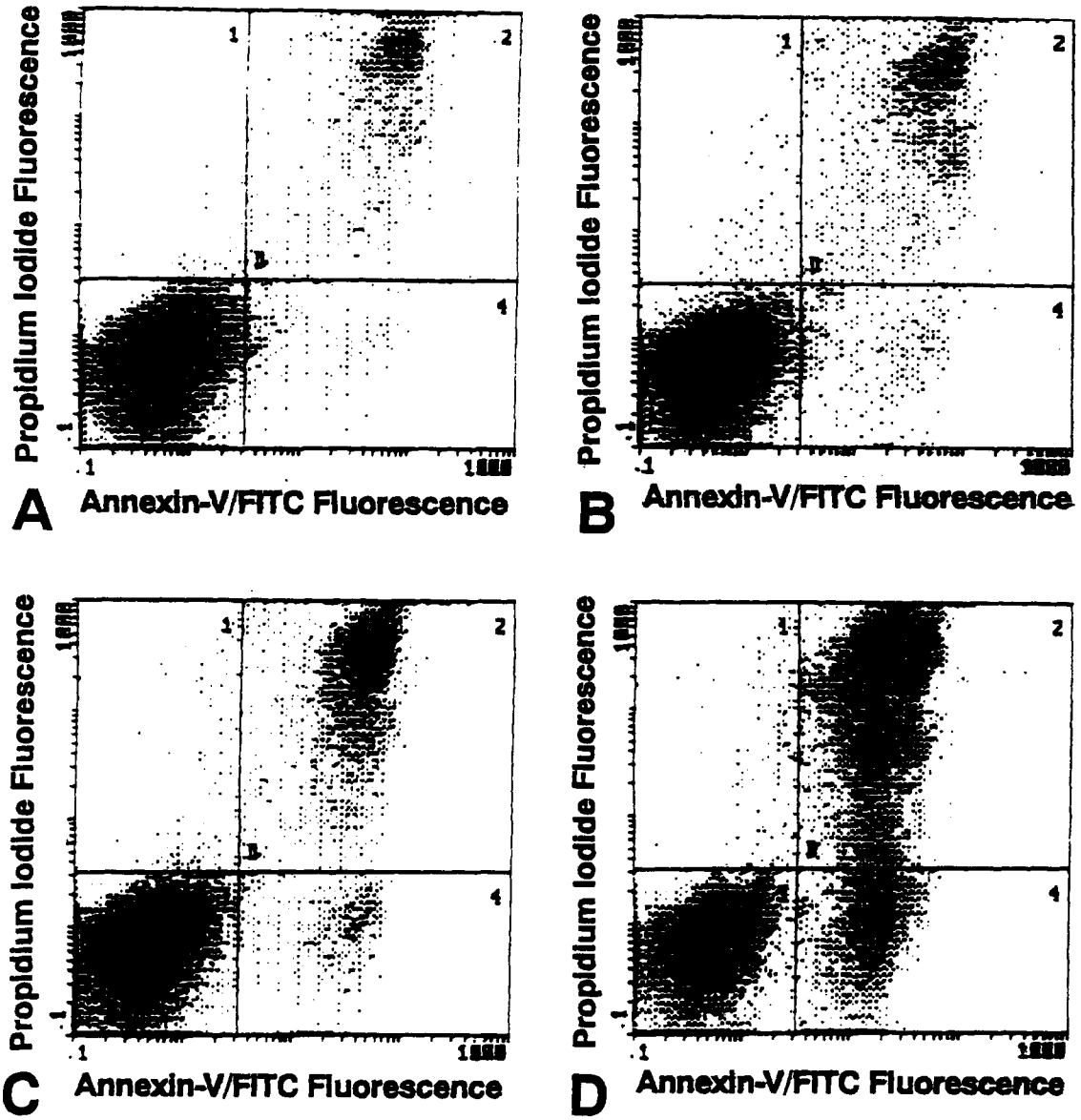

FIGURE 4 Flow cytometric analyses in U937 cells. Cells were collected after incubation for 6 h and analyzed with flow cytometry. (A) 37.0 °C; (B) 37.0 °C + 50 mM AAPH; (C) 44.0 °C; (D) 44.0 °C + 50 mM AAPH. The data are representative of three experiments.

MDA levels in both the cells and medium. Figure 6C shows the MDA levels in samples treated with hyperthermia, and the combination with hyperthermia and AAPH, were significantly higher than those of the controls. MDA levels increased markedly after 10 min, suggesting that LPO was produced immediately after hyperthermia treatment.

We also monitored the integrity of $\Delta\Psi m$ during the different treatments using the potential-sensitive dye $DiOC_6(3)$. Loss of $\Delta\Psi m$ was apparent when cells were treated with

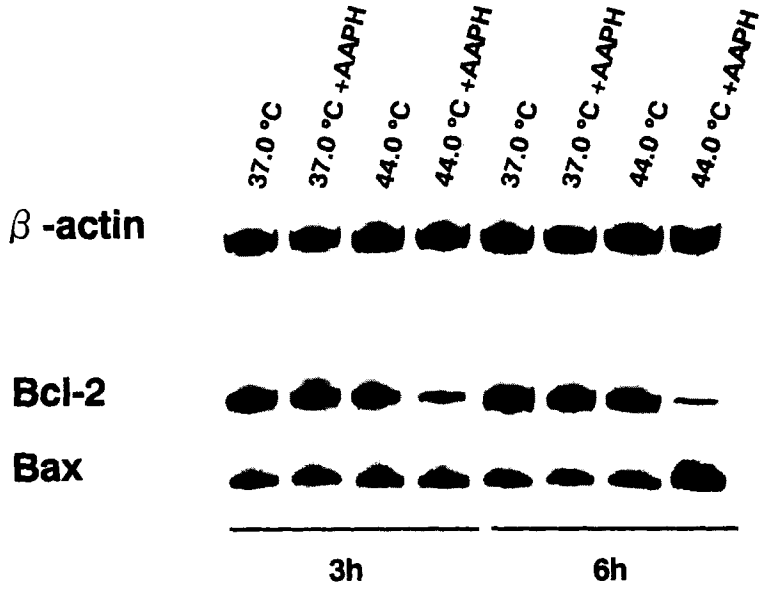

FIGURE 5 Western blotting analyses of Bcl-2 and Bax expression in U937 cells. Cells were collected after incubation for 3 h and 6 h. After being lysed in a RIPA buffer for 15 min, equal amounts of protein (30 μg) were electrophoresed, transferred to Immobilon, blocked with dry milk (5%) for 2 h, and incubated with mouse monoclonal IgG1 Bcl-2 (1:500) or rabbit polyclonal IgG Bax (1:500) for 3 h at room temperature. Bcl-2 and Bax were detected after incubation with HRP-Linked antimouse IgG and anti-Rabbit IgG (1:1000), respectively, for 1 h, followed by chromogenic visualization with BLAST 4CN Plus.

hyperthermia combined with AAPH. However, AAPH treatment or hyperthermia alone did not induce any significant loss of $\Delta\Psi m$ (Figure 6D).

Effects of Hyperthermia Combined with AAPH on $[Ca^{2+}]i$ and Expression of IP_3R1

Cells treated with AAPH, hyperthermia and the combination of hypertehrmia and AAPH and $[Ca^{2+}]i$ were examined after incubation for 6 h. Histograms of $[Ca^{2+}]i$ in 100 randomly selected cells are shown in Figure 7. AAPH alone did not induce an increase in $[Ca^{2+}]i$. The mean $[Ca^{2+}]i$ was 45.7 ± 10.6 nM (mean $\pm$ SD) and the value was similar to the controls (42.2 ± 8.1 nM). When cells were treated with hyperthermia, the number of cells showing higher $[Ca^{2+}]i$ increased; the mean $[Ca^{2+}]i$ was 138.4 ± 33.3 nM. When the cells were treated with hyperthermia combined with AAPH, $[Ca^{2+}]i$ increased significantly. The mean $[Ca^{2+}]i$ reached 176.8 ± 49.3 nM. These results indicate that hyperthermia combined with AAPH increases $[Ca^{2+}]i$, which seems to enhance the effect of hyperthermia on $[Ca^{2+}]i$.

To explore the reasons for the increase in $[Ca^{2+}]i$, expressions of IP_3R proteins were examined by Western blotting. Although hyperthermia and the combination with hypertehrmia and AAPH induced an increase in expression of IP_3R1 (Figure 8), and no expression of IP_3R2 or IP_3R3 were detected (data not shown). This finding implies the increase of $[Ca^{2+}]i$ induced by hyperthermia or the combination is related to the elevation of IP_3R1. Free radical formation due to AAPH seems to have no effect on the expression of IP_3R1.

LPO $[Ca^{2+}]i$ and Apoptosis

The inhibitory effects of antioxidants on apoptosis has demonstrated the involvment of oxidative stress in the apoptosis pathway.[6,19] Recently, Sandstrom *et al.* found that lipid hydroperoxide could induce apoptosis in human T cell lines.[11]

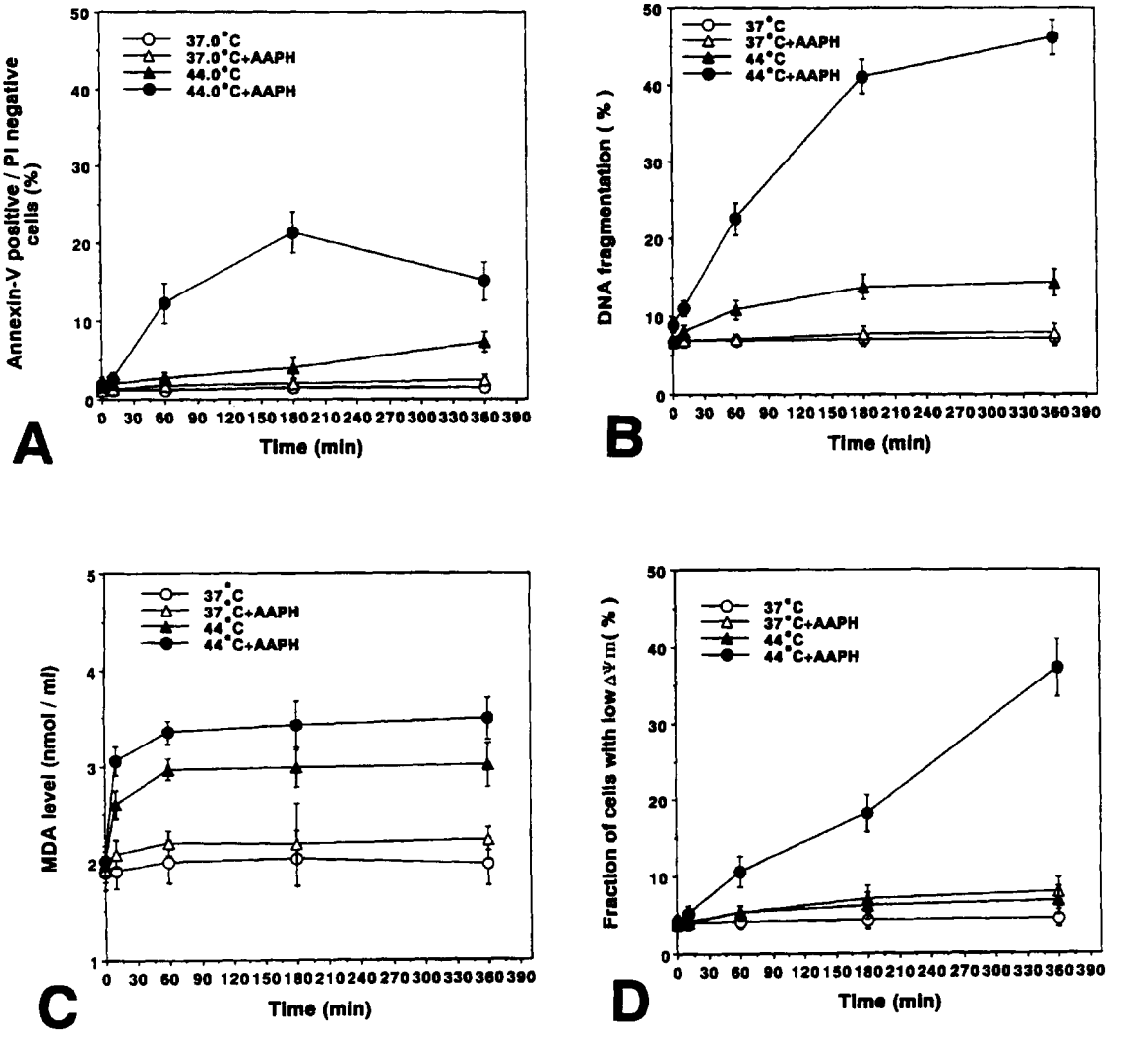

FIGURE 6 Kinetics of early apoptosis (PS externalization), DNA fragmentation, LPO and loss of $\Delta\Psi m$. Cells were collected at different incubation times after different treatments for 10 min. (A) A cell fraction from early apoptosis stained with Annexin V/FITC-positively and PI-negatively; (B) Percentage of DNA fragmentation; (C) Percentage of MDA levels; (D) Percentage of cells showing loss of $\Delta\Psi m$. Bars in figures represent means $\pm$ SD ($n = 3$).

Here, we demonstrated that Trolox showed a complete inhibition of the production of LPO induced by hyperthermia combined with AAPH (Figure 9A). Although Trolox inhibited DNA fragmentation in a dose-dependent manner, the inhibition rate at a concentration of 1 mM was only 35.6%. We also measured the effect of Trolox on the elevation of the $[Ca^{2+}]i$ induced by hyperthermia combined with AAPH in U937 cells. The results revealed that Trolox caused partial inhibition of the elevation in $[Ca^{2+}]i$ (Figure 7F), which coincided with the effect of Trolox on DNA fragmentation. When cells were treated with Trolox, the mean $[Ca^{2+}]i$ was 47.6 $\pm$ 11.1 nM (mean $\pm$ SD) and the value after treatment with hyperthermia combined with AAPH was 124.1 $\pm$ 30.6 nM. In addition, a cytosolic free Ca^{2+} chelator, BAPTA-AM, was used to examine

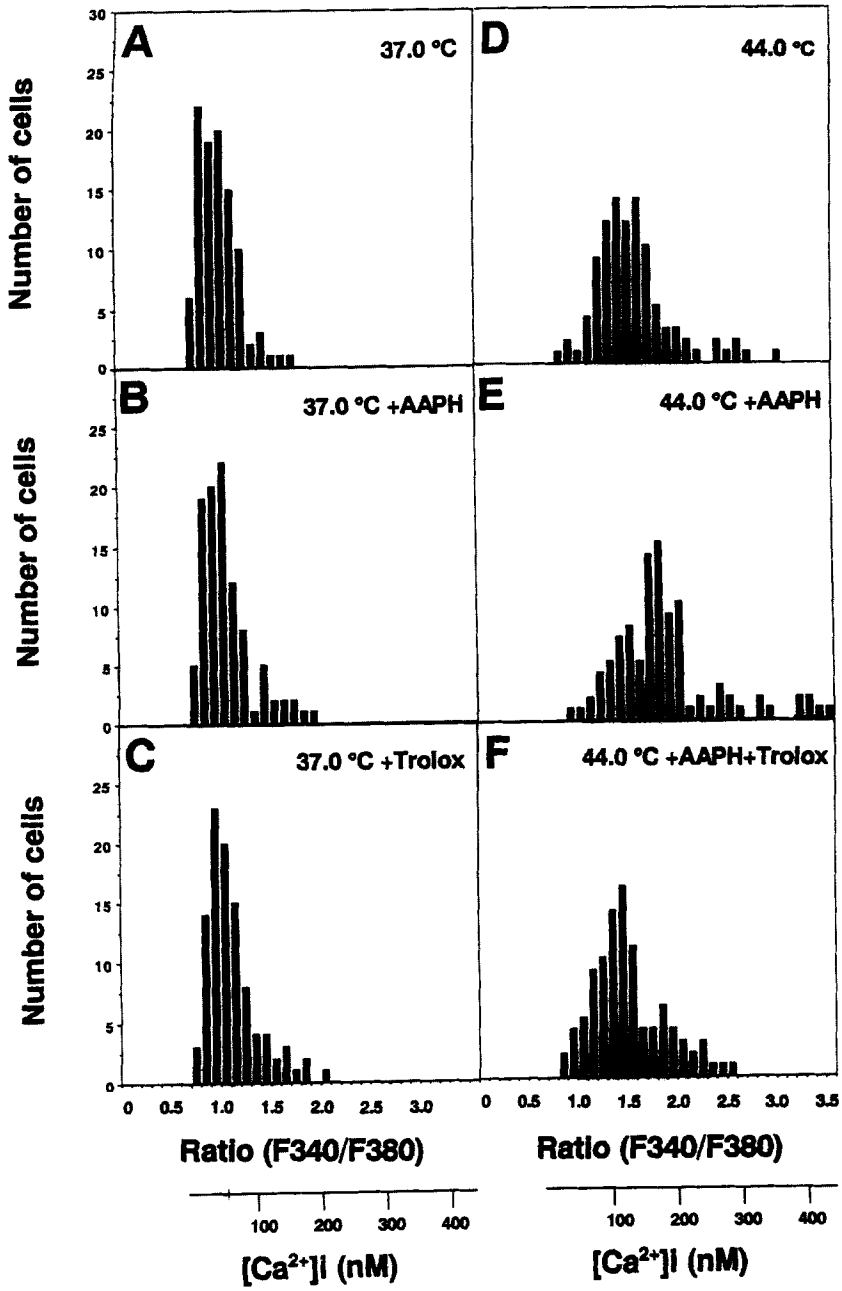

FIGURE 7 Histogram analyses of $[Ca^{2+}]i$ in U937 cells. After incubation for 6 h, the cells were harvested and loaded with fura-2/AM for 30 min and measured the fluorescent image of a single cell was made as described in 'Materials and Methods'. 100 cells were randomly selected and analyzed at a fluorescence ratio of 340 nm and 380 nm (F340/380) for a single cell. (A) 37.0 °C; (B) 37.0 °C + 50 mM AAPH; (C) 37.0 °C + 1 mM Trolox; (D) 44.0 °C; (E) 44.0 °C + 50 mM AAPH; (F) 44.0 °C + 50 mM AAPH + 1 mM Trolox.

the effect of $[Ca^{2+}]i$ on LPO and DNA fragmentation. Figure 9B shows that using BAPTA-AM resulted in the complete abrogation of the DNA fragmentation induced by hyperthermia combined with AAPH. However, BAPTA-AM failed to inhibit LPO. These data are consistent with

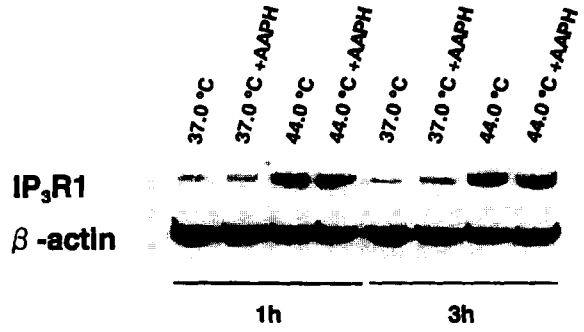

FIGURE 8 Western blotting of IP_3R1 expression in U937 cells. Cells were collected after incubation for 1 h and 3 h, respectively. After being lysed in an RIPA buffer for 15 min, equal amounts of protein (30 μg) were electrophoresed, transferred to Immobilon, blocked with dry milk (5%) for 2 h, and incubated with goat polyclonal IgG IP_3R1 (1:500) for 3 h at room temperature. IP_3R1 protein was detected after incubation with HRP-Linked anti-goat IgG (1:1000) for 1 h followed by chromogenic visualization with BLAST 4CN Plus.

the idea that LPO is upstream of Ca^{2+} fluxes during apoptosis induced by hyperthermia combined with AAPH in U937 cells, while appears to be only one reason causing the increase in $[Ca^{2+}]i$.

Cytochrome c Release and Effect of a Caspase-3 Inhibitor on Apoptosis

To study the role of mitochondria-caspase dependent pathway of apoptosis the cytochrome c release and effect of a caspase-3 inhibitor on apoptosis were examined. After 6 h incubation, the marked increase of expression of cytochrome c was observed in cytosolic extracts from cells treated with hyperthermia combined with AAPH (Figure 10).

In the presence of a caspase-3 inhibitor, Z-DEVD-FMK, for 8 h, 2 h before hyperthermia treatment and subsequent 6 h, the fraction of early apoptosis, i.e. Annexin V/FITC-positive and PI-negative cells, induced by hyperthermia combined with AAPH was $2.8 \pm 2.4\%$ (mean $\pm$ SD, $n = 3$), and was markedly suppressed as compared with that without the inhibitor ($27.9 \pm 7.7\%$ (mean $\pm$ SD, $n = 3$)).

Effects of the Combination of Hyperthermia and AAPH on Cell Growth

The growth curves of U937 cells after different treatments for 10 min are shown in Figure 11. At 37.0 °C, 50 mM of AAPH did not exert significant cytotoxicity in U937 cells. In contrast, after 6 h incubation, hyperthermia or the combination of hyperthermia and AAPH inhibited significantly the growth of cells. Although cell growth in cells treated with hyperthermia returned to normal after 21 h, a significant growth delay was still observed after combined treatment with hyperthermia and AAPH.

DISCUSSION

The present results demonstrate that hyperthermia induces apoptosis by increasing $[Ca^{2+}]i$, which is attributed to the enhancement of IP_3R1 expression and the production of LPO. Enhancement of the hyperthermia-induced apoptosis via the LPO-Ca^{2+} pathway and the additional mitochondria-caspase dependent pathway in cells treated with hyperthermia combined with AAPH has also been shown (see Figure 12).

No Induction of Apoptosis by Free Radicals Derived from AAPH at 37.0 °C

The EPR spin trapping study clearly showed that AAPH decomposed at near ambient temperatures, and that the rate of free radical production was temperature and concentration-dependent. However, despite the fact that AAPH decomposed and produces radicals at 37.0 °C, it was found to have relatively little effect on the cell growth of U937 cells at a concentration of 50 mM, and did not induce apoptosis. AAPH is a hydrophilic radical initiator which can only produce radicals in the aqueous region.[1] Therefore, hydrophilic AAPH dose not induce apoptosis in U937 cells, because no free

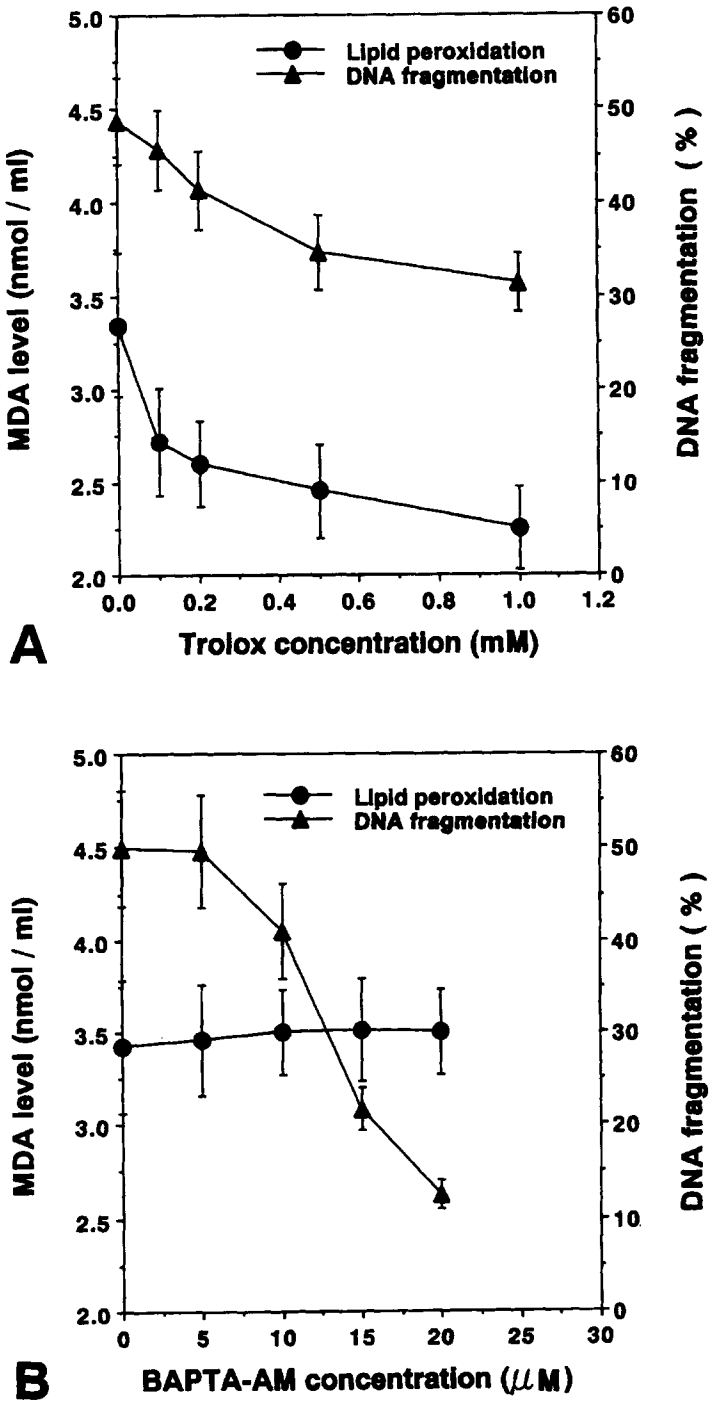

FIGURE 9 Effects of Trolox and BAPTA-AM on DNA fragmentation and lipid peroxidation. DNA fragmentation and MDA levels were quantified using a diphenylamine reagent and 2-thiobarbituric acid, respectively. (A) U937 cells were incubated for 3 h in the presence of Trolox after treatment with hyperthermia at 44.0 °C combined with 50 mM of AAPH for 10 min; (B) U937 cells were preincubated for 30 min in the presence of BAPTA-AM before treatment with hyperthermia combined with 50 mM of AAPH. BAPTA-AM was also presented in the medium during and after treatment for 3 h. Bars in figures represent means ± SD ($n = 3$).

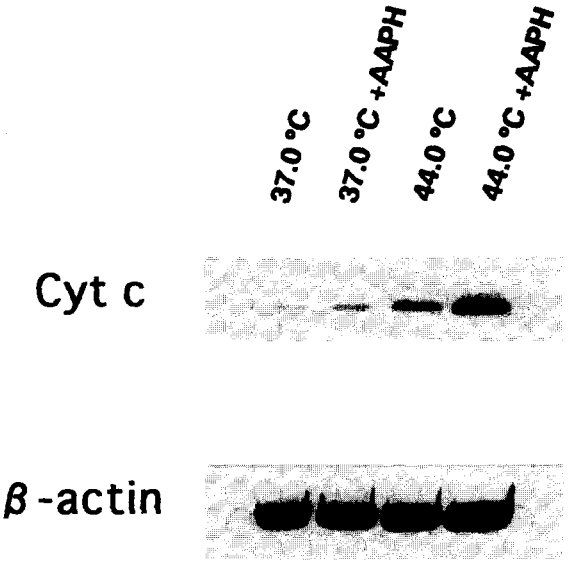

FIGURE 10 Western blotting analyses of cytochrome c expression in cytosol of U937 cells. Cells were collected after incubation for 6 h. Cytochrome c release to cytosol was monitored by Western blot according to the method described in Materials and Methods.

radicals enter the cells at 37.0 °C. In contrast, the lipophilic free radical initiator, AMVN, induces apoptosis.[3,4]

Apoptosis Induced by Hyperthermia

Although apoptosis induced by hyperthermia has been reported in human lung cancer PC10 cells[20] and human promyelocytic leukemia HL60 cells,[21] the mechanism is not clear. In this study, we demonstrated that hyperthermia could induce apoptosis in U937 cells. We also demonstrated that the production of LPO, the enhancement of IP_3R1 expression and the increase in $[Ca^{2+}]i$ after treatment with hyperthermia.

The mechanism by which LPO elicits apoptosis is unclear. It has recently been suggested that the form of cleavage which leads to the DNA fragmentation seen in apoptosis results from an accumulation of single-strand breaks.[11] Free radicals, and in particular lipid hydroperoxides, are capable of generating single-strand DNA breaks,[22] while the addition of 15-HPETE (a product of lipid hydroperoxides), to isolated A 3.01 nuclei did not elicit DNA fragmentation.[10] Such observations seem to argue against the direct effect of lipid hydroperoxides on either endonucleases or DNA. As we demonstrated, hyperthermia results in a marked increase of $[Ca^{2+}]i$ that is essential for the induction of apoptosis. Therefore, LPO induced by hyperthermia could induce apoptosis with the mediation of Ca^{2+}, rather than induce apoptosis directly.

Abundant evidence indicates that IP_3R is associated with non-mitochondrial Ca^{2+} stores, and the IP_3R1 calcium release channel is present on the endoplasmic reticulum (ER) of some cell types,[24,25] where it may regulate release of Ca^{2+}. In our experiment, we detected enhancement of IP_3R1 expression, while no IP_3R2 or IP_3R3 proteins were detected, when U937 cells were treated with hyperthermia or hyperthermia combined with AAPH. Our findings, in conjunction with other recent reports, convincingly show a causal role for IP_3R1 in U937 apoptosis and also indicate a possible mechanism of apoptosis, namely augmentation of Ca^{2+} entry through IP_3R1.

Ca^{2+} Channels in the ER

Collectively, these results suggest that intracellular Ca^{2+} plays a major role in the regulation of apoptosis induced by hyperthermia. The increase in $[Ca^{2+}]i$ is attributed to LPO and the enhancement of IP_3R1 expression.

AAPH Enhances Apoptosis Induced by Hyperthermia

Although it has been shown that AAPH enhanced hyperthermia-induced cytotoxicity,[2] the mechanism is not fully understood. The unique features of thermally generating radicals from AAPH suggested that hyperthermia combined with AAPH enhances cell death. In fact, our results showed that hyperthermia combined with AAPH could retard cell growth. Furthermore,

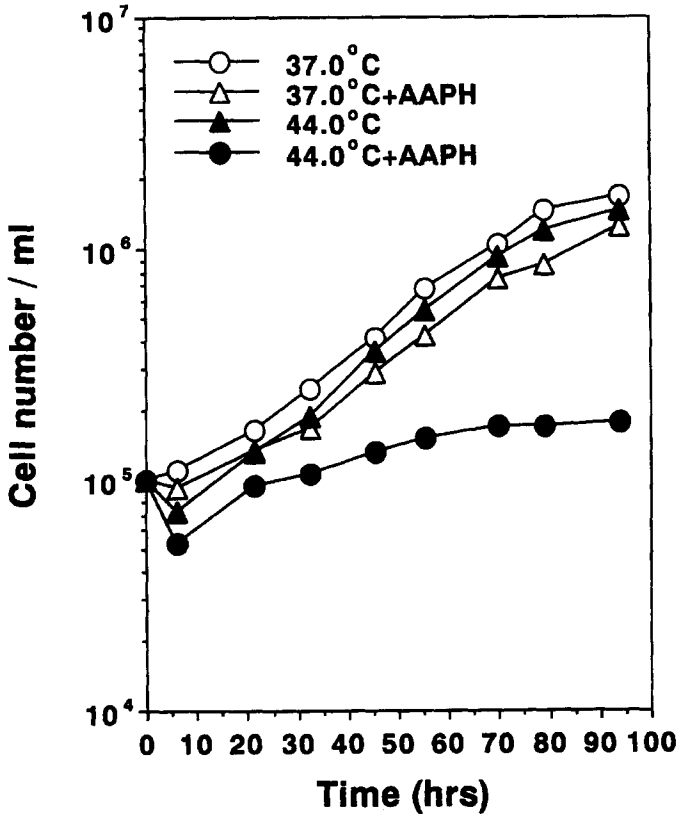

FIGURE 11 Growth curve of U937 cells after various treatments for 10 min. The data are representative of three independent experiments.

although AAPH alone could not induce apoptosis, hyperthermia combined with AAPH was capable of eliciting many significant cellular changes definitive of apoptosis, including PS externalization (Figure 4), DNA fragmentation (Figure 3) and some morphological changes (data not shown). Thus, apoptosis may be a mechanism of enhancement of the hyperthermia-induced cytotoxicity of AAPH. AAPH can be used as a hyperthermic sensitizer to enhance the cytotoxicity induced by hyperthermia.

The relationship between oxidative stress and $[Ca^{2+}]i$ has been extensively studied,[6,7] although the details of the biochemical and molecular events involved in the process are still far from clear. Here, we report for the first time that hyperthermia combined with AAPH induced an increase in $[Ca^{2+}]i$, which enhanced the effects of hyperthermia. The results of the present study indicate that these rises are secondary to the production of lipid peroxidation, as demonstrated by the inhibitory effect of antioxidants and an intracellular Ca^{2+} chelator, BAPTA-AM (Figure 9). Several possible biochemical mechanisms could account for the increase in $[Ca^{2+}]i$. First, peroxidation of the membrane lipids may be involved in the effects of AAPH on the PMCA,[9] further inducing the increase in $[Ca^{2+}]i$. Second possibility is that oxygen radicals themselves derived from the thermal decomposition of AAPH can mediate intracellular Ca^{2+} entry through voltage-operated Ca^{2+} channels.[23]

It was found that hyperthermia combined with AAPH also led to loss of $\Delta\Psi m$, indicating the function of the mitochondria was also

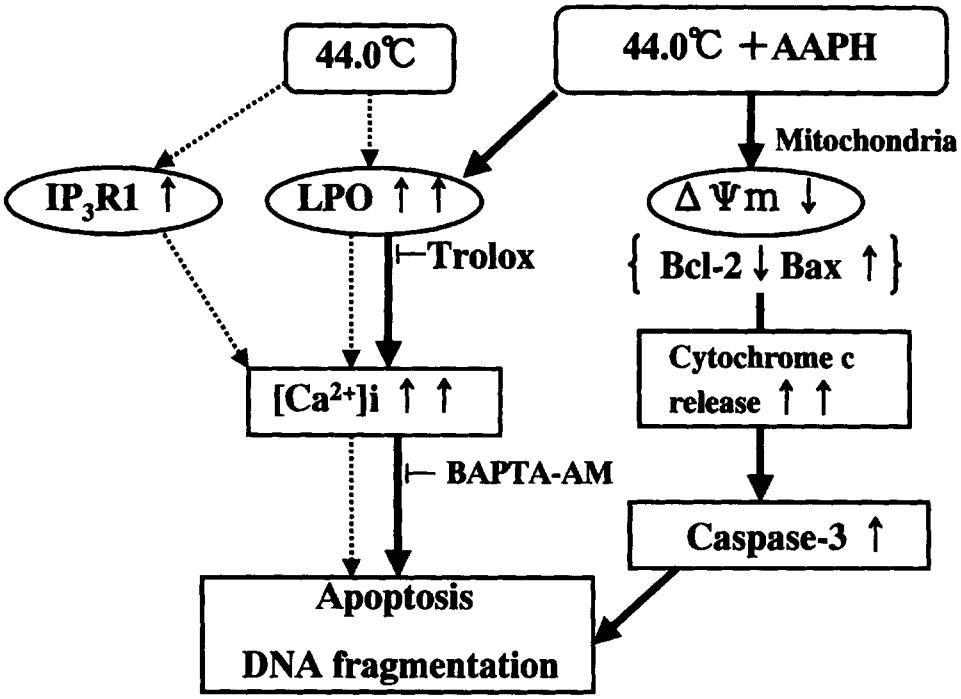

FIGURE 12 Hypothetical model showing the mechanism of enhancement of apoptosis by the combination of hyperthermia and AAPH.

damaged during this treatment course. It was recently demonstrated that apoptosis programing is associated with the release of cytochrome c from the mitochondria to the cytosol.[25] Furthermore, Tyurina *et al.* speculated that cytochrome c released to the cytosol may be involved in peroxidation of specific phospholipids.[3] Our study demonstrated significant increases in cytochrome c, and the significant suppression of apoptosis by a caspase-3 inhibitor. Therefore, hyperthermia combined with AAPH can damage the mitochondria, causing loss of $\Delta\Psi m$ and inducing the release of cytochrome c, and subsequently activate a caspase-dependent cell death pathway.

The relationship between loss of $\Delta\Psi m$ and the increase in $[Ca^{2+}]i$ remains elusive. It has recently been shown that loss of $\Delta\Psi m$ preceding nuclear apoptosis is mediated by the opening of permeability transition (PT) pores.[13] Many effectors that can induce apoptosis, including oxidative stress, can provoke PT pore opening.[26] Therefore, hyperthermia combined with AAPH could cause a loss of $\Delta\Psi m$ via PT pore opening. $\Delta\Psi m$ represents the electrochemical forces employed for ATP synthesis, as well as for other metabolic activities, including the maintenance of Ca^{2+} homeostasis within the mitochondria.[23] Due to the energy requirements of the different Ca^{2+} transport systems that control Ca^{2+} homeostasis, the loss of $\Delta\Psi m$ and subsequent ATP depletion invariably result in the disruption of intracellular Ca^{2+} homeostasis and potentially trigger a series of Ca^{2+}-dependent events that ultimately lead to cell death.

Although earlier studies reported that Bcl-2 expression was almost unaltered or slightly decreased, while Bax expression increased gradually, in human lung cancer PC-10 cells[20] during hyperthermia, in our study Bcl-2 and Bax

expression remained stable during hyperthermia, in good agreement with the results in the HL-60 human promyelocytic leukemia cell line.[21] In contrast, a significant decrease in Bcl-2 and an increase in Bax expression could be seen in cells treated with hyperthermia combined with AAPH. This result suggests that Bcl-2, as well as Bax, may be involved in the regulation of apoptosis induced by hyperthermia combined with AAPH. The addition of AAPH, following greater production of free radcials, enhanced the action of proapoptotic protein Bax and Bcl-2, and failed to protects cell from oxidative damage, further inducing apoptosis.

We used an alternative approach to describe hyperthermia combined with AAPH-induced apoptosis in terms of the relationship between the important physiological and biochemical events that occur during apoptosis, including the production of LPO, the activation of IP_3R1 and loss of $\Delta\Psi m$. Our data support the idea that hyperthermia combined with AAPH activates a Ca^{2+}-dependent pathway and a mitochondria-caspase-dependent pathway for apoptosis. In future clinical applications of AAPH or alternative azo compounds for hyperthermic cancer therapy, water soluble free radical initiators appear to be strong hyperthermic sensitizers for specifically heated-regions, as they are not so toxic and difficult to access inside cells or tissues, while free radicals derived from these compounds become highly toxic only for tissues or cells exposed to hyperthermia. The alternation of plasma membranes by hyperthermia makes cells more susceptible to the attack of free radicals due to these compounds. Our results indicate that free radicals due to AAPH did not induce apoptosis at 37.0 °C, though a lipophilic azo compound, AMVN, can induce apoptosis at 37.0 °C. Considering the characteristics of thermally generating radicals from AAPH, our observations may give insights into new potential methods of cancer treatment designed to overcome drug resistance caused by the failure to induce apoptosis.

Acknowledgements

This study was supported by the Japan–China Medical Association Nippon Foundation. This study was sopprted in part by the Grant in Aid for Scientific Research on Priority Areas (C) (12217049) from the Ministry of Education, Science, Sports and Culture, Japan.

References

[1] E. Niki (1990) Free radical initiators as source of water- or lipid soluble peroxyl radicals. *Methods in Enzymology*, **186**, 100–108.

[2] M.C. Krishna, M.W. Dewhirst, H.S. Friedman, J.A. Cook, W. Degraff, A. Samuni, A. Russo and J.B. Mitchell (1994) Hyperthermic sensitization by the radical initiator 2,2′-azo-bis(2-amidinopropane) dihydrochloride(AAPH). I. *In vitro* studies. *International Journal of Hyperthermia*, **10**, 271–278.

[3] Y.Y. Tyurina, V.A. Tyurin, G. Carta, P.J. Quinn, N.F. Schor and V.E. Kagan (1997) Direct evidence for antioxidant effect of bcl-2 in PC12 rat pheochromocytoma cells. *Archives of Biochemistry and Biophysics*, **344**, 413–423.

[4] J.P. Fabisiak, Y.Y. Tyurina, V.A. Tyurin, J.S. Lazo and V.E. Kagan (1998) Random versus selective membrane phospholipid oxidation in apoptosis: role of phosphatidylserine. *Biochemistry*, **37**, 13781–13790.

[5] R. Asmis and E.S. Wintergerst (1998) Dehydroascorbic acid prevents apoptosis induced by oxidized low-density lipoprotein in human monocyte-derived macrophages. *European Journal of Biochemistry*, **255**, 147–155.

[6] E.L. Ridgley, Z.H. Xiong and L. Ruben (1999) Reactive oxygen species activate a Ca^{2+}-dependent cell death pathway in the unicellular organism trypanosoma brucei brucei. *Biochemical Journal*, **340**, 33–40.

[7] M.M. Rimpler, U. Rauen, T. Schmidt, T. Moroy and H. de Groot (1999) Protection against hydrogen peroxide cytotoxicity in rat-1 fibroblasts provided by the oncoprotein Bcl-2: maintenance of calcium homoeostasis is secondary to the effect of Bcl-2 on cellular glutathione. *Biochemical Journal*, **340**, 291–297.

[8] C. Kimura, Q.L. Zhao, T. Kondo, M. Amatsu and Y. Fujiwara (1998) Mechanism of UV-induced apoptosis in human leukemia cells: roles of Ca^{2+}/Mg^{2+}-dependent endonuclease, caspase-3, and stress-activated protein kinases. *Experimental Cell Research*, **239**, 411–422.

[9] M. Li, T. Kondo, Q.L. Zhao, F.J. Li, K. Tanabe, Y. Arai, Z.C. Zhou and M. Kasuya (2000) Apoptosis induced by cadmium in human lymphoma U937 cells through Ca^{2+}-calpain and caspase-mitochondria-dependent pathways. *Journal of Biological Chemistry*, **275**, 39702–39709.

[10] A. Zaidi and M.L. Michaelis (1999) Effects of reactive oxygen species on brain synaptic plasma membrane Ca^{2+}-ATPase. *Free Radical Biology and Medicine*, **27**, 810–821.

[11] P.A. Sandstrom, D. Pardi, P.W. Tebbey, R.W. Dudek, D.M. Terrian, T.M. Folks and T.M. Buttke (1995) Lipid hydroperoxide-induced apoptosis: lack of inhibition by bcl-2 over-expression. *FEBS Letter*, **365**, 66–70.

[12] V.E. Kagan, M. Tsuchiya, E. Serbinova, L. Packer and H. Sies (1993) Interaction of the pyridoindole stobadine

with peroxyl, superoxide and chromanoxyl radicals. *Biochemical Pharmacology*, **45**, 393–400.

[13] A. Macho, T. Hirsch, I. Marzo, P. Marchetti, B. Dallaporta, S.A. Susin, N. Zamzami and G. Kroemer (1997) Glutathione depletion is an early and calcium elevation is a late event of thymocyte apoptosis. *Journal of Immunology*, **158**, 4612–4619.

[14] K.S. Sellins and J.J. Cohen (1987) Gene induction by gamma-irradiation leads to DNA fragmentation in lymphocytes. *Journal of Immunology*, **139**, 3199–3206.

[15] J.A. Buege and S.D. Aust (1978) Microsomal lipid peroxidation. *Methods in Enzymology*, **52**, 302–310.

[16] T. Kondo, E. Kano, Y. Habara and T. Kanno (1993) Enhancement of cell killing and increase in cytosolic calcium concentration by combined treatments with hyperthermia and TMB-8 in mouse mammary carcinoma FM3A cells. *Cell Calcium*, **14**, 621–629.

[17] V. Misik, N. Miyoshi and P. Riesz (1996) EPR spin trapping study of the decomposition of azo compounds in aqueous solutions by ultrasound: potential for use as sonodynamic sensitizers for cell killing. *Free Radical Research*, **25**, 13–22.

[18] D.M. Hockenbery, Z.N. Oltvai, X.M. Yin, C.L. Milliman and S.J. Korsmeyer (1993) Bcl-2 function in an antioxidant pathway to prevent apoptosis. *Cell*, **75**, 241–251.

[19] P. Nicotera, P. Hartzell, C. Baldi, S.A. Svensson, G. Bellomo and S. Orrenius (1986) Cystamine induces toxicity in hepatocytes though the elevation of cytosolic Ca^{2+} and the stimulation of a non-lysosomal proteolytic system. *Journal of Biological Chemistry*, **261**, 14628–14635.

[20] M. Nishita, S. Inoue, M. Tsuda, C. Tateda and T. Miyashita (1998) Nuclear translocation and increased expression of Bax and disturbance in cell cycle progression without prominent apoptosis induced by hyperthermia. *Experimental Cell Research*, **244**, 357–366.

[21] T. Takasu, J.C. Lyons, H.J. Park and C.W. Song (1998) Apoptosis and perturbation of cell cycle progression in an acidic environment after hyperthermia. *Cancer Research*, **58**, 2504–2508.

[22] T. Ochi and P.A. Cerutti (1987) Clastogenic action of hydroperoxy-5,8,11,13-icosatetraenoic acids on the mouse embryo fibroblasts C3H/10T1/2. *Proceedings of the National Academy of Sciences* USA, **84**, 990–994.

[23] P. Nicotera, G. Bellomo and S. Orrenius (1992) Calcium-mediated mechanisms in chemically induced cell death. *Annual Review of Pharmacology and Toxicolology*, **32**, 449–470.

[24] T. Jayaraman and A.R. Marks (1997) T cells deficient in inositol 1,4,5-trisphosphate receptor are resistant to apoptosis. *Molecular and Cellular Biology*, **17**, 3005–3012.

[25] X. Liu, C.N. Kim, J. Yang, R. Jemmerson and X. Wang (1996) Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell*, **86**, 147–157.

[26] P. Costantini, B.V. Chernyak, V. Petronilli and P. Bernardi (1996) Modulation of the mitochondrial permeability transition pore by pyridine nucleotides and dithiol oxidation at two separate sites. *Journal of Biological Chemistry*, **271**, 6746–6751.