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Protection by ascorbic acid from denaturation and release of cytochrome *c*, alteration of mitochondrial membrane potential and activation of multiple caspases induced by H_2O_2, in human leukemia cells☆

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Abstract

We investigated peroxide and superoxide accumulation, cytochrome *c* nature and release from mitochondria, as well as caspase activation during exposure of HL-60 cells to H_2O_2 and the protective effect of ascorbic acid. Exposure of the cells to 100 μM H_2O_2 resulted in intracellular accumulation of peroxides, denaturation of cytochrome *c* that was identified in the mitochondria and cytosol, release of native cytochrome *c* to the cytosol and fall in mitochondrial membrane potential ($\Delta\Psi_m$). Loading of cells with ascorbic acid before exposure to H_2O_2 resulted in a dose-dependent protective effect against: intracellular accumulation of peroxides, $\Delta\Psi_m$ alteration, cytochrome *c* denaturation and release. The accumulation of peroxides induced processings and activations of procaspase-8, -9 and -3. Double staining with antiserum which recognizes the p18 subunit of cleaved caspase-3 and with Hoechst had shown that a high percentage of cells exposed to 100 μM H_2O_2 stained positively with the antibody and showed features of apoptosis. Ascorbic acid loading prevented caspase activation induced by H_2O_2. We conclude that ascorbic acid protects against activation of apoptotic cascades in HL-60 cells exposed to H_2O_2 and against apoptosis.

Keywords: Ascorbic acid; Caspases; Cytochrome *c*; Free radicals

1. Introduction

It is widely known that reactive oxygen species including superoxide and H_2O_2 formed in association with a variety of oxidative stress-induced disorders, may be linked to excess cell loss. Hydrogen peroxide, an important intracellular compound that influences cellular redox state, modulates gene expression and signaling [1,2] can induce oxidative damage to membrane lipids [3], proteins [4] and DNA [5]. Other *in vitro* studies have shown degradation of cytochrome *c* by H_2O_2 [6]. Hydrogen peroxide can induce necrosis or apoptosis in cells, depending on the severity of damage to macromolecules [7,8]. The mechanisms by which it induces apoptosis in cells are not fully defined, it seems that activation of caspases is involved.

The two most well-studied pathways of caspase activation include the surface death receptor pathway and the mitochondrion-initiated pathway [9].

The first pathway involves apoptosis mediated by death receptors, such as Fas or tumor necrosis factor receptors. In the second pathway, diverse proapoptotic signals converge at the mitochondrial level. As a result of various apoptotic triggers cytochrome *c* becomes translocated from the mitochondrial intermembrane compartment into the cytosol [10]. Once released, cytochrome *c* binds to Apaf-1 and induces activation of caspase-9 [11], which in turn cleaves and activates the executioner caspases, such as caspase-3 and -7 [12]. The release of cytochrome *c* has been linked to loss of Ψ_m and permeability transition [13], although there

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Abbreviations: CLSM, confocal laser scanning microscopy; DHA, dehydroascorbic acid; DCF, 2′,7′-dichlorofluorescin; DCFH-DA, 2′,7′-dichlorofluorescin-diacetate; DEVD-AMC, Asp-Glu-Val-Asp-7-amino-4-methylcoumarine; $DiOC_6(3)$, 3,3′-dihexyloxacarbocyanine iodide; FITC, fluorescein isothiocyanate; mAb, monoclonal antibody; PBS, phosphate buffered saline; PI, propidium iodide; Ψ_m, mitochondrial membrane potential.

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are also reports providing evidence that these are independent events [14]. Recent studies have shown that H_2O_2 stimulates mitochondrial cytochrome *c* release and activates caspase-3 [15].

Ascorbic acid is important as a cofactor of several biosynthetic enzyme reactions and is widely used as an antioxidant [16]. However, ascorbic acid can act as an antioxidant or a pro-oxidant, for example, in the Fenton reaction with iron [17]. Under physiological conditions, ascorbic acid has predominantly an antioxidant role [18]. In a recent study we have shown that ascorbic acid inhibits apoptosis induced by H_2O_2 in HL-60 myeloid leukemia cells [19]. The aim of this study was to elucidate intracellular pathways involved in H_2O_2-induced activation of apoptotic processes in HL-60 cells (including the effect on degradation and release of cytochrome *c*) and the protective abilities of ascorbic acid on these pathways.

Our present data reveal that exposure of HL-60 cells to H_2O_2 is associated with denaturation of part of cytochrome *c* (which can be identified in the mitochondria and cytosol) as well as release of native cytochrome *c* to the cytosol, reduction in mitochondrial membrane potentials and activations of caspase-8, -9 and -3.

Loading of the cells with ascorbic acid decreases denaturation of cytochrome *c* and the activation of the H_2O_2-induced apoptotic cascade.

2. Materials and methods

2.1. Reagents

Iscove's Modified Dulbecco's Medium was purchased from Gibco. Fetal bovine serum was purchased from HyClone Laboratories. Ascorbic acid-sodium salt, bisbenzimide (Hoechst 33258 dye) and fluorescein isothiocyanate (FITC)-conjugated F(ab′) 2 fragment of goat anti-mouse immunoglobulin were purchased from Sigma Chemical Co. Purified mouse-anti-cytochrome *c* monoclonal antibody (mAb) clone 7H.2C12 that recognizes the denatured form of human cytochrome *c*, clone 6H2.B4 that recognizes the native form of human cytochrome *c* and mouse anti-human caspase-8 mAb were purchased from Pharmingen. Anti-caspase-9 was a generous gift from XIMENA OPITZ-ARAYA. A fluorogenic substrate for caspase-9 (Ac-LEHD-AML) and the caspase-3 inhibitor Acetyl-Asp-Glu-Val-Asp-aldehyde (Ac-DEVD-CHO) were from Calbiochem. A fluorogenic substrate for caspase-3 (CPP32), DEVD-AMC was purchased from Biomol Research Lab. The polyclonal antiserum, CM1, which recognizes the p18 subunit of cleaved caspase-3 but not the zymogen [20] was provided by IDUN Pharmaceutical. Anti-caspase-3 polyclonal antibody which reacts with the p20 subunit and the precursor of caspase-3 was purchased from Santa Cruz Biotechnology. Indocarbocyanine Cy3 antibody was purchased from Jackson ImmunoRes. Lab. Mito Tracker Red CMXRos, 2′,7′-dichlorofluorescin-diacetate (DCFH-DA) and 3,3′-dihexyloxacarbocyanine iodide [$DiOC_6(3)$] were purchased from Molecular Probes. Gel Mount was purchased from Biomeda. Annexin V-FITC was purchased from R&D Systems.

2.2. Cell culture and treatment with H_2O_2

HL-60 cells were grown in Iscove's Modified Dulbecco's Medium supplemented with 10% heat inactivated fetal bovine serum, 2 mM L-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, at 37° in a humidified incubator with 5% CO_2. Cells were pre-incubated for 15 min in the presence or absence of 50–250 μM DHA as previously described by us [19,21]. Following pre-incubation the cells were exposed to various concentrations of H_2O_2 (as detailed in Sections 2 and 3).

2.3. Detection of peroxides

Intracellular oxidation of DCFH-DA by active oxygen species to fluorescent dichlorofluorescin (DCF) was used to quantitate peroxide and superoxide generation in cells. An adaptation of the method of Jayanthi *et al*. was used [22]. Cells were pre-incubated (1×10^6/mL) in incubation buffer in the presence or absence of 50–250 μM DHA for 10 min. Following pre-incubation the cells were washed and 40 μM DCFH-DA were added in medium, the cells were kept in the dark for 15 min at room temperature. Cells were then washed in darkness, resuspended in Hank's Balanced Salt Solution and plated at a density of 3750 cells per well of a 96-well plate. To the wells were added 100 μM H_2O_2 and DCF fluorescence was measured over a 2 hr time period with a spectrofluorometer (FL-600 microplate Fluorescence Reader, Biotek Belgium) with excitation at 488 nm and emission at 525 nm. The fluorescence increase with time was expressed as relative fluorescence values normalized to the initial reading.

2.4. Analysis of H_2O_2-denatured cytochrome *c* by flow cytometry

HL-60 cells were pre-incubated in incubation buffer in the absence or presence of 250 μM DHA and exposed to 100 μM H_2O_2 for 0–16 hr. Following incubation the cells were washed, fixed with 3.7% paraformaldehyde and treated with acetone at −20° for 10 min. After two washings, cells were incubated with anti-cytochrome *c* mAb (clone 7H8.2C12 diluted 1:50) for 30 min at 4°. They were then exposed to the secondary FITC-conjugated F(ab′)2 fragment of goat anti-mouse IgG. Cells were analyzed by flow cytometry with a FACScan apparatus (Beckton Dickinson). The corresponding control consisted of identical staining except for the use of an irrelevant mouse IgG of identical isotype (Dako) instead of the anti-cytochrome

c antibody. Intensity of fluorescence was expressed as mean channel fluorescence.

2.5. Cytochrome c confocal microscopy analysis

2.5.1. Hydrogen peroxide-denatured cytochrome c

HL-60 cells were pre-incubated in buffer in the absence or presence of 250 μM DHA and exposed to 100 μM H_2O_2 for 0–6 hr as described above. Following incubation the cells were labeled with Mito Tracker Red (20 min at 37°; 250 nM) and cytocentrifuge preparations were made. The cells were fixed with methanol and acetone at −20° for 10 min as previously described [23], blocked with 2% bovine serum albumin and 10% normal sheep serum (prepared in PBS) for 20 min at room temperature and incubated for 1 hr with 1:50 anti-cytochrome *c* mAb (clone 7H8.2C12). After three washes with PBS, the cells were revealed by using solutions of secondary antibody conjugated with FITC. Slides were visualized using a Zeiss confocal laser scanning microscope (CLSM). The Zeiss CLSM 410 is equipped with a 25 mW Krypton–Argon laser and a 10 mW Helium–Neon laser. Images were stored on an optical disk drive and printed using a Codonics NP 1600 printer.

2.5.2. Native cytochrome c

HL-60 cells pre-incubated in buffer in the absence or presence of 250 μM DHA were exposed to 100 μM H_2O_2 for 4 hr. Following incubation the cells were labeled with Mito Tracker Red as described above and cytocentrifuge preparations were made. The cells were fixed with 3.7% paraformaldehyde for 30 min and permeabilyzed with 0.1% Triton X-100 in PBS for 10 min, both at room temperature. The cells were blocked with 2% bovine serum albumin and 10% normal sheep serum for 20 min, followed by incubation for 1 hr with 1:50 anti-cytochrome *c* mAb (clone 6H2.B4). After three washes with PBS, the cells were revealed by using solutions of secondary antibody conjugated with FITC and 0.05% Hoechst. Slides were visualized using a CLSM.

2.6. Analysis of mitochondrial transmembrane potential

A previously described method was used [24]. HL-60 cells were pre-incubated in the presence or absence of 250 μM DHA and exposed to 100 μM H_2O_2 for 0–15 hr. Following incubation the cells were incubated with 40 nM 3,3′-dihexyloxacarbocyanine iodide [$DiOC_6(3)$] for 15 min at 37° in the dark. The cells were washed and resuspended in PBS. Stained cells (10,000) were analyzed with a flow cytometer. Propidium iodide (PI; 10 μL of 1 mg/mL stock) was added 30 s before analysis. Cells were gated to exclude cellular debris associated with necrosis and the median values of green fluorescence from the subpopulation of cells that were negative for PI staining were determined using WINMDI software.

2.7. Fluorogenic assay for caspase-9 and -3 activities

HL-60 cells were pre-incubated in buffer in the presence of 0–250 μM DHA and exposed to 0–200 μM H_2O_2 for 0–8 hr. Following incubation cells were collected, washed, resuspended in 50 mM Tris–HCl buffer pH 7.4, 1 mM EDTA, 10 mM EGTA and lysed by three successive freeze–thaw cycles at dry ice/37°. Cell lysates were centrifuged at 20,000 g for 5 min, and the supernatants were stored at −70°. The protein concentration of each sample was estimated using the Bio-Rad protein assay. For caspase-9 and -3 activities a total of 50 μg protein was incubated with 50 μM LEHD-AMC or DEVD-AMC respectively, at 37°, for 30 min, and the release of 7-amino-4-methylcoumarine (AMC) was monitored by a spectrofluorometer using an excitation wavelength of 360 nm and an emission wavelength of 460 nm. In some experiments using caspase-3 inhibitor (Z-DEVD-CHO), it was directly added to the medium 2 hr before the addition of H_2O_2 [25].

2.8. Western blot analysis

HL-60 cells were pre-incubated in buffer in the presence of 0–250 μM DHA and exposed to 100 μM H_2O_2 for 1–6 hr. For caspase-8, -9 and -3 immunoblottings, total cell lysates were prepared. Cells were collected and washed twice in ice-cold PBS, and then lysed with a solubilizing solution containing 150 mM NaCl, 10 mM Tris–HCl (pH 7.6), 1% Triton X-100, 5 mM EDTA, 2 mM PMSF and 10 μg/mL aprotinin on ice for 30 min. The extracts were cleared by centrifugation.

For cytochrome *c* assay, soluble cytosolic fraction was prepared as described [26]. Cells were collected, washed and then the cell pellet was suspended in buffer containing 20 mM HEPES–KOH (pH 7.5), 10 mM KCl, 1.5 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM PMSF and 250 mM saccharose. After sitting on ice for 20 min, the cells were disrupted by douncing 20 times with a tight-fitting pestle (Dounce type homogenizer). The suspension was centrifuged at 750 *g* for 10 min at 4° to remove the nuclei. The supernatant was further centrifuged at 15,000 *g* for 15 min at 4° (Sorvall RC5C centrifuge). The resulting supernatant was used as soluble cytosolic fraction. Equal amounts of protein (50 μg) were subjected to electrophoresis on a 12% SDS–polyacrylamide gel and then electrophoretically transferred to a nitrocellulose membrane. Each blot was stained with Ponceau stain to confirm equal protein loading from lane to lane. Nonspecific binding was blocked with 3% skim milk in Tris-buffered saline containing 0.05% Tween-20. For caspases-8, -9 and -3 immunoblottings, the membranes were incubated with anti-caspase-8 mAb, anti-caspase-9 Ab or caspase-3 polyclonal Ab (each Ab was diluted 1:1000). For cytochrome *c* immunoblotting the membrane was incubated with anti-cytochrome *c* mAb (clone 7H.2C12 diluted 1:1000).

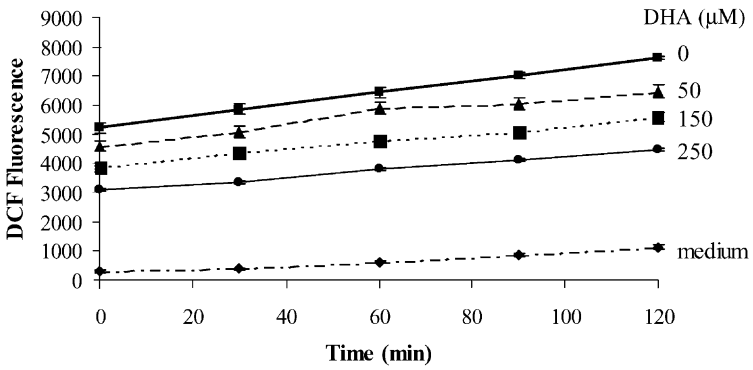

Fig. 1. Time course changes in DCF fluorescence in HL-60 cells pre-incubated in buffer in the presence or absence of increasing concentrations of DHA before exposure to 100 μM H_2O_2. Results expressed are of one representative experiment. Similar results were observed in two additional experiments.

Following incubation the membranes were exposed to horseradish peroxidase-conjugated anti-mouse IgG (or anti-rabbit IgG, for caspase-3) (for 1 hr at room temperature). Bound antibodies were visualized following chemiluminescence detection on autoradiographic film.

2.9. Immunostaining with anti-caspase-3 Ab and morphology studies

HL-60 cells were incubated in medium or pre-incubated in buffer in the absence or presence of 250 μM DHA and exposed to 100 μM H_2O_2 for 6 hr. Following incubation conventional cytocentrifuge preparations were made [19,21], cells were fixed by incubating in 3.7% formaldehyde in PBS for 15 min. Fixed cells were washed twice with ice cold PBS, blocked for 1 hr in PBS containing 2% bovine serum albumin, 0.2% non fat milk powder, 2% normal goat serum and 0.4% Triton X-100.The cells were then incubated at room temperature for 1 hr with CM1 antiserum (diluted 1:2500), washed three times and then exposed to the secondary Cy3 antibody and 0.05% Hoechst (for 1 hr at room temperature) [22]. After three washes, the slides were mounted using Gel Mount. Staining analyses was performed using a CLSM.

2.10. Apoptosis assay

Apoptosis was measured by flow cytometry after concurrent staining with fluorescein-conjugated annexin V and PI as previously described [26]. Statistical significance was determined by using the Student's *t*-test.

3. Results

3.1. Effect of ascorbic acid on cellular accumulation of peroxides and superoxide in HL-60 cells exposed to H_2O_2

To investigate the time course of intracellular peroxide and superoxide accumulation an oxidation-sensitive fluorescenct probe DCFH-DA was used. HL-60 cells were pre-incubated in the presence of 0–250 μM DHA, loaded with DCFH-DA and exposed to 100 μM H_2O_2. Fig. 1 indicates that an immediate marked increase in fluorescence intensity occurred following exposure of the cells to H_2O_2, the fluorescence increased with time. Pre-incubation with DHA reduced intracellular peroxide levels in a dose-dependent manner. The fluorescence intensity in cells that were pre-incubated with 150 or 250 μM DHA, before exposure to 100 μM H_2O_2 for 2 hr, was 27 and 42% lower,

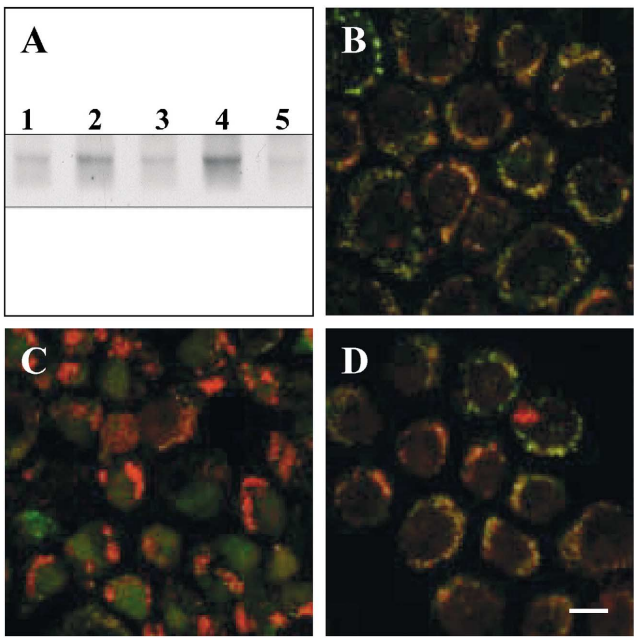

Fig. 2. Effect of DHA on the release of cytochrome *c* to the cytosol induced by H_2O_2. (A) Western blot analysis. HL-60 cells were incubated in medium (lane 1) or pre-incubated in buffer in the absence (lanes 2 and 4) or presence of 250 μM DHA (lanes 3 and 5) before exposure to100 μM H_2O_2, for 2 hr (lanes 2 and 3) or 4 hr (lanes 4 and 5). Cytosolic extracts were prepared and then analyzed as described in Section 2. The WB data are representative of two experiments. (B–D) Immunostaining with anti-cytochrome *c* Ab (clone 6H2.B4) of HL-60 cells exposed to H_2O_2. Effect of DHA. HL-60 cells were incubated in medium (B) or pre-incubated in buffer in the absence (C) or presence (D) of 250 μM DHA and exposed for 4 hr to 100 μM H_2O_2 (C and D). The cells were stained with Mito Tracker Red (red color) and anti-cytochrome *c* mAb that recognizes the native cytochrome *c*. The scale bar represents 10 μm.

respectively ($P < 0.05$), than in cells not pre-incubated with DHA.

3.2. Hydrogen peroxide induces mitochondrial release and denaturation of cytochrome c—protective effect of ascorbic acid

We measured cytochrome *c* release from mitochondria to cytosol following incubation of the cells with 100 μM H_2O_2. Two complementary techniques were used: Western blot analysis and immunohistochemistry. Cytosolic cytochrome *c* protein levels were measured by Western blot analysis. Fig. 2A indicates that cytochrome *c* release was detected already at 2 hr and increased at 4 hr (Fig. 2A, lanes 2 and 4, respectively). Pre-incubation of the cells with 250 μM DHA before exposure to H_2O_2 inhibited cytochrome *c* release (Fig. 2A, lanes 3 and 5).

Next we investigated, by immunohistochemical methods (Fig. 2B–D), the release of native cytochrome *c* from mitochondria to the cytosol, we asked whether there is correlation between cytochrome *c* release and apoptosis and whether DHA has a protective effect. To asses native cytochrome *c* expression in HL-60 cells, the cells were exposed to 100 μM H_2O_2 for 4 hr, washed and stained with Mito Tracker dye and anti-cytochrome *c* mAb (clone 6H2.B4 that recognizes the native form of cytochrome *c*) and with Hoechst 33258 dye (to identify apoptotic nuclear morphology). Temporal correlation between the changes of red Mito Tracker dye and the subcellular localization of cytochrome *c* were determined by CLSM. Fig. 2B shows that in cells incubated in medium cytochrome *c* was found in a punctate pattern (yellow color), in keeping with its normal mitochondria location. Following exposure of the cells to H_2O_2 for 4 hr the fluorescence due to cytochrome *c* increased homogeneously in the cytosol (Fig. 2C) (green color), reflecting the release of this protein from the mitochondria. A high percentage of cells exposed to H_2O_2 that contained cytochrome *c* in the cytosol showed chromatin condensation and nuclear fragmentation typical of apoptosis (data not shown). Pre-incubation with 250 μM DHA, previous to exposure of the cells to H_2O_2, partially protected against cytochrome *c* release (Fig. 2D) and against apoptosis (data not shown).

It is generally accepted that under *in vitro* conditions excess H_2O_2 degrades cytochrome *c* and other hemoproteins [6], other studies have shown that aged mitochondria have high levels of free H_2O_2 [27]. Thus we investigated whether exposure of HL-60 cells to H_2O_2 will induce cytochrome *c* denaturation and whether pre-incubation with DHA will have a protective effect. Flow cytometric studies (performed with anti-cytochrome *c* mAb clone 7H8.2C12, that recognizes the denatured form of cytochrome *c*) have shown that HL-60 cells exposed for 4 hr to 100 μM H_2O_2 express high levels of denatured cytochrome *c* and that pre-incubation with 250 μM DHA decreased the appearance of denatured cytochrome *c* (Fig. 3A).

Using confocal microscopy we sought to identify the subcellular localization of H_2O_2-denatured cytochrome *c* (Fig. 3B–E). Following exposure of the cells to H_2O_2 the cells were washed and a double staining was performed

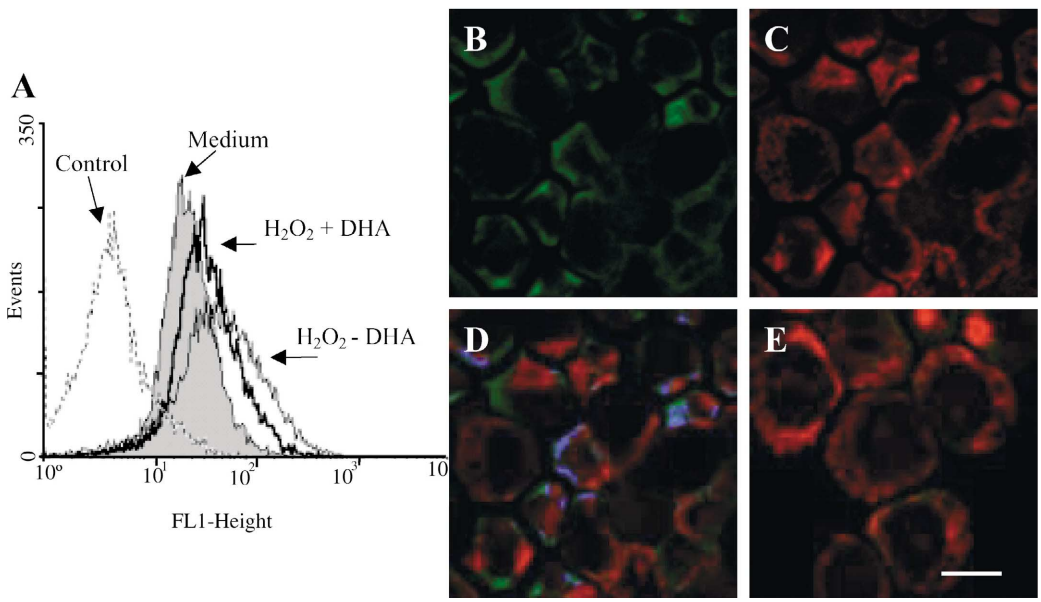

Fig. 3. Effect of DHA on denaturation of cytochrome *c* induced by H_2O_2. (A) Flow cytometry studies of the expression of denatured cytochrome *c* in cells exposed to H_2O_2. HL-60 cells were pre-incubated in the presence or absence of 250 μM DHA and exposed to 100 μM H_2O_2 for 4 hr, washed and stained with purified anti-cytochrome *c* mAb (clone 7H8.2C12) that recognizes the denatured form of cytochrome *c*, as described in Section 2. An irrelevant Ab was used as control. (B–E) Localization studies of denatured cytochrome *c*. Cells were pre-incubated in buffer in the absence (B–D) or presence of 250 μM DHA (E) and treated for 4 hr with 100 μM H_2O_2. Fluorescence was analyzed by confocal microscopy. The cells were stained with anti-cytochrome *c* mAb clone 7H.2C12 (green color) (B) and with Mito Tracker Red (red color) (C). For colocalization analysis a region of maximal colocalization was selected in the graph and the green and red images were overlaid with blue regions representing colocalization (D). The scale bar represents 10 μm.

with Mito Tracker dye and mAb that recognizes the denatured form of cytochrome *c*. Control cells (incubated in medium) only exhibited the red color, with no contribution of denatured cytochrome *c*-dependent fluorescence (green) (data not shown). This was because the anti-cytochrome *c* mAb does not recognize the native structure of cytochrome *c* and the cells do not contain denatured cytochrome *c*. When cells were exposed for 4 hr to 100 μM H_2O_2 the fluorescence due to denatured cytochrome *c* could be detected in the cytosol (green color, Fig. 3B and D) and in the mitochondria (blue color, as the green color of denatured cytochrome *c* colocalized with the red color of the Mito Tracker, Fig. 3D). Fig. 3E indicates cells that were pre-incubated with 250 μM DHA before their exposure to H_2O_2, only exhibit the red color of the Mito Tracker, with minimal denatured cytochrome *c* fluorescence, indicating that ascorbic acid loading protects from denaturation of cytochrome *c* induced by H_2O_2.

3.3. Ascorbic acid decreases reduction in mitochondrial membrane potential ($\Delta\Psi_m$) in cells exposed to H_2O_2

We measured $\Delta\Psi_m$ in cells exposed to 100 μM H_2O_2 for various periods of time. Fig. 4 shows the measurement of $\Delta\Psi_m$ using $DiOC_6(3)$ and PI. Cells excluding PI were gated and the green $DiOC_6(3)$ fluorescence histogram was generated. A decrease in $\Delta\Psi_m$ was observed after exposure of the cells to100 μM H_2O_2 for 5 hr (Fig. 4A). Fig. 4B indicates that the decrease in $\Delta\Psi_m$ was first detected at 3 hr at which time there was a 3-fold increase in the percentage of $DiOC_6(3)$ low cells as compared to control cells, not treated with H_2O_2 ($P < 0.05$). At 15 hr a 4-fold increase in the percentage of $DiOC_6(3)$ low cells was observed ($P < 0.05$). Pre-incubation with 250 μM DHA resulted with a partially protective effect against decrease in $\Delta\Psi_m$ (Fig. 4A and B).

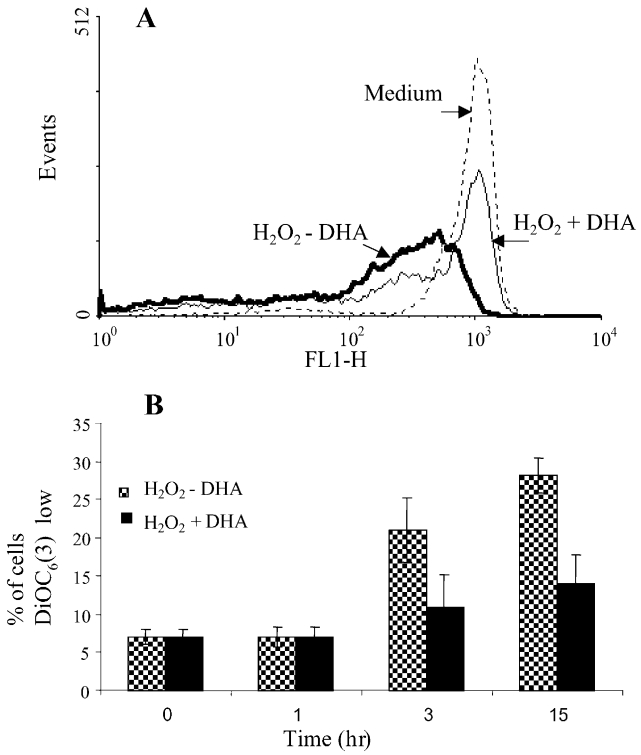

Fig. 4. DHA decreases reduction in $\Delta\Psi_m$ following exposure of cells to 100 μM H_2O_2. (A) Example of the measurement of $\Delta\Psi_m$ in cells exposed to H_2O_2 for 5 hr, using $DiOC_6(3)$. (B) Kinetics of changes in $\Delta\Psi_m$. Data are the mean ± SE of three independent experiments.

3.4. Activations of caspase-8 and -9 by H_2O_2—protective effect of ascorbic acid

As caspase-8 and-9 were proposed to be initiator caspases, we investigated whether H_2O_2 activates these two caspases and whether ascorbic acid has a protective effect against their activation.

We examined procaspase-8 cleavage by Western blotting using an anti-human caspase-8 mAb which recognizes both the procaspase and the cleaved 20-kDa fragment of caspase-8. Fig. 5A reveals that incubation of HL-60 cells

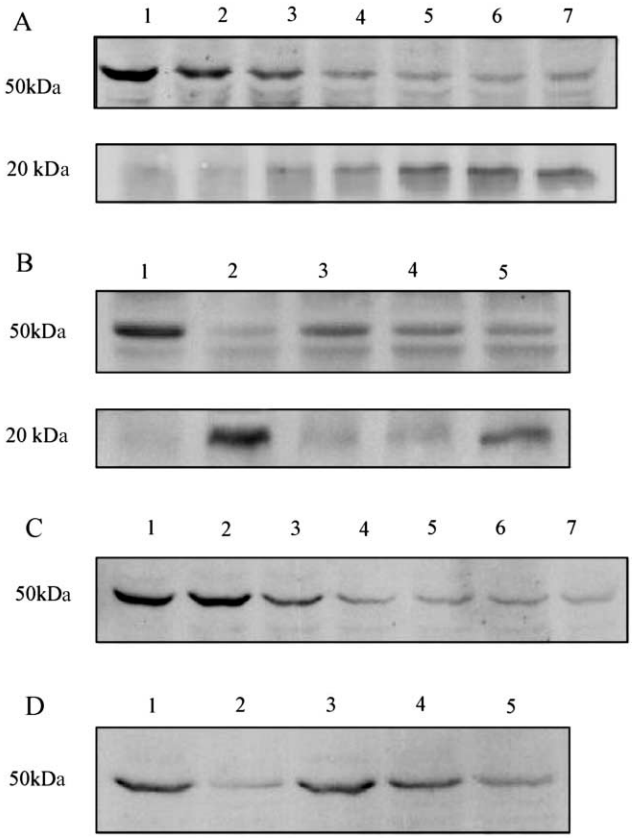

Fig. 5. H_2O_2-induced processings of (A and B) procaspase-8 and (C and D) procaspase-9. (A and C) Time-dependent studies. HL-60 cells were incubated in medium (lane 1) or exposed to 100 μM H_2O_2 for 1 hr (lane 2), 2 hr (lane 3), 3 hr (lane 4), 4 hr (lane 5), 5 hr (lane 6) or 6 hr (lane 7) and then analyzed by Western blot as described in Section 2. (B and D) Effect of ascorbic acid loading. Cells were incubated in medium (lane 1) or pre-incubated in buffer in the absence (lane 2) or presence of 250 μM (lane 3), 150 μM (lane 4) or 50 μM DHA (lane 5) previous to their exposure for 6 hr to 100 μM H_2O_2 (lanes 2–5), then analyzed by Western blots for processings of procaspase-8 and -9. The WB data are representative of three experiments.

with 100 μM H_2O_2 for 1–6 hr induced a time-dependent reduction of procaspase-8 and the appearance of the active form of the caspase. The processing of procaspase-8 was first observed at 1 hr following treatment with H_2O_2, at which time a slight reduction was observed (Fig. 5A, lane 2). The appearance of the active form of caspase-8 could be detected at 3 hr after H_2O_2 treatment (Fig. 5A, lane 4). We next analyzed the protective effect of ascorbic acid on caspase-8 activation. HL-60 cells were pre-incubated with increasing doses of DHA before exposure to 100 μM H_2O_2 for 6 hr. Following treatment with H_2O_2 procaspase-8 was cleaved (Fig. 5B, lane 2), pre-incubation with 50–250 μM DHA decreased procaspase-8 cleavage in a dose-dependent manner (Fig. 5B). Pre-incubation with 250 μM DHA markedly reduced procaspase-8 cleavage (Fig. 5B, lane 3) while lower doses of DHA only partially decreased the cleavage of the procaspase.

In the next stage, we examined caspase-9 activation. Fig. 5C revealed that incubation of HL-60 cells with 100 μM H_2O_2 for 1–6 hr induced a time-dependent reduction of procaspase-9. The processing of procaspase-9 was first observed at 2 hr following treatment with H_2O_2, at which time a slight reduction was observed (Fig. 5C, lane 3). A marked reduction of the procaspase occurred at 3 hr after H_2O_2 treatment (Fig. 5C, lane 4). To investigate the protective effect of ascorbic acid, HL-60 cells were

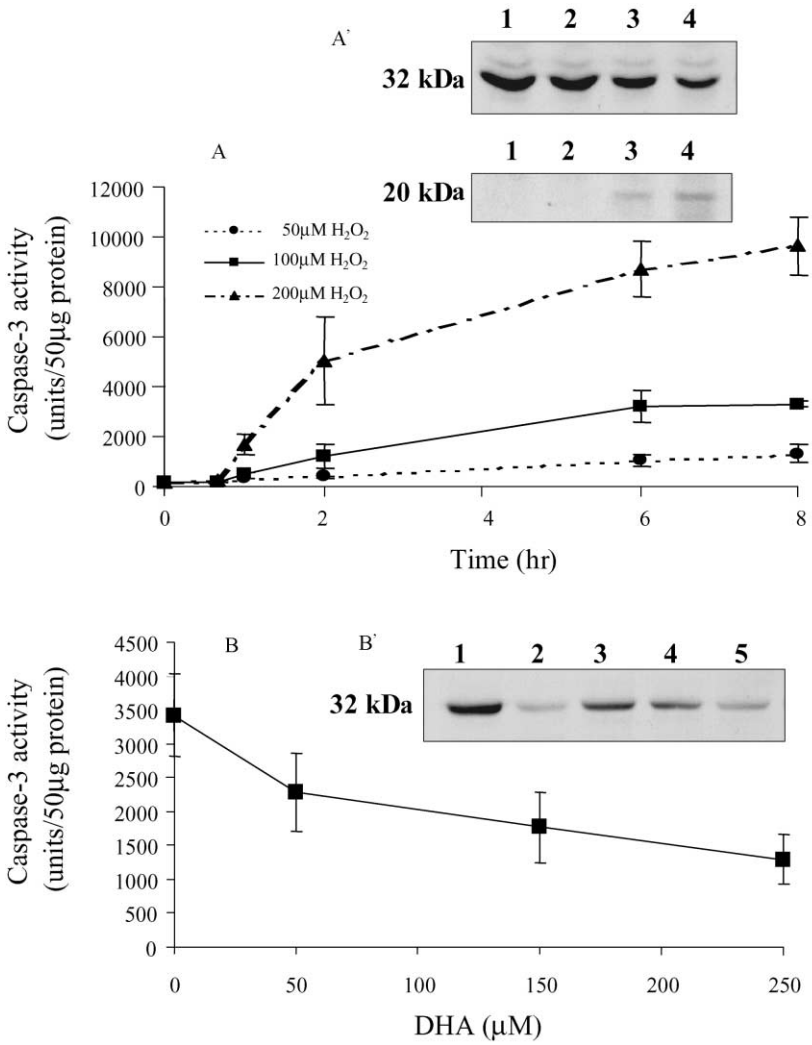

Fig. 6. Caspase-3 activation induced by H_2O_2. (A) Time-dependent studies. Cells (1×10^6/mL) were seeded into dishes and incubated with 0, 50, 100 or 200 μM H_2O_2 for 20 min–8 hr at 37° under 5% CO_2 and then lysates were prepared. Caspase-3 activity in these lysates was measured using DEVD-AMC as substrate. Data represent the mean ± SE of three experiments. (A′, inset in A) Western blot analysis of active caspase-3. Cells were incubated in medium (A′, lane 1) or exposed to 100 μM H_2O_2 for 1–3 hr (lanes 2–4, respectively). Lysates were prepared and samples containing 50 μg protein were resolved in 12% SDS gels and electroblotted onto nitrocellulose membranes. The membranes were probed with anti-caspase-3 rabbit polyclonal Ab as described in Section 2. (B) Protective effect of increasing concentrations of DHA against activation of caspase-3 induced by 100 μM H_2O_2. Fluorescence studies. Cells were pre-incubated with increasing concentrations of DHA and exposed to 100 μM H_2O_2 for 6 hr. Following incubation lysates were prepared and caspase-3 activity was measured using DEVD-AMC as substrate. Results represent the mean ± SE of seven experiments. (B′, inset in B) Western blot analysis of procaspase-3. Cells were incubated in medium (lane 1), or pre-incubated in the absence (lane 2) or presence of 250 μM (lane 3), 150 μM (lane 4) or 50 μM DHA (lane 5) before exposure to H_2O_2, as described in (B). Lysates were prepared and samples containing 50 μg protein were resolved in 12% SDS gels and electroblotted onto nitrocellulose membranes. The membranes were probed with anti-caspase-3 rabbit polyclonal Ab.

pre-incubated with increasing doses of DHA before exposure to 100 μM H_2O_2 for 6 hr. Fig. 5D (lane 2) indicates that following treatment of the cells with H_2O_2 procaspase-9 was cleaved, while pre-incubation of the cells with 50–250 μM DHA inhibited procaspase-9 cleavage in a dose-dependent manner (Fig. 5D, lanes 3–5). To confirm caspase-9 activation we used a specific fluorogenic substrate for caspase-9. Cell lysates from HL-60 cells that were treated with 100 μM H_2O_2 for 6 hr were incubated with Ac-LEHD-AML and the increase in fluorescence due to enzymatic cleavage of the peptides was measured with a fluorometer. A 2.6-fold increase in caspase-9 activation was observed (relative fluorescence increased from 338 to 894 arbitrary units) following exposure of the cells to H_2O_2. Pre-incubation with 250 μM DHA resulted with 60% inhibition in caspase-9 activation.

3.5. Activation of caspase-3 by H_2O_2

DEVD-AMC is a specific substrate for caspase-3 that mimics the PARP cleavage site by caspase-3. To test caspase-3 activation, cell lysates from HL-60 cells that were treated with H_2O_2 for various periods of time were incubated with the substrate, and the increase in fluorescence due to enzymatic cleavage of the peptides was measured with a fluorometer. Fig. 6A indicates that treatment of the cells with 50–200 μM H_2O_2 strongly enhances caspase-3 activity and that the activity was dependent on the concentration of H_2O_2 and time of exposure. In response to 50 μM H_2O_2 a 2-fold increase in caspase-3 activity was observed already after 1 hr ($P < 0.05$), and it continued to increase up to 6 hr (6.6-fold, $P < 0.05$). Prolongation of the incubation time to 8 hr did not affect the activity. With 100 and 200 μM H_2O_2 a 2.6- and 10.6-fold increase, respectively, in caspase-3 activity was observed after 1 hr and maximum augmentation at 6 hr (20- and 55.6-fold augmentation, respectively, $P < 0.05$) and persisted until 8 hr (the time measured in these experiments). To confirm that the caspase activity observed is specific to caspase-3, we employed the use of a caspase-3-specific inhibitor, Z-DEVD-CHO. Treatment of cells with the inhibitor before the addition of 50 μM H_2O_2 reduced the degree of caspase-3 activation (data not shown).

Activation of caspase-3 by H_2O_2 was further confirmed by Western blot analysis. Upon activation, the 32-kD CPP32 zymogen is cleaved into 20-kD and 11-kD fragments [28]. Time-dependent studies have shown that following exposure of HL-60 cells to 100 μM H_2O_2, part of procaspase-3 disappeared and the cleaved fragments of caspase-3 could be detected at 2 hr and their amount increased at 3 hr (inset in Fig. 6A, lanes 3 and 4, respectively). To investigate whether ascorbic acid loading has a protective effect against caspase-3 processing, HL-60 cells were pre-incubated in the presence of various concentrations of DHA and exposed to 100 μM H_2O_2 for 6 hr. Fig. 6B indicates that pre-incubation with 50–250 μM DHA before exposure to H_2O_2 resulted in a 2.1–2.6-fold decrease in caspase-3 activation ($P < 0.05$). The inset in Fig. 6B (Western blot analysis) reveals reduction of procaspase-3 upon incubation with H_2O_2 (lane 2) which was markedly

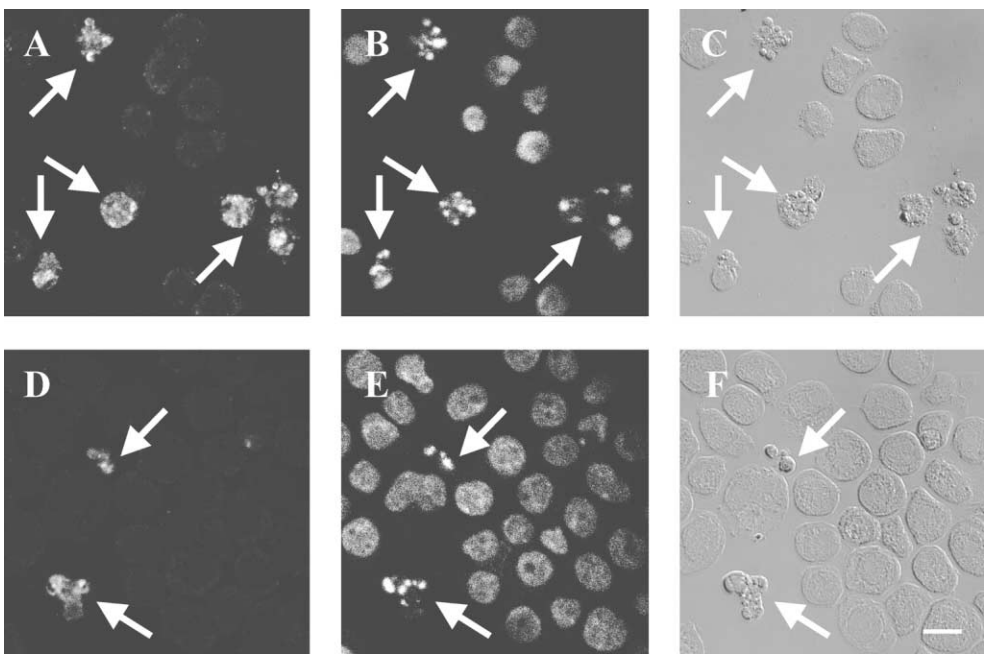

Fig. 7. Immunostaining and nuclear morphology of HL-60 cells exposed for 6 hr to 100 μM H_2O_2. Effect of DHA. HL-60 cells were pre-incubated in the absence (A–C) or presence of 250 μM DHA (D–F), previous to their exposure to H_2O_2. Following incubation immunostaining was performed with CM1 Ab (A and D), arrows indicate CM1 labeled cells (A and B). Labeling with Hoechst 33258 dye (B and E), and Nomarsky differential contrast microscopy analysis (C and F) enable identification of cells with chromatin condensation and nuclear fragmentation typical of apoptosis (see arrows). Note that CM1 preferentially immunostains cells which show apoptotic nuclear morphology. The scale bar represents 10 μm.

blocked by pre-incubation of the cells with 250 μM DHA before exposing to 100 μM H_2O_2 (lane 3) and partially blocked by 150 or 50 μM DHA (lanes 4 and 5, respectively). These results paralleled with the decrease in DEVD-AMC cleavage observed in Fig. 6B.

3.6. Immunostaining of apoptotic HL-60 cells exposed to H_2O_2—effect of ascorbic acid loading

In order to investigate whether caspase-3 activation induced by H_2O_2 correlates with the level of apoptosis

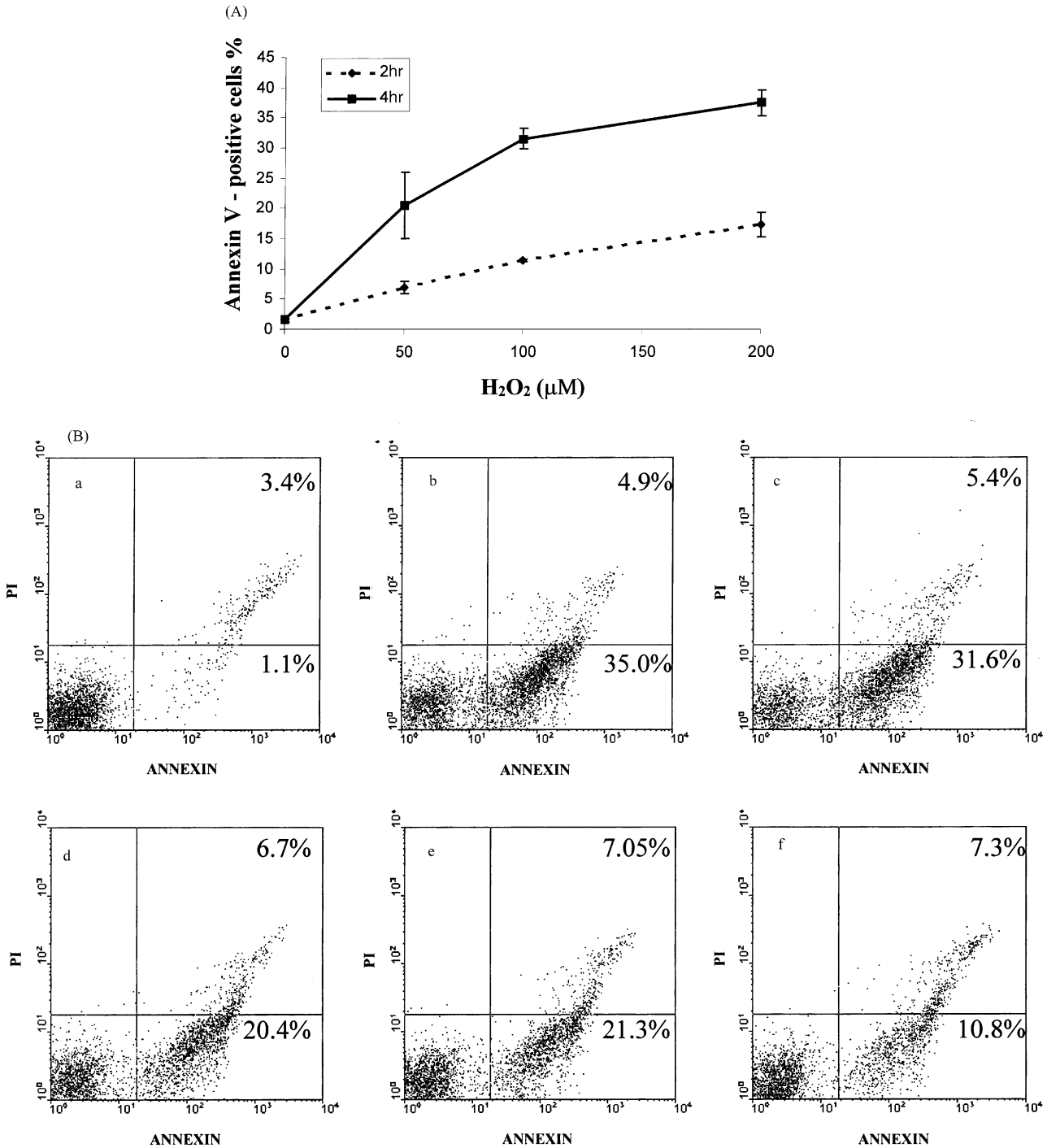

Fig. 8. Induction of apoptosis by exposure to H_2O_2 and effect of DHA. (A) Effect of H_2O_2 concentrations. Cells were incubated in the presence of 50–200 μM H_2O_2 for 2 or 4 hr and flow cytometric analysis of apoptosis was determined by binding of annexin V and uptake of PI. Data present the mean ± SE of three independent experiments. (B) Effect of DHA concentrations. Cells were incubated in medium (a) or pre-incubated with 0, 50, 150, 250 or 500 μM DHA (b–f, respectively) before exposure to 100 μM H_2O_2 for 4 hr (b–f). Apoptosis was determined as described in (A). The percentage of annexin V-positive PI-negative cells is indicated in the lower right quadrangle and annexin V-positive PI-positive cells in the upper right quadrangle. The *x*-axis shows log annexin V fluorescence intensity, *y*-axis indicates PI fluorescence intensity.

in HL-60 cells, we incubated the cells for 6 hr with 100 μM H_2O_2 and performed double staining with CM1 Ab (rabbit polyclonal antiserum which recognizes the p18 subunit of cleaved caspase-3 but not the procaspase [20]) and with Hoechst 33258 dye (to identify apoptotic nuclear morphology). Fig. 7A shows that a high percentage of cells exposed to H_2O_2 stained positively with CM1 Ab. Hoechst staining (Fig. 7B) and morphology analysis performed by Nomarsky differential interference contrast microscopy (Fig. 7C) indicate that cells that were stained positively with CM1 Ab show chromatin condensation and nuclear fragmentation typical of apoptosis. Upon incubation of the cells with 250 μM DHA previous to their exposure to 100 μM H_2O_2 a protective effect of DHA was observed against activation of caspase-3 (Fig. 7D) and against apoptosis (Fig. 7E and F).

3.7. Inhibition of apoptosis induced by H_2O_2 by loading cells with ascorbic acid

Phosphatidylserine, which is normally confined to the inner leaflet of the plasma membrane, is exported to the outer plasma membrane leaflet during apoptosis. We assessed phosphatidylserine externalization following exposure of the cells to increasing concentrations of H_2O_2 for 2 and 4 hr. Fig. 8A shows that a dose-dependent increase in annexin V-positive cells occurred upon exposure of the cells to H_2O_2 for 2 hr. Prolongation of the exposure time to 4 hr resulted with a marked increase in annexin V-positive cells. In a previous study we have shown the protective effect of ascorbic acid against apoptosis induced by H_2O_2 (morphological studies) [19]. Fig. 8B(d–f) shows a decrease in annexin V-positive (PI-negative) cells upon pre-incubation of the cells with 150–500 μM DHA (but not with 50 μM DHA (Fig. 8B(c)) before their exposure to100 μM H_2O_2 for 4 hr, confirming the results of our previous studies [19].

4. Discussion

Cytochrome *c* is a well known component of the mitochondrial respiratory chain. For many years the biological function of cytochrome *c* was assumed to be confined to electron transfer from cytochrome c_1 to cytochrome oxidase. Recently it was found that cytochrome *c*, when released from mitochondria to cytosol activates a programmed cell death cascade (reviewed in [29]). Other studies have shown that culture of cerebellar granule cells in low potassium medium (conditions that induce apoptosis) is accompanied by release of cytochrome *c*. The released cytochrome *c* was found to be an intact functionally active protein that accumulates in the cytosol and after progressive accumulation it is degraded by cytosolic caspases [30].

We studied the location and nature of cytochrome *c* during apoptosis induced by H_2O_2 in HL-60 cells. We show that H_2O_2 induces denaturation of cytochrome *c* (flow cytometry and confocal microscopy studies), moreover, we show that the denatured cytochrome *c* could be detected in the mitochondria and in the cytosol. To the best of our knowledge our findings provide the first evidence that following exposure of cells to H_2O_2, cytochrome *c* becomes denatured inside the mitochondria. The entire cytochrome *c* located in the mitochondria is not degraded by H_2O_2 as our data also indicate that H_2O_2 induces the release of native cytochrome *c* from mitochondria to the cytosol and reduction in mitochondrial Ψ_m.

Yang *et al*. have shown that in HL-60 cells undergoing apoptosis induced by staurosporine, cytochrome *c* is released before a reduction in Ψ_m occurs [31]. Our results are in line with Yang *et al*., as we have shown that released cytochrome *c* could already be detected in the cytosol after 2 hr (Fig. 2) while $\Delta\Psi_m$ was observed only after 3 hr (Fig. 4) following exposure of the cells to H_2O_2.

Reduction of Ψ_m and release of cytochrome *c* from mitochondria appear to be key events during apoptosis. Studies have shown that released cytochrome *c* induces activation of caspase-9 which in turn cleaves and activates the executioner caspases [11,12].

The present study shows that treatment of cells with H_2O_2 activates both the mitochondrial pathway as well as the cytosolic pathway as caspase-8 and -9 became activated. The activation of caspase-8 preceded the activation of caspase-9, as the processing of pro-caspase-8 was first observed at 1 hr following treatment with H_2O_2 and that of procaspase-9 at 2 hr (Fig. 5).

Apoptosis is accomplished by the initiation of an ''executioner'' machinery, involving caspase-3 and related proteases which are present in the cytoplasm as dormant proenzymes. In the present study we have shown that exposure of the cells to H_2O_2 activates caspase-3 (the activation was examined by fluorescence studies and further confirmed by Western blot analysis and by immunostaining with an antibody that recognizes the subunit of cleaved caspase-3).

In a recent study Guaiquil *et al*. have shown that ascorbic acid inhibits cell death induced by oxidative stress in glutathione-depleated HL-60 myeloid leukemia cells [32], while we have shown that ascorbic acid inhibits apoptosis induced by H_2O_2 or X-irradiation in HL-60 cells [19,21]. The present study shows that loading of HL-60 cells with ascorbic acid provides them a partial protection from cytochrome *c* denaturation and release. In addition loading of HL-60 cells with ascorbic acid declines procaspase-8,-9 and -3 cleavages in a dose-dependent manner. Our findings are in line with Chan *et al*. [33] who have shown that hyperosmotic shock elicits a stress response in mammalian cells, activates a family of cysteine proteases termed caspase-3 and the activation could be blocked by antioxidants such as ascorbic acid, α-tocopherol, dithiothreitol, β-mercaptoethanol and glutathione. Other studies performed by Brown *et al*. have indicated that ascorbic

acid protects against apoptosis or necrosis induced by oxidative stress in alveolar type II cells [34].

Taken together our previous [19] and present results, we conclude that ascorbic acid decreases the intracellular oxidative level in cells, thus enabling them to tolerate increased levels of oxidative stress before activation of the apoptotic cascades that induce apoptosis.

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