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Differential Regulation of Hydrogen Peroxide and Fas-Dependent Apoptosis Pathways by Dehydroascorbate, the Oxidized Form of Vitamin C

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ABSTRACT

Dehydroascorbate (DHA), the oxidized form of vitamin C (ascorbate), enhanced antioxidant defenses of human T cells preferentially importing DHA over ascorbate. In itself, DHA did not affect cytosolic or mitochondrial reactive oxygen intermediate levels as monitored by flow cytometry using oxidation-sensitive fluorescent probes. DHA at 200–1,000 μ*M* stimulated activity of pentose phosphate pathway enzymes glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and transaldolase, elevated intracellular glutathione levels, and inhibited H_2O_2-induced changes in mitochondrial transmembrane potential and cell death. With respect to the CD4 antigen, DHA selectively enhanced cell-surface expression of the Fas receptor and increased susceptibility of Jurkat and H9 human T cells to Fas-mediated cell death. The data identify DHA as a selective regulator of H_2O_2- and Fas-dependent apoptosis pathways. *Antioxid. Redox Signal.* 4, 357–369.

INTRODUCTION

REACTIVE OXYGEN INTERMEDIATES (ROIs) have long been considered as toxic by-products of aerobic existence, however, evidence is now accumulating that controlled levels of ROIs modulate various aspects of cellular function and are necessary for signal-transduction pathways, including those mediating T-cell activation (26, 30, 38) and apoptosis (7, 13, 35, 37). Increased production of ROIs was demonstrated in tumor necrosis factor-(TNF) (29, 45, 63) and Fas-mediated cell death (7, 9, 25, 34, 71, 78, 82). Disruption of the mitochondrial membrane potential has been proposed as the point of no return in apoptotic signaling (65, 73, 82). Recent data from this laboratory indicate that elevation of mitochondrial transmembrane potential ($\Delta\Psi_m$), *i.e.,* mitochondrial hyperpolarization, occurs in the early phase of Fas-induced apoptosis of Jurkat human leukemia T cells and normal human peripheral blood lymphocytes (9). Mitochondrial hyperpolarization precedes phosphatidylserine (PS) externalization and a disruption of $\Delta\Psi_m$ in Fas- (9) and H_2O_2-induced apoptosis (56). These observations were confirmed and extended to p53 (36), TNFα (24), and staurosporine-induced apoptosis (62). Elevation of $\Delta\Psi_m$ is independent from activation of caspases and represents an early event in apoptosis (9, 36). Whereas H_2O_2-induced apoptosis is dependent on formation of ROI in mitochondria, Fas signaling can be regulated on the levels of Fas receptor expression and assembly of the caspase 8-activating death signaling complex (10, 47, 57, 76, 83). Intracellular antioxidants, ascorbate, reduced glutathione (GSH), and thioredoxin have emerged as important regulators of various apoptosis signal-processing pathways (52).

Humans and other primates lack gulonolactone oxidase, a key enzyme for ascorbic acid biosynthesis and, therefore, it must be provided from external sources (49). Even in ascorbic acid-synthesizing species, the majority of cells need ascorbic acid from the outside (23). Vitamin C is absorbed

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from the gastrointestinal tract in the form of ascorbic acid and circulates in the blood as ascorbate at pH 7.4. Ascorbic acid, or vitamin C, is widely used as an antioxidant (11, 14, 46). However, vitamin C also has prooxidant properties and may cause apoptosis of human lymphocytes and myelogenous leukemia cell lines (2, 54). Apoptosis, a form of programmed cell death, is indispensable for normal development and homeostasis within multicellular organisms (81). Defects in programmed cell death may underlie the etiology of neurodegenerative diseases, cancer, autoimmune diseases, and the acquired immune deficiency syndrome (48, 68).

Prooxidant and proapoptotic effects of vitamin C may be related to hydroxylation (70) and/or formation of ascorbyl radicals (60). By contrast, dehydroascorbate (DHA), the oxidized form and major transport form of vitamin C, does not influence cell viability by itself. Blood cells primarily import DHA via the hexose transporter GLUT-1 (74). Within the cell, DHA is regenerated into ascorbate at the expense of GSH (46). However, ascorbate cannot regenerate GSH from its oxidized form, GSSG. GSSG is reduced to GSH at the expense of NADPH, which is produced by the pentose phosphate pathway (PPP) (44). In fact, a fundamental function of PPP is to maintain GSH in a reduced state, thereby protecting sulfhydryl groups and cellular integrity from emerging oxygen radicals.

The PPP comprises two separate, oxidative and nonoxidative, phases (44). Reactions in the oxidative phase are irreversible, whereas all reactions of the nonoxidative phase are fully reversible. The two phases are functionally connected. The nonoxidative phase converts ribose 5-phosphate to glucose 6-phosphate for utilization by the oxidative phase and thus, indirectly, contributes to generation of NADPH. Different enzymes are rate-limiting in the two phases. The oxidative phase primarily depends on glucose 6-phosphate dehydrogenase (G6PD) (80), whereas transaldolase (TAL) is the rate-limiting enzyme for the nonoxidative phase (28). TAL (7) and G6PD (61) regulate NADPH production and, thereby, they influence GSH levels, $\Delta\Psi_m$, and susceptibility to apoptosis signals (7–9). Unless reduced back to ascorbate, DHA is rapidly hydrolyzed into 2,3-diketo-L-gulonate and decarboxylated to L-xylonate and L-lyxonate (33). In turn, these five-carbon sugars can enter the nonoxidative branch of the PPP (3, 12).

This study provides evidence that DHA stimulates the activity of PPP enzymes TAL, G6PD, and 6-phosphogluconate dehydrogenase (6PGD), elevates intracellular GSH levels, and increases resistance of Jurkat and H9 human T-cell lines to H_2O_2-induced cell death. By contrast, pretreatment with DHA increased cell-surface expression of the Fas receptor and enhanced susceptibility to Fas-mediated cell death. Therefore, DHA may operate as a selective regulator of ROI- and Fas-dependent apoptosis pathways.

MATERIALS AND METHODS

Cell culture and apoptosis assays

Jurkat human T cells were cultured in RPMI 1640 medium, supplemented with 10% fetal calf serum, 2 m*M* L-glutamine, 100 IU/ml penicillin, and 100 μg/ml gentamicin. H9 cells were cultured under similar conditions. For each experiment, ascorbic acid and dehydroascorbic acid (both from Sigma, St. Louis, MO, U.S.A.) were freshly resuspended in H_2O and the pH was adjusted to 7.4 with KOH. At physiological pH 7.4, ascorbic acid and dehydroascorbic acid dissociate to ascorbate and DHA, respectively. Prior to assays, Jurkat cells were fed with fresh medium, seeded at a density of 2×10^5 cells/ml, and treated with ascorbate or DHA at concentrations between 100 and 800 μ*M*. After pretreatment with DHA for 15 min to 36 h, cells were washed and cell death was induced with 100 μ*M* H_2O_2 or 100 ng/ml Fas antibody CH-11. Apoptosis was monitored by observing cell shrinkage and nuclear fragmentation, and quantified by flow cytometry after concurrent staining with fluorescein-conjugated annexin V (annexin V-FITC, R & D Systems, Minneapolis, MN, U.S.A.; FL-1) and propidium iodide (FL-2) as previously described (8). Staining with phycoerythrin-conjugated annexin V (annexin V-PE; R & D Systems) was used to monitor PS externalization (FL-2) in parallel with measurement of ROI levels and $\Delta\Psi_m$, using dihydrorhodamine 123 (DHR), 5,6-carboxy-2′,7′-dichlorofluorescein (DCF), or 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$) fluorescence. PS externalization was monitored with annexin V-FITC (FL-1) in parallel with measurement of ROI levels using ethidium (FL-2), as previously described (9). Thus, annexin V-PE or annexin V-FITC was matched with emission spectra of potentiometric and oxidation-sensitive fluorescent probes. Specific combinations are described in each figure legend.

Flow cytometric analysis of ROI production and $\Delta\Psi_m$

The production of ROIs was estimated fluorometrically using oxidation-sensitive fluorescent probes 5,6-carboxy-2′,7′-dichlorofluorescein-diacetate (DCFH-DA), DHR, and hydroethidine (HE; Molecular Probes, Eugene, OR, U.S.A.) as previously described (7). Following apoptosis assay, cells were washed three times in 5 m*M* HEPES-buffered saline, pH 7.4, incubated in HEPES-buffered saline with 0.1 μ*M* DHR for 2 min, 1 μ*M* DCFH-DA for 15 min, or 1 μ*M* HE for 15 min, and samples were analyzed using a Becton–Dickinson FACStar Plus flow cytometer equipped with an argon ion laser delivering 200 mW of power at 488 nm. Fluorescence emission from DCF (green) or DHR (green) was detected at a wavelength of 530 ± 30 nm. Fluorescence emission from oxidized HE, ethidium (red), was detected at a wavelength of 605 nm. Dead cells and debris were excluded from the analysis by electronic gating of forward and side scatter measurements. Whereas R123, the fluorescent product of DHR oxidation, binds selectively to the inner mitochondrial membrane, ethidium and DCF remain in the cytosol of living cells. $\Delta\Psi_m$ was estimated by staining with 40 n*M* $DiOC_6$ (Molecular Probes), a cationic lipophilic dye (53, 67, 82), for 15 min at 37°C in the dark before flow cytometry (excitation: 488 nm, emission: 525 nm recorded in FL-1). Fluorescence of $DiOC_6$ is oxidation-independent and correlates with $\Delta\Psi_m$ (67). $DiOC_6$ staining was complete after a 15-min incubation. $DiOC_6$ fluorescence was diminished three- to fourfold by 5

μ*M* carbonyl cyanide *m*-chlorophenylhydrazone (mClCCP; Sigma) and 10-fold or more by 50 μ*M* mClCCP as previously described (9). $\Delta\Psi_m$ was also quantitated using a potential-dependent J-aggregate-forming lipophilic cation, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide (JC-1) (64). JC-1 selectively incorporates into mitochondria, where it forms monomers (fluorescence in green, 527 nm) or aggregates, at high transmembrane potentials (fluorescence in red, 590 nm) (16, 64). Cells were incubated with 1 μ*M* JC-1 for 15 min at 37°C before flow cytometry. Co-treatment with a protonophore, 5 μ*M* mClCCP, for 15 min at 37°C resulted in decreased DHR, $DiOC_6$, and JC-1 fluorescence and served as a positive control for disruption of $\Delta\Psi_m$ (9).

Flow-cytometric analysis of receptor expression

Fas-receptor expression was monitored by flow cytometry using ZB4 monoclonal IgG_1 Fas antibody (Upstate Biotechnologies, Lake Placid, NY, U.S.A.), following staining with fluorescein-conjugated goat anti-mouse IgG antibody (Boehringer, Indianapolis, IN, U.S.A.). As a negative antibody control, cells were stained with C4 anti-actin IgG_1 monoclonal antibody (Boehringer). As a control antigen, cells were co-stained with quantum red-conjugated (Cy-5) CD4 monoclonal antibody Q4120 (Sigma) belonging to the same IgG_1 subclass as ZB4.

PPP enzyme activities

TAL activity was tested in the presence of 3.2 m*M* D-fructose 6-phosphate, 0.2 m*M* erythrose 4-phosphate, 0.1 m*M* NADH, 10 μg of α-glycerophosphate dehydrogenase/triosephosphate isomerase at a 1:6 ratio at room temperature by continuous absorbance reading at 340 nm for 6 min (55). The enzyme assays were conducted in the activity range of 0.001–0.01 U/ml. G6PD was measured in the presence of 120 m*M* Tris, pH 7.7, 10 m*M* $MgCl_2$, 2 m*M* glucose 6-phosphate, 0.9 m*M* NADP, and 0.1 U/ml 6PGD (59). 6PGD activity was determined in 120 m*M* Tris, pH 7.7, 10 m*M* $MgCl_2$, 0.9 m*M* NADP, 2 m*M* 6-phosphogluconate (59).

Glutathione levels

Total glutathione content was determined by the enzymatic recycling procedure essentially as described by Tietze (69). Cells (10^6) were resuspended in 50 μl of 4.5% 5-sulfosalicylic acid. The acid-precipitated protein was pelleted by centrifugation at 4°C for 10 min at 15,000 *g*. The total protein content of each sample was determined using the Lowry assay (39). GSH content of the aliquot assayed was determined by comparison with reference curves generated with known amounts of GSH (7). Reduced (GSH) and oxidized (GSSG) glutathione were measured by reverse-phase ion-exchange HPLC using UV detection at 365 nm (21). In brief, 10^6 cells were deproteinized in the presence of 10% perchloric acid and 1 m*M* bathophenanthrolinedisulfonic acid. After repeated freezing and thawing, samples were centrifuged at 15,000 *g* for 3 min. Fifty microliters of 100 m*M* monoiodoacetic acid in 0.2 m*M* *m*-cresol purple was added to 500 μl of supernatant. Samples were neutralized by addition of 480 μl of 2 *M* KOH and 2.4 *M* $KHCO_3$ and incubated in the dark at room temperature for 10 min. Then 1 ml of 1% fluorodinitrobenzene was added, and the samples were incubated in the dark at 4°C overnight. After centrifugation and filtering, 100 μl of supernatants was injected into the HPLC equipped with a photodiode array detector (Waters Alliance System, Milford, MA, U.S.A.) and a Waters Spherisorb NH_2 column (4.6 × 250 mm; 10 μm) .

Measurement of ascorbate

Jurkat cells (4×10^6) were treated with 400–800 μ*M* DHA for 15 min to 48 h. After incubation, cells were washed with phosphate-buffered saline and the pellets were deproteinized in 5% metaphosphoric acid. The lysate was centrifuged for 10 min at 14,000 *g*. Twenty-five microliters of each supernatant was injected on a C_{18} column (Waters Nova-Pak; 3.9 × 150 mm, 4 μm). Ascorbate was detected at 232 nm wavelength using HPLC (27).

Western blot analysis

Forty micrograms of total cell lysate in 10 μl per well was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electroblotted to nitrocellulose as previously described (7). Nitrocellulose strips were incubated in 100 m*M* Tris, pH 7.5, 0.9% NaCl, 0.1% Tween 20, and 5% skim milk with the primary antibodies, anti-protein disulfide isomerase (PDI) polyclonal antibody SPA-890 (StressGen, Victoria, BC, Canada), anti-Fas monoclonal antibody ZB4 (Upstate Biotechnologies), antibody 170 directed to human TAL (6), and anti-actin monoclonal antibody C4 (Boehringer) at room temperature overnight. After washing, the blots were incubated with biotinylated secondary antibodies and, subsequently, with horseradish peroxidase-conjugated avidin (Jackson Laboratories, West Grove, PA, U.S.A.). Between incubations, the strips were washed in 0.1% Tween 20, 100 m*M* Tris, pH 7.5, and 0.9% NaCl. The blots were developed with a substrate comprised of 1 mg/ml 4-chloronaphthol and 0.003% H_2O_2. Fas, PDI, TAL, and actin protein levels of control and DHA-treated cells were quantified by densitometry (Model GS-700, Bio-Rad, Hercules, CA, U.S.A.). Alternatively, low-abundance proteins, *e.g.,* Fas, were visualized using a chemiluminescence detection system (Amersham Pharmacia Biotech Inc; Piscataway, NJ, U.S.A.).

Statistics

Alterations in cell survival, PPP enzyme activities, and GSH levels were analyzed by Student's *t* test. Changes were considered significant at $p < 0.05$.

RESULTS

Contrasting effects of DHA on H_2O_2- and Fas-induced apoptosis pathways

Lymphoid cells preferentially import DHA over ascorbate. Whereas ascorbate at concentrations above the normal serum level of 50 μ*M* (2, 54, 60) induces apoptosis, DHA alone, up

to 1 m*M*, does not affect cell viability (56). Surprisingly, DHA pretreatment had opposing effects on H_2O_2- and Fas-induced cell-death pathways. Jurkat and H9 human leukemic T cells were preincubated with 200–800 μ*M* DHA for 48 h and washed with fresh media, and then apoptosis was induced with 100 μ*M* H_2O_2 or 100 ng/ml CH-11 Fas IgM antibody, as previously described (7, 9). As noted earlier, pretreatment with 400 μ*M* or more DHA for 48 h protected Jurkat (Fig. 1a) and H9 cells (Fig. 1b) from H_2O_2-induced apoptosis. In contrast, preincubation with DHA dramatically enhanced Fas-induced cell death in both cell lines (Fig. 1).

Effect of DHA on $\Delta\Psi_m$

Disruption of $\Delta\Psi_m$ has been proposed as the point of no return in apoptotic signaling (65, 73, 82). $\Delta\Psi_m$ was monitored with respect to externalization of PS, an early event in programmed cell death (42, 75). $\Delta\Psi_m$ was assessed with potentiometric fluorescent probes, $DiOC_6$ (53, 67) and JC-1 (16, 64). As expected, incubation with H_2O_2 or Fas antibody resulted in PS externalization, and $\Delta\Psi_m$ was diminished in annexin V-positive cells (Fig. 2a). In correlation with previous results (9, 56), $DiOC_6$ fluorescence was increased in annexin V-negative cells (Fig. 2a), suggesting that an elevation of $\Delta\Psi_m$, *i.e.,* mitochondrial hyperpolarization, preceded PS externalization in Jurkat cells. Control cells stained with JC-1 showed green fluorescence, whereas H_2O_2-treated and Fas-stimulated cells gained red fluorescence (Fig. 2b), consistent with elevation of $\Delta\Psi_m$. DHA pretreatment for 48 h attenuated the H_2O_2-induced augmentation of $\Delta\Psi_m$ in annexin V-negative cells (Fig. 2a). By contrast, DHA stimulated Fas-induced mitochondrial hyperpolarization of annexin V-negative cells and accelerated PS externalization (Fig. 2a). Thus, the impact of DHA on H_2O_2- and Fas-induced cell death occurred prior to changes in $\Delta\Psi_m$.

DHA stimulates the PPP and GSH levels and diminishes mitochondrial ROI content in Jurkat and H9 cells

Mitochondrial membrane permeability and $\Delta\Psi_m$ are subject to regulation by an oxidation–reduction equilibrium of

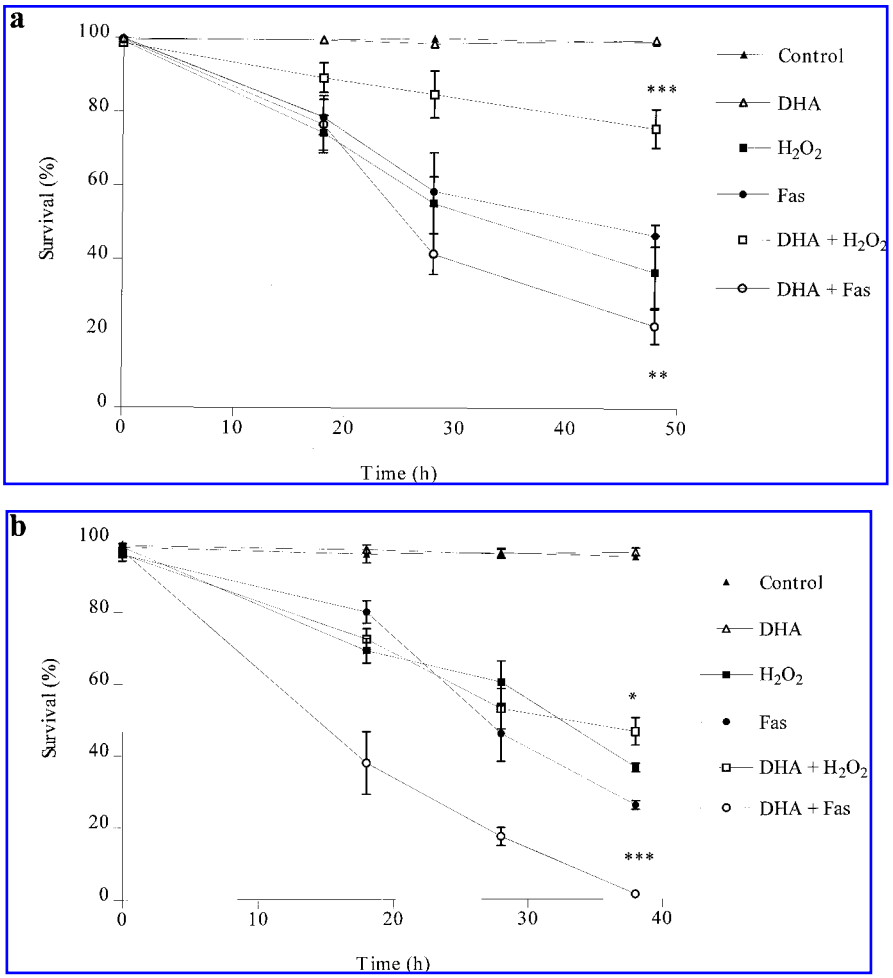
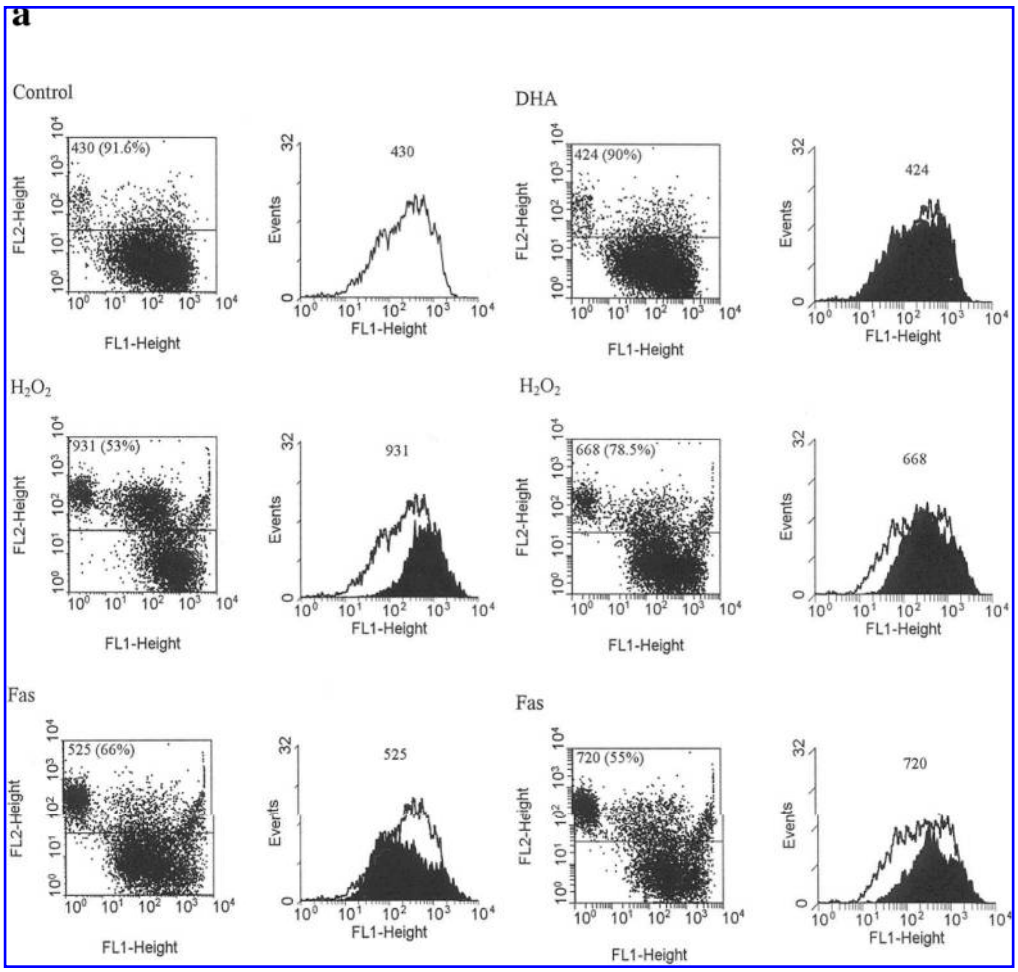


FIG. 1. Contrasting effects of DHA on H_2O_2- and Fas-induced cell death in Jurkat (a) and H9 cells (b). Cells were preincubated in the presence or absence of 400 μ*M* DHA for 36 h, washed, and then stimulated with 100 μ*M* H_2O_2 or 100 ng/ml CH-11 anti-Fas monoclonal antibody for 18–48 h. Percent survival was quantified by trypan blue exclusion. Data represent means ± SEM of three independent experiments. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.



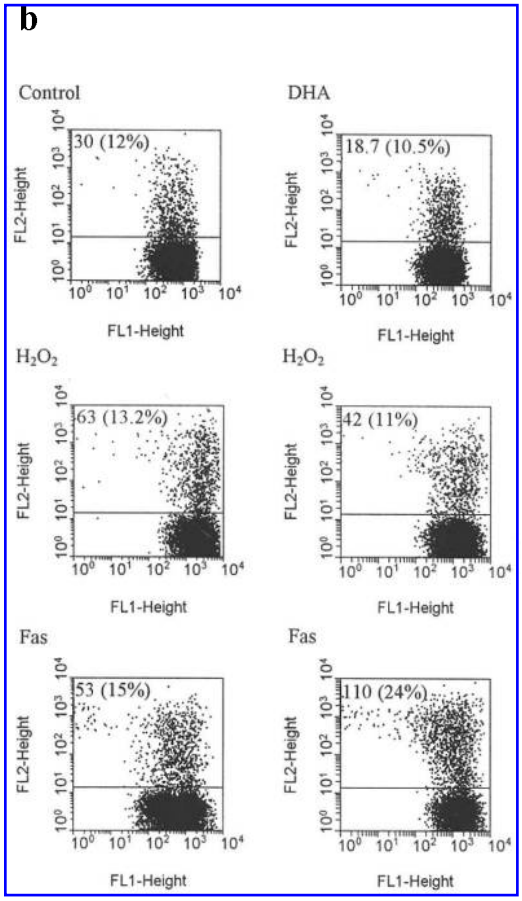


FIG. 2. Effect of DHA on H_2O_2- and Fas-induced changes in $\Delta\Psi_m$ and cell death. (**a**) Jurkat cells were preincubated with or without 400 μM DHA for 36 h. Cells were washed and exposed to 100 μM H_2O_2 or 100 ng/ml CH-11 Fas IgM antibody for 24 h, and analyzed by flow cytometry. Dead cells and debris were gated out by forward and side scatter measurements. PS externalization, an early event of apoptosis, and $\Delta\Psi_m$ were concurrently monitored by staining with annexin V-PE (FL-2) and $DiOC_6$ (FL-1), respectively (dot plots, **left columns**). Mean channel number of $DiOC_6$ fluorescence and percentage of annexin V-PE-negative cells (in parentheses) are indicated in the upper left corner of each dot plot. H_2O_2 and Fas treatment increased $DiOC_6$ fluorescence in annexin V-PE (FL-2) negative cells and decreased $DiOC_6$ fluorescence in annexin V-PE-positive cells. H_2O_2- and Fas-induced increase of $\Delta\Psi_m$ is shown by overlay of $DiOC_6$ fluorescence of annexin V-negative populations (histograms, **right columns**). Open curves correspond to control cells, whereas shaded curves represent H_2O_2-, Fas-, and/or DHA-treated cells. *x*-axis shows log FL-1 fluorescence intensity; *y*-axis indicates cell number (events). Values over curves indicate mean channel of $DiOC_6$ fluorescence. (**b**) JC-1 fluorescence (FL-2) of H_2O_2-, Fas-, and/or DHA- treated cells. Cells were incubated in the presence or absence of 400 μM DHA for 36 h. After washing, apoptosis was induced with 100 μM H_2O_2 or 100 ng/ml Fas antibody for 24 h, and cells were analyzed by flow cytometry. Dead cells and debris were gated out by forward and side scatter measurements. JC-1 selectively incorporates into mitochondria, where it forms monomers (fluorescence in green, FL-1) or aggregates, at high transmembrane potentials (fluorescence in red, FL-2). Mean channel number and percentage (in parentheses) of high-potential aggregates are indicated in the upper left corner of each dot plot.

ROIs, pyridine nucleotides (NADH/NAD + NADPH/NADP), and GSH levels (15). Increased ROI and diminished GSH levels have been shown to enhance both H_2O_2- and Fas-induced apoptosis (9, 56). Regeneration of GSH from its oxidized form depends on NADPH produced in the PPP. As DHA can stimulate the PPP and GSH synthesis (56), these effects were expected to influence H_2O_2- and Fas-induced apoptosis similarly. In accordance with previous findings, DHA pretreatment dose-dependently stimulated activity of PPP enzymes, G6PD, 6PGD, and TAL, and elevated intracellular GSH levels (Fig. 3). Short-term (<8 h) incubation with DHA had no effect on GSH levels (56), whereas pretreatment with DHA for 24–48 h increased intracellular GSH levels in both cells lines (Fig. 3b and d). In Jurkat cells, maximum GSH elevation, from a baseline of 7.65 ± 1.2 ng/μg of protein to 25.36 ± 3.05 ng/μg ($p < 0.001$), was observed after 48 h of stimulation with 800 μ*M* DHA. In H9 cells, 24 h of stimulation with 400 μM DHA caused the elevation of intracellular GSH from 3.1 ± 0.27 ng/μg of protein to 11.43 ± 2.16 ng/μg ($p < 0.001$). Limited increases of GSH in H9 cells between DHA concentrations of 600 and 800 μ*M* may be due to lower PPP enzyme activities and accumulation of toxic metabolities with respect to Jurkat cells.

The effect of DHA on ROI levels was assessed using oxidation-sensitive fluorescent probes, DHR and HE, as previously described (9). DHR is nonfluorescent, uncharged, and readily taken up by cells, whereas R123, the product of DHR oxidation, is fluorescent and positively charged and binds selectively to the inner mitochondrial membrane of living cells (58). Fluorescence of this dye is an indicator of mitochondrial ROI production and maintenance of the membrane integrity and transmembrane potential. HE is oxidized into ethidium by ROIs and remains in the cytosol (84). Thus, R123 fluorescence correlates with mitochondrial ROI levels, whereas ethidium fluorescence reflects cytosolic ROI levels. Short-term (1–6 h) treatment with DHA alone did not increase R123 (Fig. 4A) or ethidium fluorescence (Fig. 4B), thus excluding the possibility of DHA-induced oxidative stress. Although H_2O_2, by itself, is not toxic, it is highly diffusible and capable of inducing apoptosis upon transformation to ROI in mitochondria. As the first line of defense against ROI involves GSH, metabolic stimulation of the PPP and GSH levels by DHA is likely to be responsible for inhibition of H_2O_2-induced apoptosis. Elevation of GSH content alone was expected to diminish rather than enhance processing of Fas-

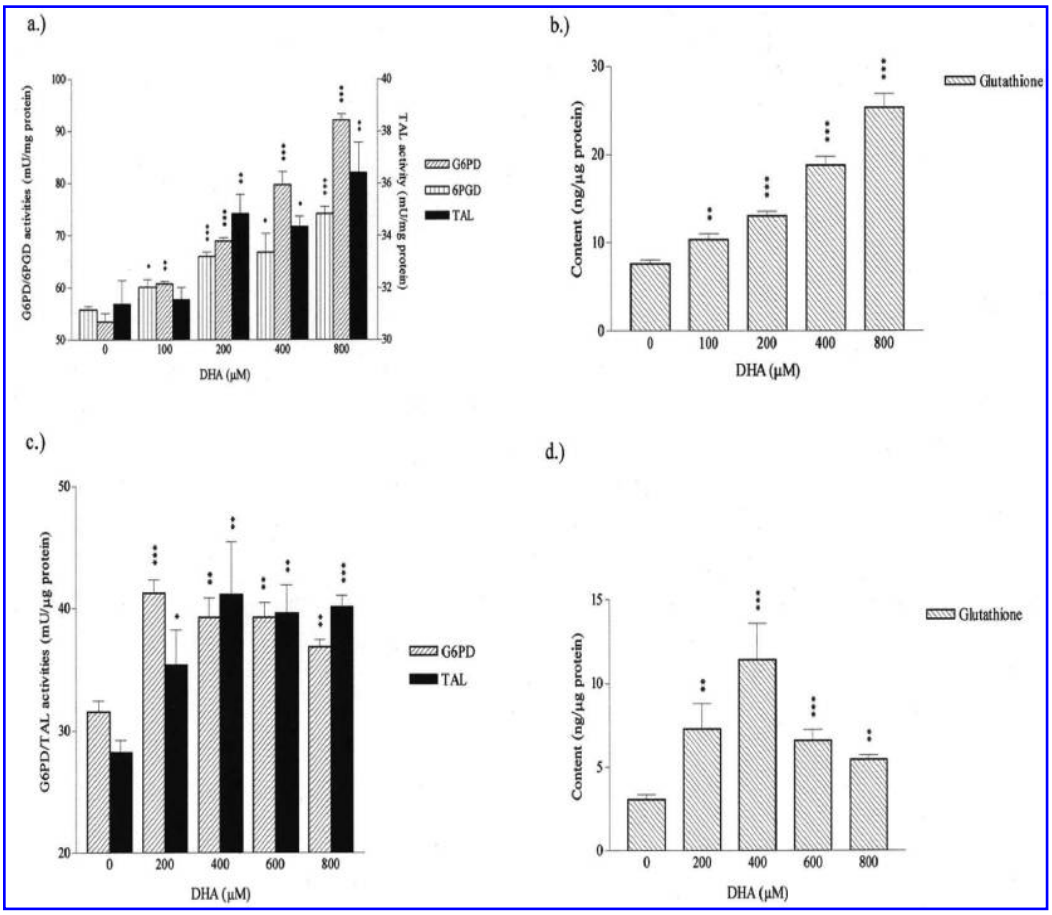


FIG. 3. DHA-induced changes in G6PD, 6PGD, and TAL activities and glutathione levels in Jurkat (a and b) and H9 cells (c and d). Cells were preincubated with 100–800 μ*M* DHA for 48 h. Data represent means ±SEM of four to six independent experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

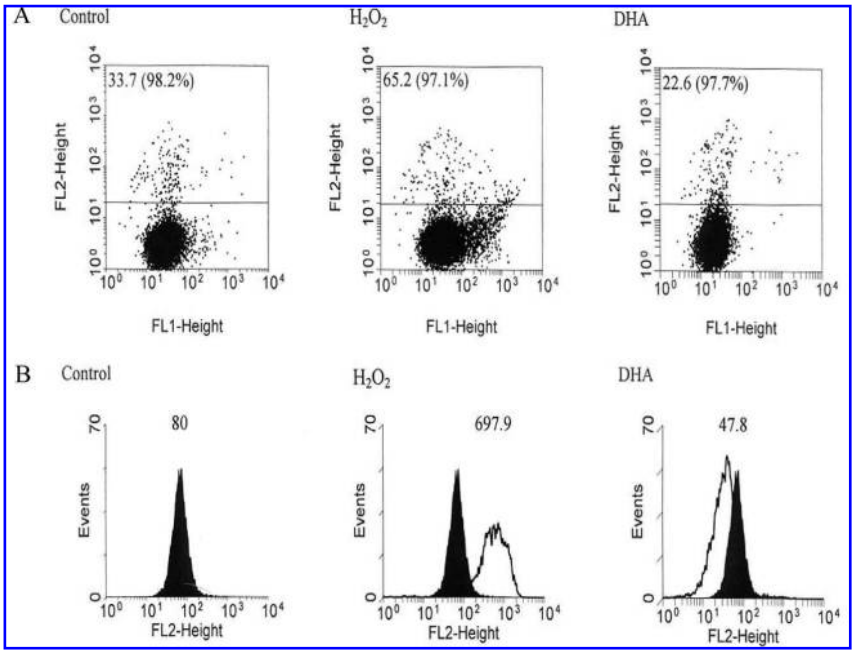


FIG. 4. Effect of DHA and H_2O_2 on ROI levels. (**A**) Jurkat cells were incubated with 100 μ*M* H_2O_2 or 400 μ*M* DHA for 6 h and analyzed by flow cytometry. Mitochondrial ROI levels and cell death were monitored by staining with DHR (FL-1) and annexin V-PE (FL-2), respectively. (**B**) Jurkat cells were preincubated with 100 μ*M* H_2O_2 or 400 μ*M* DHA for 6 h and analyzed by flow cytometry using HE. Mean channel number of ethidium fluorescence is indicated over each histogram. Data are representative of four independent experiments.

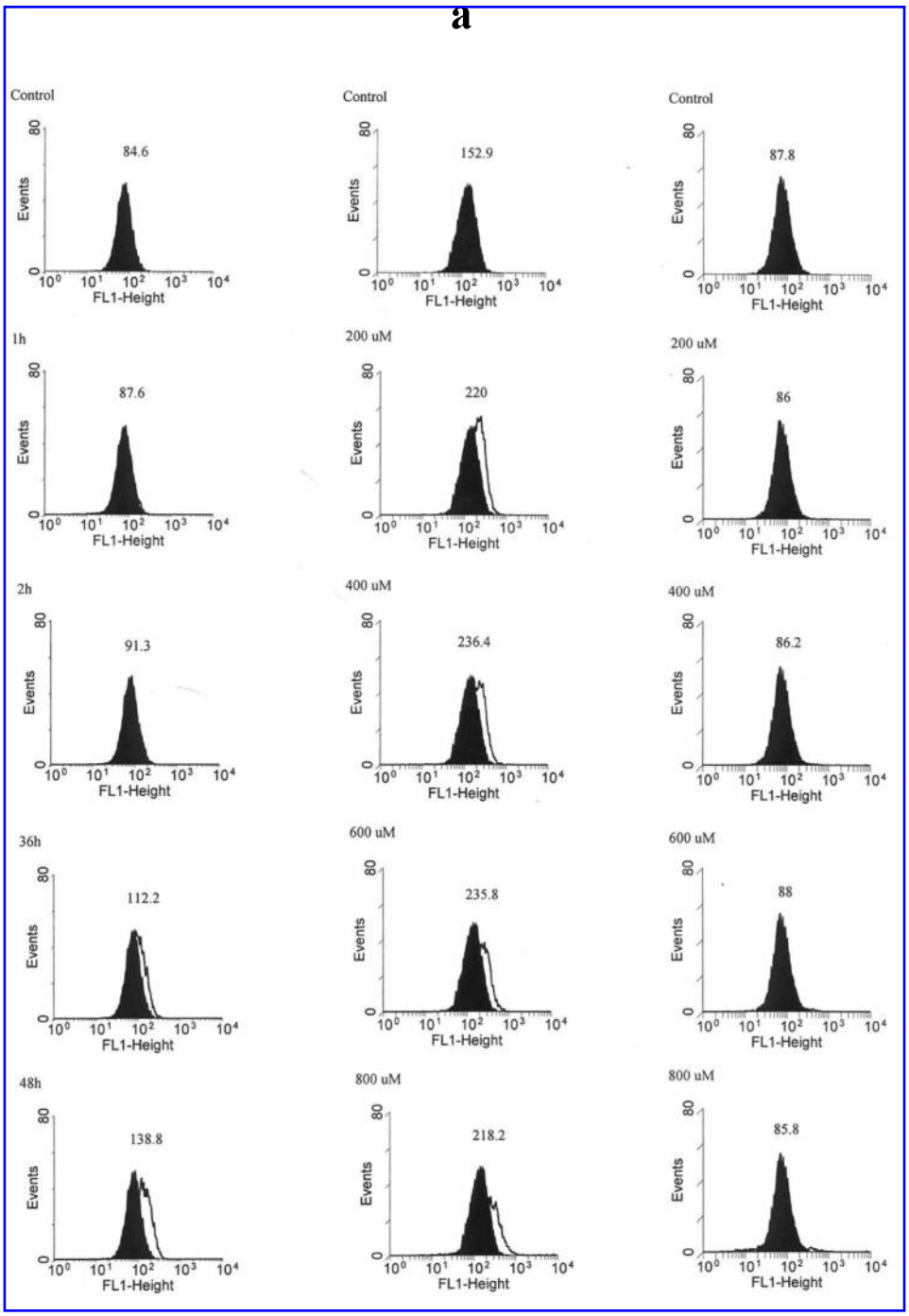
mediated apoptosis at the level of caspase 8 activation, or disruption of $\Delta\Psi_m$ (9).

DHA stimulates surface expression of the Fas receptor

Sensitivity to Fas apoptosis can be regulated upstream of caspase 8 activation, by redistribution of death receptors to the cell surface (10, 47, 57, 76, 83) or progression through the cell cycle (1, 18, 40). Therefore, we next examined the effect of DHA treatment on Fas-receptor expression and cell-cycle progression. Fas-receptor expression was assessed with IgG_1 monoclonal antibody ZB4. As controls, monoclonal IgG_1 antibodies to actin (negative control antigen) and CD4 antigen were utilized. Incubation of Jurkat and H9 cells with 200–800 μ*M* DHA for 36 h significantly increased cell-surface Fas-receptor expression (Fig. 5). In Jurkat cells, highest elevation of Fas expression was observed after 36 h of stimulation with 400 μ*M* DHA. In H9 cells, Fas expression peaked after 24 h or stimulation with 600 μ*M* DHA. CD4 expression was not affected by DHA (Fig. 5). Western blot analysis showed no difference in Fas, PDI, or TAL protein levels with respect to actin between control and DHA-treated Jurkat and H9 cells. After 48 h of treatment with DHA, Fas expression was reduced by 24 ± 7.6% in H9 and 18 ± 10.8% in Jurkat cells. However, these changes were not statistically significant. Representative western blots are shown in Fig. 6. Therefore, increased cell-surface Fas-receptor staining was not a consequence of augmented protein expression.

DISCUSSION

Ascorbate protects cell membranes from external oxidants by reacting directly with ROIs or regenerating vitamin E from α-tocopheroxyl radical (11, 51) and has a GSH-sparing effect (46). However, vitamin C cannot be efficiently transported into blood cells. Moreover, ascorbate concentrations above the normal serum level of 50 μ*M* induce oxidative stress and apoptosis of lymphoid and myeloid cells (2, 54, 60). Interestingly, blood cells and related tumor cell lines preferentially import DHA over ascorbate (23). DHA has long been considered as a product of ascorbate oxidation (66, 79). Nevertheless, DHA can increase GSH levels through stimulation of the PPP (56). Thus, the intracellular presence of DHA may not solely represent an accumulation of the oxidized form of vitamin C, but it may have a role in influencing GSH levels through the PPP. Products of DHA metabolism, 2,3-diketo-L-gulonate and its decarboxylation products, L-xylonate and L-lyxonate, can enter the nonoxidative branch of PPP (3, 12, 33). Activity of the PPP is vitally important by providing NADPH for biosynthetic reactions and maintenance of GSH in a reduced state and, thus, regulating the redox homeostasis of the cell. Accordingly, treatment of Jurkat cells with DHA increased G6PD, 6PGD, and TAL activities. These changes were accompanied by a severalfold increase in GSH level and protection from H_2O_2-induced cell death. Carbon flux via the PPP has been implicated in regulating expression of other glucose-metabolizing enzymes, pyruvate kinase (20), glucose 6-phosphatase, and phosphoenolpyruvate carboxyki-



nase (43). Although xylulose 5-phosphate has been proposed as a key intermediate, its impact on the PPP and potential involvement of xylulose 5-phosphate metabolites remain to be determined (20, 43).

GSH and GSSG levels were not affected by pretreatment of Jurkat cells with DHA for <24 h. Along the same line, DHA did not influence intracellular ROI levels as estimated by DHR and HE fluorescence. DHA treatment, up to 1 m*M*, did not affect cell viability. Our data correlate with observations by others showing that DHA has no prooxidant effects, and it may directly neutralize ROIs by undergoing a peroxidative decarboxylation reaction (19). Thus, the possibility that increased PPP enzyme activities were triggered by a DHA-induced oxidative stress could be clearly excluded.

Stimulation by DHA of activities of NADPH-generating enzymes, G6PD and 6PGD, may be responsible for elevation of GSH levels. Genetically enforced augmentation of G6PD activities can increase GSH levels and resistance to apoptosis signals (6, 8, 61). In turn, increased GSH can directly stimulate expression of γ-glutamylcysteine synthetase, the rate-limiting enzyme of *de novo* GSSG synthesis (72), and further support GSH production. Thus, inhibition of H_2O_2-induced

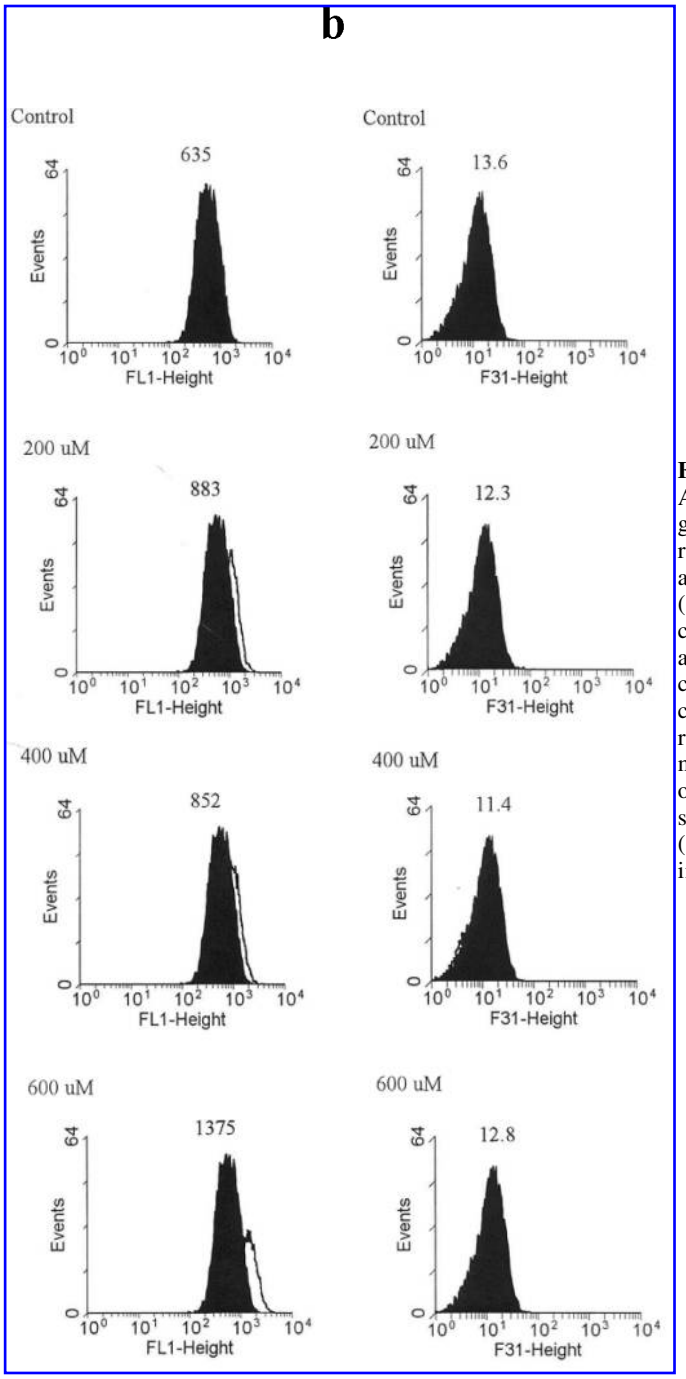
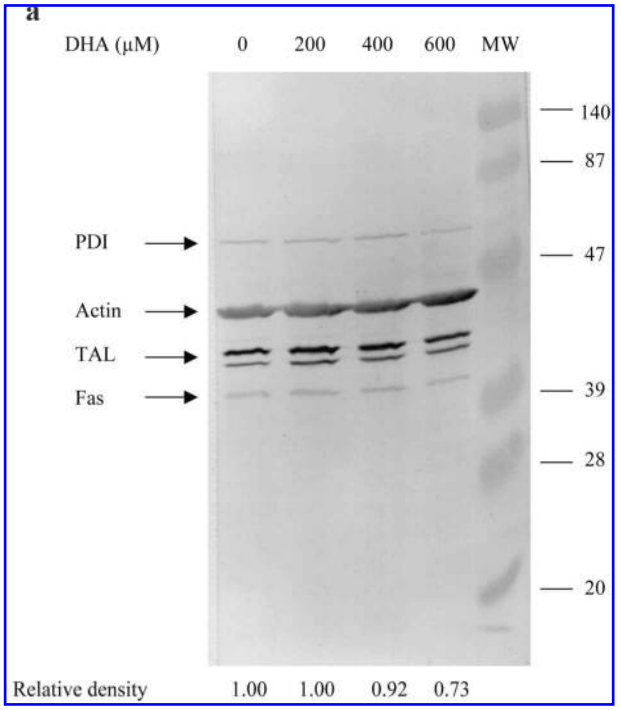


FIG. 5. Effect of DHA on cell surface Fas expression. After DHA treatment, cells were stained with FITC-conjugated anti-Fas monoclonal IgG_1 antibody ZB4 (FL-1, fluorescein) or Cy-5-conjugated anti-CD4 monoclonal IgG1 antibody Q4120 (FL-31, quantum-red). (**a**) Time course (**left column**) and dose dependence of DHA-induced changes on cell-surface Fas expression (**middle column**), and CD4 expression on Jurkat cells exposed to increasing concentrations of DHA for 48 h (**right column**). Shaded curves correspond to control cells, whereas open curves represent DHA-treated cells. Values over curves indicate mean channel of fluorescence. Expression in the presence of DHA is represented by overlays (open curves). (**b**) Cell-surface expression of Fas (**left column**) and CD4 antigens (**right column**) on H9 cells following exposure to increasing concentrations of DHA for 48 h.

apoptosis by DHA was consistent with elevation of intracellular GSH. DHA treatment caused higher elevation of GSH content and a more pronounced inhibition of H_2O_2-induced apoptosis in Jurkat with respect to H9 cells. These cell type-specific differences are likely to be related to higher maximum G6PD activities in DHA-treated Jurkat cells (92.1 ± 1.2 mU/mg of protein at 800 μ*M* DHA) as compared with H9 cells (41.2 ± 2.1 mU/mg of protein at 200 μ*M* DHA; Figs. 3a and c; $p << 0.0001$). Lower G6PD activities may make H9 cells less amenable to the protective effect of DHA and more susceptible to H_2O_2-induced cell death in general.

Surprisingly, increased GSH levels did not protect from Fas-mediated apoptosis. In contrast, after DHA preincubation, Fas-induced mitochondrial membrane hyperpolarization and cell death were dramatically accelerated in both H9 and Jurkat cells. DHA increased cell-surface Fas-receptor expression in both cell lines. The increased surface expression was not related to an elevation of total Fas protein levels, thus excluding increased Fas gene expression. As a control antigen, surface expression of CD4 was not affected by DHA.

The native structure of the Fas receptor is dependent on formation of disulfide bonds. Point mutations affecting cys-



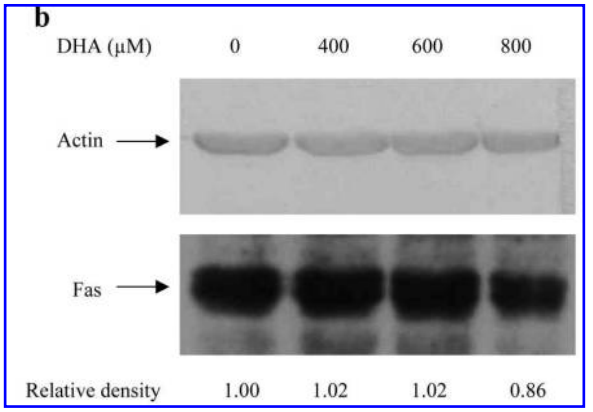


FIG. 6 (a) Effect of DHA treatment on expression of PDI (55 kDa), TAL (38 kDa), Fas (36 kDa), and actin (42 kDa) in H9 cells. After incubation with 400, 600, or 800 μ*M* DHA, protein levels were determined by western blot analysis. Forty micrograms of total protein from each cell lysate was loaded per lane. The same blot was successively analyzed with Fas (ZB4), PDI, TAL, and actin antibodies and developed using 4-chloronaphthol. MW, molecular weight marker. The relative density values represent expression of Fas with respect to actin in response to DHA treatment. **(b)** Effect of DHA treatment on expression of Fas in Jurkat cells. The Fas antigen was detected with chemiluminescence followed by visualization of actin on the same blot using 4-chloronaphthol. Relative expression of Fas with respect to actin was quantified by computerized densitometry.

teine residues of Fas and the related TNF receptor are incompatible with apoptotic signaling (31, 50). Folding into the correct three-dimensional structure through cross-linking of cysteine residues is catalyzed by PDI (22). PDI activity is particularly relevant for folding of proteins with cysteine-rich domains, such as TNF and Fas (22). Although GSSG has also been proposed as an oxidant cofactor for PDI, recent evidence shows that the glutathione system provides reducing rather than oxidizing power for protein folding in the endoplasmic reticulum (5, 17). Interestingly, PDI also catalyzes the reduction of DHA to ascorbate (77). There is evidence that DHA may be an oxidant cofactor of PDI-mediated folding in the endoplasmic reticulum (5). Although expression of Fas was stimulated by DHA, cell-surface CD4 density was not affected in DHA-treated cells. Of note, the extracellular domain of the Fas receptor has 20 cysteine residues (32), whereas that of CD4 only contains six cysteines (41). The presence of cysteine-rich domains may explain selective up-regulation of Fas expression by DHA. In summary, the data identify DHA and its sugar metabolites as potential targets for development of selective apoptosis regulators.

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ABBREVIATIONS

annexin V-FITC, annexin V-PE, phycoerythrin-conjugated annexin V; fluorescein-conjugated annexin V; DCF, 5,6-carboxy-2′,7′-dichlorofluorescein; DCFH-DA, 5,6-carboxy-2′,7′-dichlorofluorescein-diacetate; DHA, dehydroascorbate; DHR, dihydrorhodamine 123; $DiOC_6$, 3,3′-dihexyloxacarbocyanine iodide; G6PD, glucose 6-phosphate dehydrogenase; GSH, reduced glutathione; GSSG, oxidized glutathione; HE, hydroethidine; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide; mClCCP, carbonyl cyanide *m*-chlorophenylhydrazone; PDI, protein disulfide isomerase; 6PGD, 6-phosphogluconate dehydrogenase; PPP, pentose phosphate pathway; PS, phosphatidylserine; ROI, reactive oxygen intermediate; TAL, transaldolase; TNF, tumor necrosis factor; $\Delta\Psi_m$, mitochondrial transmembrane potential.

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