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Protective Effect of Docosahexaenoic Acid against Hydrogen Peroxide-Induced Oxidative Stress in Human Lymphocytes

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ABSTRACT. Oxidatively stressed lymphocytes exhibit decreased proliferative response to mitogenic stimulation. Although several sensitive targets involved in lymphocyte suppression have already been identified, little is known about the influence of oxidative stress on cyclic nucleotide phosphodiesterases (PDE) (EC 3.1.4.17), thought to play a major role in the control of cyclic AMP (cAMP) level, a well-recognized negative effector of lymphoproliferation. Although the polyunsaturated fatty acid content of membrane phospholipids is thought to be directly related to the extent of oxidant-induced lipid peroxidation, some n-3 fatty acids also seem to have antioxidant effects, depending on the concentration used and the overall redox status of the cells in question. Results of the present study showed that human peripheral blood mononuclear cells (PBMC) as well as rat thymocytes were relatively resistant to a short-term exposure (10 min) to hydrogen peroxide (H_2O_2). Indeed, H_2O_2-induced lipid peroxidation, estimated by malondialdehyde (MDA) production, was only 2-fold increased by H_2O_2 concentrations lower than 2 mM, whereas a larger increase (10-fold) could be observed in PBMC at the highest dose (5 mM). Previous enrichment of PBMC with 5 μM docosahexaenoic acid (22:6n-3), brought to the cells as a fatty acid–albumin complex (ratio 1), significantly reduced MDA production induced by low doses of H_2O_2, the protective effect no longer being observed at the highest doses. In contrast, eicosapentaenoic acid (20:5n-3) did not have any protective effect. Cytosolic PDE activities of both human PBMC and rat thymocytes were significantly inhibited (40–50%) after H_2O_2 treatment of the cells, whereas particulate PDE activities were not modified. Different responses of PDE activities to H_2O_2 treatment were observed when PBMC were first enriched with 22:6n-3 prior to H_2O_2 addition. In 22:6n-3-treated cells, the H_2O_2-induced inhibition of both cAMP- and cGMP-PDE cytosolic activities was abolished, whereas the particulate activities were increased by the highest H_2O_2 concentration used (5 mM). At the same time, the glutathione peroxidase (glutathione: oxidoreductase, EC 1.11.1.9) (GSH-Px) activity of PBMC and thymocytes was only marginally inhibited by H_2O_2 addition (20%), and pretreatment of the cells with 22:6n-3 did not modify the slight inhibitory effect of H_2O_2. Collectively, these results suggest that lymphocytes are relatively resistant to H_2O_2-induced lipid peroxidation due to their high GSH-Px content, and that low doses of 22:6n-3 are able to prevent some of the H_2O_2-induced alterations such as lipid peroxidation and PDE inhibition. Docosahexaenoic acid might thus offer some protection against oxidant-induced lymphocyte suppression. BIOCHEM PHARMACOL **57**;9:1021–1030, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. hydrogen peroxide; lipid peroxidation; docosahexaenoic and eicosapentaenoic acids; glutathione peroxidase; cyclic nucleotide phosphodiesterase; activated human lymphocytes

Reactive oxygen intermediates (ROIs) have been implicated in certain disease processes and aging [1]. Among ROIs, H_2O_2† has been shown to alter the immune responses of lymphocytes in inflammatory microenvironments. At sublethal concentrations, H_2O_2 inhibits the proliferation and effector function of human T cells without cellular damage [2]. It has been hypothesized that H_2O_2 may trigger cellular signaling pathways which are also utilized by physiological T cell activators, leading to enhancement or suppression of T cell responses depending on the level of oxidative stress. One of the best characterized T cell responses to H_2O_2-induced oxidative stress is the rapid increase in tyrosine phosphorylation either through activation of protein tyrosine kinases [3] or inhibition of protein tyrosine phosphatase [4]. In addition, H_2O_2 has also been shown to modulate the activity of several serine/threonine kinases important for T cell signal transduction. In Jurkat cells, submillimolar H_2O_2 concentrations were

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† *Abbreviations:* H_2O_2, hydrogen peroxide; GSH-Px, glutathione peroxidase; MDA, malondialdehyde; PBMC, peripheral blood mononuclear cells; PDE, cyclic nucleotide phosphodiesterase; PLD, phospholipase D; TBA, thiobarbituric acid; and cAMP, cyclic AMP.

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shown to induce a truncated process of protein kinase C activation involving stimulation of cytosolic activity without translocation to membranes [4], whereas pharmacological doses were inhibitory. The partial activation of cytosolic protein kinase C may in turn stimulate mitogen-activated protein kinases, which are also well-recognized targets of H_2O_2 in various cell types including smooth muscle [5] and Jurkat cells [4, 6]. Ultimately, the activation of multiple kinase cascades would result in activation of transcriptional factors such as nuclear factor-κB [7] or AP-1 [8]. Among the second messengers involved in the control of lymphocyte activation, cAMP is a well-recognized negative effector of the proliferative response, and the inhibitors of the cAMP-degradating phosphodiesterases are potent suppressors of immune function [9]. We have already shown that the mitogenic activation of rat thymocytes [10] or human lymphocytes [11] induces a rapid increase in PDE activity within the first minutes of activation. The stimulation of PDE activity is hypothesized to be a necessary step to maintain the low cAMP level required for an optimal lymphocyte response. However, little is known about the influence of oxidative stress on the cAMP-regulating pathways in lymphocytes. Thus, one aim of the present study was to investigate whether PDE activity of human lymphocytes and rat thymocytes was sensitive to an oxidative stress induced by pharmacological concentrations of H_2O_2. Oxidants such as hydroperoxides and free radicals produce cell injury by oxidative modifications of membrane lipids and proteins [12]. The polyunsaturated fatty acid moieties of membrane phospholipids are particularly susceptible to oxidant injury because of the relative reactivity of the bis-allylic hydrogen atoms within the fatty acid double bond systems [13]. It has been shown that altering the fatty acid composition of membrane phospholipids by culturing cells in supplemented medium modulates the susceptibility of the cells to oxidative damage. However, the relationship between the extent of unsaturation of cellular fatty acids and oxidant susceptibility is complex. For example, it has been shown that supplementation of culture media with 18:2n-6 and 18:3n-6 fatty acids protects endothelial cells from H_2O_2-mediated cell detachment, whereas supplementation with 18:1n-9 is less effective [14]. Most human nutritional studies indicate that supplementation of the diets with n-3 polyunsaturated fatty acid (PUFA) enhanced cellular lipid hydroperoxide production, as evidenced by increased urinary MDA excretion, but they usually involved high dietary intakes [15]. On the other hand, it is becoming increasingly clear that n-3 PUFA may exhibit both pro- and antioxidant activities depending on the experimental conditions and the doses used. Using diamide-treated platelets as an *in vitro* model of oxidative stress, characterized by reduced glutathione and vitamin E levels and increased arachidonic acid peroxidation, Calzada *et al.* [16] have shown that low concentrations of 20:5n-3 were able to normalize platelet vitamin E and peroxidation. The second aim of the present study was to investigate whether low concentrations of 22:6n-3, which is abundant in fish oil together with 20:5n-3, were able to protect human lymphocytes and rat thymocytes against H_2O_2-induced lipid peroxidation evaluated by MDA formation, and against H_2O_2-induced alteration of PDE activities. As GSH-Px is a key enzyme controling cellular redox status and a known target of 22:6n-3 in platelets [17] and lymphocytes [18], we also measured its activity in H_2O_2-stressed lymphocytes either in the presence or absence of 22:6n-3.

MATERIALS AND METHODS

Chemicals

Lymphocyte preparation medium, RPMI 1640 (with HEPES), fatty acid-free human albumin, fatty acids (20:5n-3, 22:6n-3), β-NADPH tetrasodium salt, *t*-butyl hydroperoxide, and glutathione reductase (Type III) were obtained from Sigma-Chimie. Glutathione (reduced) was from Boehringer Mannheim. [8-^{3}H] cGMP (710 GBq/mmol), [8-^{3}H] cAMP (962 GBq/mmol), and [U-^{14}C] adenosine (20 GBq/mmol) were from Amersham. [U-^{14}C] guanosine (19 Gbq/mmole) was from NEN DuPont.

Cell Preparations

HUMAN **PBMC.** Peripheral blood was obtained from healthy subjects who had not taken any medication for 2 weeks prior to blood donation (Etablissement de Transfusion Sanguine, Lyon). Venous blood was drawn into citrate–phosphate–dextrose anticoagulant, pH 5.6. The platelet-rich plasma was removed after initial centrifugation (120 *g* for 18 min), and red blood cells were passively sedimented for 30 min at 37° after addition of dextran (1% final concentration) prepared in buffered saline pH 7.4 containing 1 mM EDTA. The leukocyte-rich supernatant was then removed, layered onto Histopaque (density = 1.077), and centrifuged at 600 *g* for 15 min at 20°. The resulting mononuclear fraction was washed with PBS and then washed twice with RPMI 1640 by low-speed centrifugations in order to more thoroughly eliminate the contaminating platelets. Cell viability checked with trypan blue staining was routinely higher than 95%. Flow cytometry analyses of cell preparations after staining with specific monoclonal antibodies showed that about 65–70% of the isolated cells were CD3+ T cells (T_3 Coulter clone), 4–6% were CD19+ B cells (B_4 Coulter clone), 16–24% were CD11b+ monocytes (MO1 Coulter clone), and 4–6% were CD41a+ platelets (GP IIb IIIa, Immunotech).

RAT THYMOCYTES**.** Thymocytes were isolated from thymus glands of male Sprague–Dawley rats as previously described [10]. Briefly, thymus glands were cleaned of adherent connective tissues and gently dilacerated in a loose-fitting glass/glass homogenizer. Cell suspensions were filtered through a nylon gauze. Thymic lymphocytes were then separated by gradient centrifugation on lymphocyte separation medium, washed with 0.15 M NaCl and sus-

pended in RPMI 1640 medium. Cell viability was consistently greater than 95%.

Fatty Acid Preparations

Free fatty acids were stored at −20° in ethanol under nitrogen. Aliquots of the ethanolic solution were evaporated to dryness under reduced pressure. RPMI 1640 medium containing 5 μM human albumin was added to give a final fatty acid concentration of 5 μM and an albumin to fatty acid ratio of 1. The mixtures were incubated at 37° under nitrogen for 3 hr.

Experimental Incubations

Human PBMC or rat thymocytes were preincubated for 60 min at 37° in RPMI medium (2×10^7 cells/mL) containing the fatty acids complexed to human serum albumin. Control cells were preincubated under the same conditions without fatty acids. At the end of the preincubation period, increasing concentrations of H_2O_2 were added and cells were incubated for a further 10 min at 37°. They were then pelleted and washed. For MDA determinations, pelleted cells were suspended in NaCl and immediately frozen until the assays. For enzymatic activity determinations, pelleted PBMC were washed three times with Phillips' buffer and disrupted by glycerol lysis according to Caldwell *et al.* [19] as previously described [11]. After glycerol treatment, the cells were pelleted at 900 *g* for 10 min, resuspended in lysis buffer (20 mM HEPES, 25 mM sucrose, 0.1 mM EGTA, 0.05 mM phenylmethylsufonyl fluoride, 10 U/mL aprotinin, 2 μg/mL pepstatin A, pH 7.4) and stored frozen at −80°. After thawing, the cells were homogenized in a glass/teflon homogenizer (40 strokes at maximal speed) and homogenates were centrifuged at 100,000 *g* for 60 min. Thymocytes were lysed in hypotonic buffer (10 mM Tris–HCl, 1 mM $MgCl_2$, pH 7.2) in the presence of protease inhibitors (2 μg/mL pepstatin A, 40 U/mL aprotinin, 1 mM phenylmethylsulfonyl fluoride) and homogenized in a glass/glass homogenizer. The homogenate was mixed with 4 volumes of 0.25 M sucrose and centrifuged at 100,000 *g* for 60 min as previously reported [9]. Supernatant and particulate fractions from both sources were stored at −80° until enzyme assays.

Incorporation of Fatty Acids into Cell Phospholipids

In experiments designed to control the 22:6n-3 enrichment of cell phospholipids, cells were preincubated for 60 min at 37° in RPMI medium (2×10^7 cells/mL) containing 22:6n-3 complexed to human serum albumin as described above. Lipids were extracted as previously described [20] and diheptanoyl phosphatidylcholine was added as an internal standard at the time of lipid extraction. Lipid extracts were chromatographed on TLC plates developed with hexane/diethyl ether/acetic acid (70:30:1 by vol.), which left phospholipids at the origin. Phospholipids were scraped off from the plate and directly transmethylated with 14% boron trifluoride in methanol, at 100° for 90 min under N_2 atmosphere. Fatty acid methyl esters were quantified by gas-liquid chromatography using a 0.25 mm × 60 m SP 2380 capillary column (Supelco) and helium as a carrier gas. The oven temperature was programmed from 145 to 210° at 0.9°/min. Fatty acids were identified by retention time comparisons with standard fatty acid methyl esters. Data are expressed as mean values ± SE.

Malondialdehyde Determination

MDA was determined by RP-HPLC as the MDA–TBA complex according to the method of Therasse and Lemonier [21]. Briefly, thawed cell suspensions were treated with 10 mM TBA dissolved in 0.1 M phosphate buffer (pH 3) and incubated for 1 hr at 95° in the presence of acetic acid and butylated hydroxytoluene as an antioxidant. After extraction with ethylacetate, the MDA–TBA adduct was separated on a 25-cm column packed with Nucleosil C_{18} (5 μm). The mobile phase water/methanol (80:20, v/v) was pumped at a flow rate of 0.8 mL/min. The MDA–TBA adduct was detected and quantified at 532 nm.

Cyclic Nucleotide Phosphodiesterase Assays

PDE activities of supernatant and pellet fractions were measured according to a two-step radioisotopic method modified from Thompson *et al.* [22] by Prigent *et al.* [23]. Briefly, samples were incubated at 30° with 5 mM $MgCl_2$, 0.25 μM cAMP or cGMP, 925 Bq [8-^{3}H] cAMP or [8-^{3}H] cGMP, 0.5 mg/mL BSA, and 3.75 mM 2-mercaptoethanol in 40 mM Tris–HCl buffer, pH 8. Enzyme dilutions and incubation times were adjusted so that no more than 20% of the substrate was hydrolyzed under the assay conditions. The reaction was terminated by boiling for 1 min at 100°. Samples were then incubated at 30° with snake venom (0.6 mg/mL). The reaction was stopped by addition of 1 mL AG1X2 resin slurry suspended in 15 mM acetic acid for the cAMP–PDE assay or in 115 mM formic acid for the cGMP–PDE assay. Adenosine and guanosine recoveries were measured in each assay by means of [U-^{14}C] adenosine or guanosine, simultaneously added with the snake venom. Results are expressed as pmol of cAMP or cGMP hydrolyzed/min/mg protein. Proteins were assayed according to Bradford [24] using BSA as a standard.

Glutathione Peroxidase Assay

GSH-Px activity was determined according to the method of Paglia and Valentine [25] as modified by Chaudière and Gérard [26]. The assay mixture consisted of 50 mM Tris–HCl buffer, pH 7.6, 1 mM EDTA, 0.125 mM NADPH, 1 U/mL glutathione reductase and GSH (1 mM for PBMC and 3 mM for rat thymocytes). Cell supernatants were preincubated for 2 min at 37° in the assay mixture, and the reaction was initiated by the addition of 0.366 mM *t*-

TABLE 1. Fatty acid composition of membrane phospholipids in control and 22:6n-3-treated human PBMC and rat thymocytes

	Composition (mol%)†			
Fatty acid*	Control PBMC‡	22:6n-3-treated PBMC	Control thymocytes	22:6n-3-treated thymocytes
16:0	20.89 ± 0.79	17.47 ± 1.66	26.35 ± 0.34	26.22 ± 0.71
18:0	19.05 ± 1.09	19.90 ± 0.71	15.34 ± 0.35	15.38 ± 0.23
18:1n-9	11.92 ± 0.61	11.62 ± 0.32	10.71 ± 0.15	10.68 ± 0.21
18:2n-6	9.89 ± 0.44	9.81 ± 0.31	7.06 ± 0.20	7.11 ± 0.26
20:4n-6	20.48 ± 0.65	22.57 ± 1.21	20.03 ± 0.39	19.80 ± 0.53
20:5n-3	0.35 ± 0.04	0.40 ± 0.04	0.17 ± 0.03	0.19 ± 0.05
22:5n-3	2.11 ± 0.09	2.76 ± 0.29	0.21 ± 0.40	0.20 ± 0.01
22:6(n-3)	2.99 ± 0.21	4.90 ± 0.48§	0.48 ± 0.01	0.92 ± 0.04‖
Σ n-3	5.45 ± 0.22	8.06 ± 0.77§	0.73 ± 0.08	1.19 ± 0.92‖

Human PBMC and rat thymocytes (2×10^7 cells/mL) were preincubated for 60 min at 37° in RPMI medium containing 5 μM 22:6n-3 complexed to 5 μM human serum albumin as described in Materials and Methods. Results are expressed in mol% of total fatty acids.

*Values do not add up to 100% because some minor fatty acids are not listed.

†Values are means ± SE of 5 (PBMC) or 6 (thymocytes) separate experiments.

‡Data for PBMC are from Ref. 20.

§Indicates a significant difference from control cells at $P < 0.05$ by the Student's *t*-test.

‖Indicates a significant difference from control cells at $P < 0.01$ by the Student's *t*-test.

butylhydroperoxide. Absorbance at 340 nm was recorded in a Beckman DU8 spectrophotometer. Results are expressed as nmol NADPH oxidized/min/mg protein using an extinction molar coefficient of $6.22/cm^2/\mu mol$ for NADPH. Inhibition of 90–95% was consistently observed in the presence of 0.2 mM mercaptosuccinic acid, which indicates a selenium-dependent GSH-Px activity.

Data Analyses

Data, expressed as mean values ± SE, were analyzed using the STATVIEW II program for Macintosh according to one-way or two-way analysis of variance with balanced mixed models, as indicated in the legends of the figures. Calculated F values are given. Differences were considered as significant when the calculated *P* value was less than 0.05.

RESULTS

Docosahexaenoic Acid Incorporation into Cell Phospholipids

Total phospholipids of control rat thymocytes contained about 7-fold fewer n-3 fatty acids than phospholipids from control human PBMC (Table 1). However, both types of cells can be readily enriched with a given fatty acid by incubation in appropriate medium. Thus, a short incubation (60 min) of either human PBMC or rat thymocytes with 5 μM 22:6n-3 in the presence of 5 μM serum albumin was sufficient to induce a 1.6- and 1.9-fold increase, respectively, of its proportion in cell phospholipids. Both types of cells had a similar proportion of arachidonic acid in their phospholipids, which was not altered by 22:6n-3-enrichment.

Effects of n-3 Fatty Acids on H_2O_2-induced Lipid Peroxidation

When human PBMC were incubated with increasing concentrations of H_2O_2 for 10 min, a dose-dependent MDA production which reflects lipid peroxidation was observed (Fig. 1A). The MDA level was significantly increased from 5 pmol/10^6 cells in controls incubated without H_2O_2 to 11 pmol/10^6 cells in the presence of 3 mM H_2O_2, a larger 10-fold increase being observed for 5 mM H_2O_2. Under the same experimental conditions, rat thymocytes proved to be more resistant to H_2O_2 oxidative effects as 5 mM H_2O_2, which increased the MDA level up to 60 pmol/10^6 cells in PBMC, only induced a modest MDA increase (13 pmol/10^6 cells) in thymocytes. When human PBMC were preincubated with 5 μM 22:6n-3 bound to lipid-free human serum albumin (fatty acid to albumin ratio = 1) prior to H_2O_2 treatment, MDA production was maintained around the control level up to 1.5 mM H_2O_2. Thus, a significant reduction in MDA production as compared with control cells was observed for 1 and 1.5 mM H_2O_2 concentrations (Fig. 1B), this 22:6n-3-induced protection against H_2O_2-induced lipid peroxidation no longer being observed at higher doses (not shown). In contrast, 20:5n-3 treatment of the cells did not modify H_2O_2-induced MDA production at the lowest H_2O_2 doses (Fig. 1C) and even potentiated it at the highest doses (not shown). A previous enrichment of thymocytes with 22:6n-3 had no significant effect on MDA production (not shown).

It has been checked that the pharmacological H_2O_2 concentrations used in these experiments did not induced significant cell mortality. Although H_2O_2 treatment was stopped after 10 min, cell survival determined by the trypan blue exclusion test was monitored for 1 hr after H_2O_2 addition. Data obtained in a typical experiment represen-

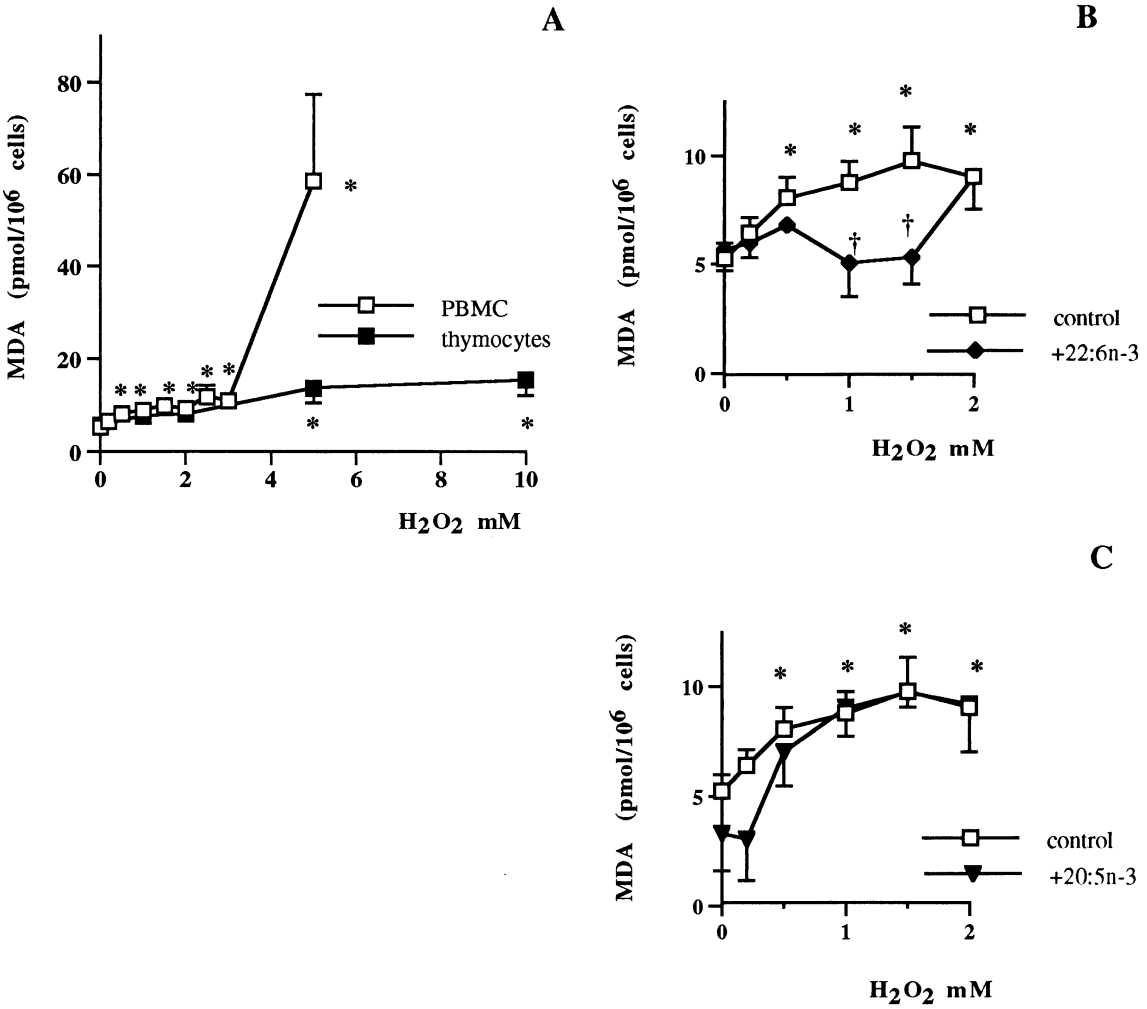

FIG. 1. H_2O_2-induced lipid peroxidation of human PBMC (A–C) and rat thymocytes (A). Control PBMC (open squares) and rat thymocytes (filled squares) or cells preincubated for 1 hr with 22:6n-3 (filled diamonds, B) or 20:5n-3 (filled triangles, C) were incubated for a further 10 min at 37° in the presence of increasing H_2O_2 concentrations ranging from 0.2 to 10 mM (A) or 0.2 to 2 mM (B, C). At the end of the incubation period, cells were pelleted, washed, and resuspended in 9% NaCl. Cell suspensions were treated with thiobarbituric acid as described in Materials and Methods. The MDA–TBA adduct was resolved by HPLC on a nucleosil C_{18} column and quantified by comparison to a standard curve generated through reaction of TBA with 1-,1′-3-,3′-tetramethoxypropane (TMP). Results are expressed as pmol MDA per 10^6 cells and are means ± SE of three to five separate experiments. Data were evaluated by a one way (A) or a two-way analysis of variance. In B, F for H_2O_2 was 2.79 (H_2O_2-treated vs untreated: $P < 0.04$) and F for 22:6n-3 was 6.72 (22:6n-3-treated vs untreated: $P < 0.02$). In C, F for H_2O_2 was 6.43 (H_2O_2-treated vs untreated: $P < 0.001$) and F for 20:5n-3 was 1.77 (20:5n-3-treated vs untreated: NS). When statistical significance was found, means were further compared by the Fisher PLSD test. * indicates a significant effect of H_2O_2 as compared with control cells ($P < 0.05$), and † indicates a significant effect of 22:6n-3 enrichment at a given H_2O_2 concentration ($P < 0.05$).

tative of two performed with human PBMC and rat thymocytes are shown in Table 2. Higher than 95% cell viability was consistently observed with H_2O_2 concentrations as high as 5 mM up to 50-min incubation. For the incubation period used in the present study, none of the H_2O_2 concentrations tested were toxic for rat thymocytes or for human PBMC.

Effects of H_2O_2 and 22:6n-3 on PDE Activities

The cytosolic PDE activity of human mononuclear cells was more sensitive to H_2O_2-induced oxidative stress (Fig. 2) than the particulate activity (not shown). The cytosolic cAMP–PDE activity was significantly and dose dependently inhibited from 33 to 48% by H_2O_2 concentrations increasing from 0.5 to 2 mM (Fig. 2A). However, the highest H_2O_2 concentration used (5 mM), which markedly increased MDA production, did not further increase the H_2O_2-induced inhibition of cytosolic PDE activities. At the same time, the cytosolic cGMP–PDE activity was inhibited by 24 to 42% (Fig. 2B). In contrast, particulate cAMP– and cGMP–PDE activities were not modified by H_2O_2 treatment of the cells (not shown). Different responses of PDE activities to H_2O_2 treatment were observed when cells were first enriched with 22:6n-3 prior to H_2O_2 addition. In 22:6n-3-treated cells, the H_2O_2-induced inhibition of both cAMP– and cGMP–PDE cytosolic activities

TABLE 2. Effect of H_2O_2 on the viability of human PBMC and rat thymocytes

time (min)	Incubation Cells	% living cells H_2O_2 (mM) 0.5	1	2	5	10
10	PBMC	98.0	98.3	96.2	98.5	ND
	Thymocytes	100	98.2	98.4	98.4	98.0
20	PBMC	97.3	97.6	98.1	97.6	ND
	Thymocytes	100	96.2	98.1	ND	ND
30	PBMC	97.7	98.3	97.7	98.5	ND
	Thymocytes	99.3	98.4	97.1	97.8	96.0
40	PBMC	98.3	95.8	98.3	96.5	ND
	Thymocytes	99.0	ND	96.9	ND	ND
50	PBMC	95.0	98.3	96.5	98.3	ND
	Thymocytes	100	98.3	96.6	96.6	85.3
60	PBMC	95.6	95.4	96.6	96.6	ND
	Thymocytes	99.5	97.3	96.15	92.5	84.9

Human PBMC and rat thymocytes suspended at 2.5×10^7 cells/mL in RPMI medium were incubated at 37° with various H_2O_2 concentrations. For each H_2O_2 concentration, aliquot fractions of cell suspensions were withdrawn at the indicated time and the percentage of living cells measured by the trypan blue exclusion test. Data are from a representative experiment repeated at least twice. ND, not determined.

was abolished. On the other hand, the particulate PDE activities of 22:6n-3-treated cells were significantly increased by 5 mM H_2O_2, from 7.92 ± 1.06 to 12.18 ± 1.28 pmol/min/mg prot (+54%, $P < 0.05$) for cAMP–PDE and from 11.31 ± 1.45 to 17.54 ± 1.53 pmol/min/mg prot (+55%, $P < 0.05$) for cGMP–PDE.

Although rat thymocytes were less sensitive to H_2O_2-induced lipid peroxidation than human PBMC their cytosolic PDE activities were more markedly inhibited by H_2O_2 than those of PBMC (Fig. 3). cAMP–PDE activity was already significantly inhibited (−38%) by 1 mM H_2O_2, with a maximal 66% inhibition for 10 mM H_2O_2, whereas cGMP–PDE activity was less sensitive (−56% for 10 mM H_2O_2). A pretreatment of the cells with 22:6n-3 did not affect their PDE sensitivity to H_2O_2-induced inhibition. At the same time, the particulate PDE activities remained insensitive to H_2O_2 both in control and in 22:6n-3-enriched cells (not shown).

Effects of H_2O_2 and 22:6n-3 on GSH-Px Activity

The seleno-dependent GSH-Px activity of both human PBMC, which proved to be sensitive to high H_2O_2 concentrations in terms of lipid peroxidation, and the more resistant rat thymocytes, was only marginally affected by increasing H_2O_2 concentrations (Fig. 4, A and B). In human PBMC, a maximal 23% inhibition was observed for 2 mM H_2O_2, which was not increased further at the highest dose used. In rat thymocytes, GSH-Px activity was maximally decreased by about 20% at H_2O_2 concentrations from 1 to 10 mM. In both cases, pretreatment of the cells with 5 μM 22:6n-3 did not modify the slight inhibitory effect of H_2O_2.

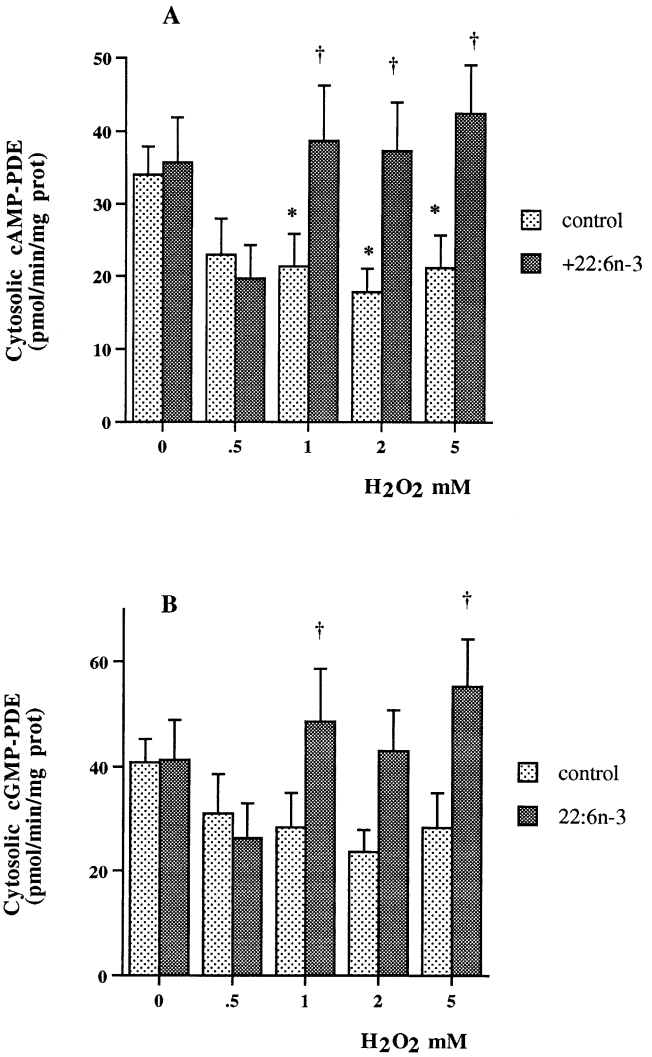

FIG. 2. Effects of H_2O_2 and 22:6n-3 on cytosolic cAMP (A) and cGMP (B) phosphodiesterase activities of human PBMC. Control cells preincubated for 1 hr with 5 μM human serum albumin alone or 22:6n-3-enriched cells preincubated for 1 hr with 5 μM 22:6n-3 complexed to 5 μM human serum albumin were incubated for a further 10 min at 37° in the presence of increasing H_2O_2 concentrations. At the end of the incubation period, cells were pelleted and lysed as described in Materials and Methods. PDE activities were assayed on aliquots of 105,000 *g* supernatant fractions. Results are expressed as pmol cyclic nucleotides hydrolyzed per min per mg proteins and are means ± SE of seven separate experiments, each performed in triplicate. Data were analyzed by one-way ANOVA and the means compared by the Fisher PLSD test. * indicates a significant difference with controls and † a significant difference between 22:6n-3-treated and untreated cells at a given H_2O_2 concentration.

DISCUSSION

It is now well recognized that oxidative stress may affect immune function, both pro-oxidant and antioxidant states being required sequentially at different times during lymphocyte activation [27]. Acutely applied oxidative stress usually induced lymphocyte suppression through alteration of the mitogen-induced transmembrane signaling [2, 8].

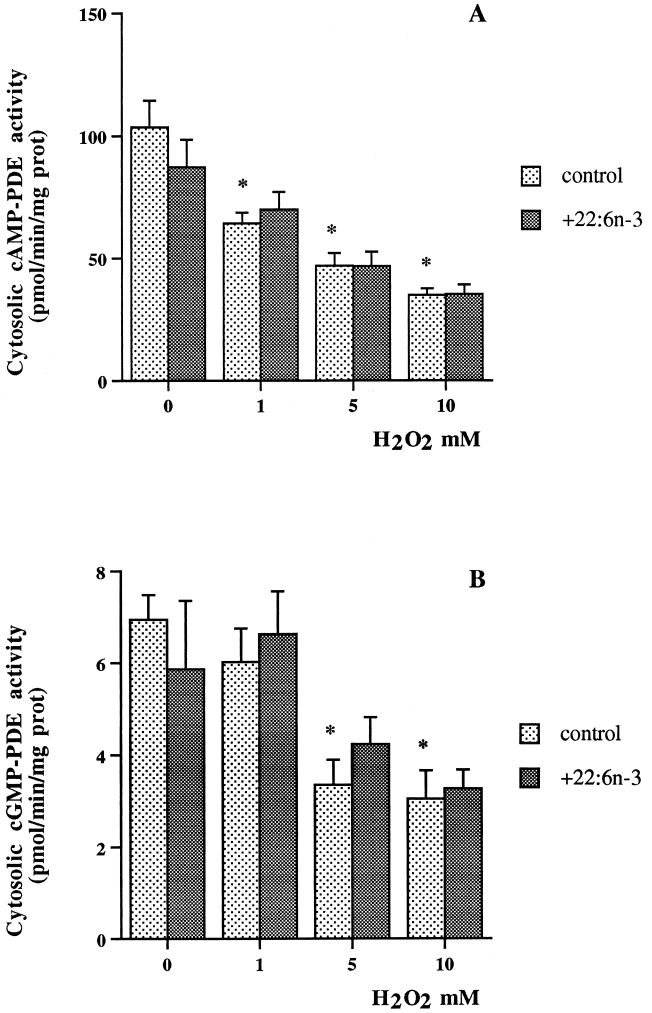

FIG. 3. Effects of H_2O_2 and 22:6n-3 on cytosolic cAMP (A) and cGMP (B) phosphodiesterase activities of rat thymocytes. Control cells preincubated for 1 hr with 5 μM human serum albumin alone or 22:6n-3-enriched cells preincubated for 1 hr with 5 μM 22:6n-3 complexed to 5 μM human serum albumin were incubated for a further 10 min at 37° in the presence of increasing H_2O_2 concentrations. At the end of the incubation period, cells were pelleted and lysed as described in Materials and Methods. PDE activities were assayed on aliquots of 105,000 g supernatant fractions. Results are expressed as pmol cyclic nucleotides hydrolyzed per min per mg proteins and are means ± SE of five separate experiments, each performed in triplicate. Data were analyzed by ANOVA and the means compared by the Fisher PLSD test. * indicates a significant difference with controls.

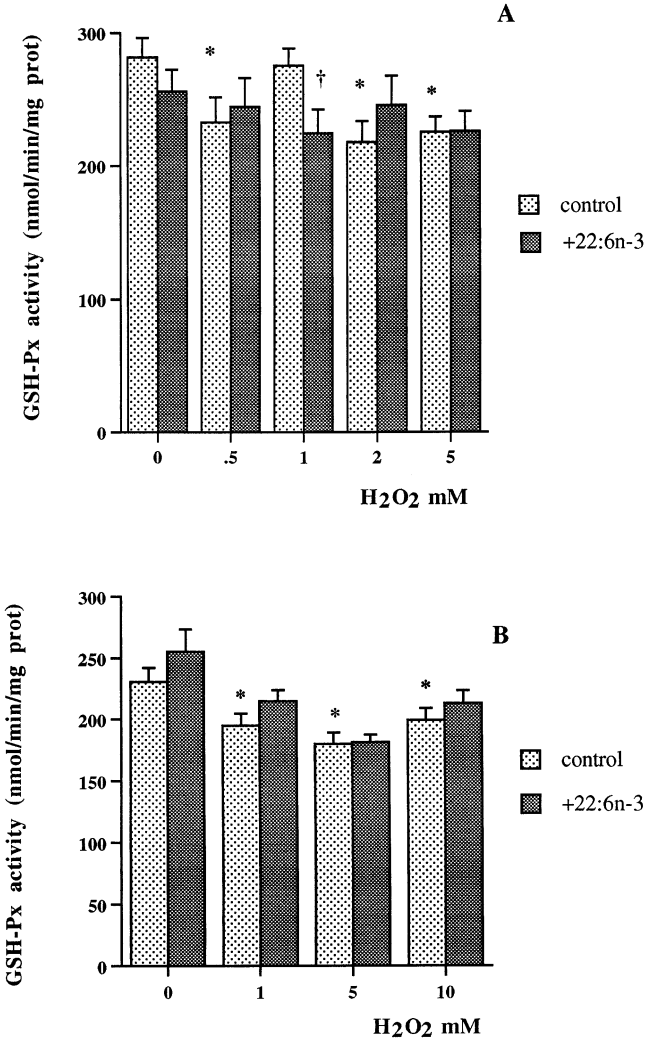

FIG. 4. Effects of H_2O_2 and 22:6n-3 on GSH-Px activity of human PBMC (A) and rat thymocytes (B). Control cells preincubated for 1 hr with 5 μM human serum albumin alone or 22:6n-3-enriched cells preincubated for 1 hr with 5 μM 22:6n-3 complexed to 5 μM human serum albumin were incubated for a further 10 min at 37°. At the end of the incubation period, cells were pelleted and lysed as described in Materials and Methods. GSH-Px activity was assayed on aliquots of 105,000 g supernatant fractions. Results are expressed as nmol NADPH oxidized per min per mg proteins and are means ± SE of seven (A) or five (B) separate experiments, each performed in quadruplicate. Data were analyzed by one-way ANOVA and the means compared by the Fisher PLSD test. * indicates a significant difference with controls. † indicates a significant difference between 22:6n-3-treated and untreated cells at a given H_2O_2 concentration.

However, all the biochemical targets likely to be involved have not been clearly delineated. To study the influence of oxidative stress on lymphocyte lipid peroxidation and the possible consequences on PDE and GSH-Px thought to play an important role in the control of immune function, we chose to use short-term exposure of the cells to increasing H_2O_2 concentrations. It is known that, when added directly to cell culture medium, H_2O_2 concentration is quickly decreased by cellular catalase and GSH-Px [28]. Furthermore, it has been shown that in Chinese hamster cells exposed to H_2O_2, DNA alterations reached a maximum as early as 5 min [29]. Thus a short-time exposure (10 min) of lymphocytes to H_2O_2 was used in the present experiments. One of the first conclusions which can be drawn from the present study is that lymphocytes are relatively resistant to exogenously applied oxidative stress. Pharmacological concentrations of H_2O_2 up to 3 mM, which did not alter cell viability, only induced a 2-fold increase in lipid peroxidation in human PBMC, whereas a 10-fold increase in MDA production was observed at the

highest dose used (5 mM). Rat thymocytes proved to be even more resistant to H_2O_2-induced lipid peroxidation, as only a 3-fold increase in MDA production was obtained with 10 mM H_2O_2. The reasons for the differential susceptibility to oxidative stress observed between human PBMC and rat thymocytes are unknown. The induction of lipid peroxidation by H_2O_2 is generally thought to be due to its ability to stimulate $OH^{\cdot}$ formation through the transition metal-catalyzed Haber–Weiss reaction [28]. It may be speculated that rat thymocytes have a lower content of transition metals than PBMC or that the antioxidant capabilities of thymocytes are not sufficient to maintain transition metals in their active reduced form. Highly polyunsaturated fatty acids such as 20:5n-3 and 22:6n-3, known to be very sensitive to peroxidation in aqueous solutions [30], have been shown to increase lipid radical formation induced by oxidant stress when incorporated into lipids of cell membranes [31]. Although rat thymocytes incorporate 22:6n-3 into their phospholipids as efficiently as human PBMC, they have, however, a 7-fold lower basal content of n-3 fatty acids than PBMC, and this difference was maintained after enrichment. Thus, the differences in fatty acid composition of phospholipids between these two types of cells could explain the differential susceptibility to oxidative stress that we have observed. In the present study, 22:6n-3, used under experimental conditions which induce a doubling of its relative proportion in cell phospholipids, did not significantly increase MDA production of PBMC induced by the highest doses of H_2O_2, and even lowered it at the lowest concentrations used (<2 mM), whereas it had no significant effect on rat thymocytes. In contrast, 20:5n-3 used under the same experimental conditions did not exhibit a significant protective effect when human PBMC were treated by low doses of H_2O_2 and markedly potentiated MDA production induced by the highest doses of H_2O_2. In human dietary studies, discrepant results have been reported regarding the influence of fish oil supplementation on lipid peroxidation, some studies describing either an increased MDA plasma level [32] or a decreased MDA production in platelets [33]. In platelets oxidatively stressed by diamide, submicromolar concentrations of 20:5n-3 have been shown to suppress the diamide-induced MDA level [15]. It might be hypothesized that, in the present experiments, the dose of 20:5n-3 used (5 μM, fatty acid to albumin ratio = 1) was already too high to observe a protective effect against H_2O_2-induced lipid peroxidation.

Treatment of human PBMC by non-toxic pharmacological doses of H_2O_2 significantly inhibited their cytosolic PDE activities, cAMP–PDE being more sensitive to the oxidative stress than cGMP–PDE, whereas both cAMP– and cGMP–PDE particulate activities were not altered. This point deserves further analysis with respect to the different PDE enzymes present in the subcellular fractions of human lymphocytes. Several groups have shown that more than 80% of the cAMP–PDE activity of lymphocyte lysates can be attributed to the cGMP-inhibited PDE3 and cAMP-specific rolipram inhibitable PDE4 [34, 35]. Furthermore, Tenor *et al.* [36] have shown that in CD4+ and CD8+ T lymphocytes, most of the PDE3 was membrane-associated while most of the PDE4 was cytosolic. Thus, it may be speculated that the PDE4 family of enzymes is more sensitive to oxidative stress than PDE3. In good agreement with this hypothesis are the results obtained with rat thymocytes. In these cells, the cytosolic cAMP–PDE activity proved to be more sensitive to H_2O_2 inhibition than the cAMP–PDE activity of human PBMC, with 66% inhibition for 5 mM H_2O_2 in thymocytes versus 48% in PBMC. It is noteworthy that PDE4 accounts for 75% of total cytosolic cAMP–PDE activity in rat thymocytes [9, 10]. It has also been demonstrated that both thymocytes and human lymphocytes are devoid of calcium- and calmodulin-dependent PDE1 [9, 10, 34–36]. In thymocytes, cGMP hydrolysis is mainly carried out by the cGMP-activated PDE2, the cGMP-specific PDE5 being far less abundant [9]. Conversely, PDE5 is prominent in human lymphocytes and PDE2 only present in trace amounts. Since cytosolic cGMP–PDE activity was more inhibited in thymocytes than in human PBMC, it may be suggested that PDE2 is more sensitive to H_2O_2 inhibition than PDE5. A direct inhibitory effect of H_2O_2 on the activity of some PDE enzymes has already been reported in an acellular system [37]. However, indirect mechanisms involving H_2O_2 interactions with other oxidant-sensitive targets may be envisaged.

22:6n-3 enrichment significantly protected human PBMC against PDE inhibition induced by the highest doses of H_2O_2 (1–5 mM). However, the extent of PDE inhibition does not seem to be closely correlated to the extent of lipid peroxidation. Although 22:6n-3 was able to prevent MDA production induced by 1 mM H_2O_2, such a protective effect was no longer observed at the highest concentrations, and yet 22:6n-3 was still able to prevent H_2O_2-induced PDE inhibition. It is likely that in the presence of high H_2O_2 doses, other mechanisms unrelated to lipid peroxidation could be involved. It has been demonstrated that H_2O_2 could stimulate PLD in endothelial cells [38] or rat fibroblasts [39], and thus induce an accumulation of phosphatidic acid. Our group has demonstrated that phosphatidic acid can directly stimulate PDE4 activity in an acellular system [40, 41]. Further experiments suggest that phosphatidic acid might also activate the other forms of PDE through phosphatidic acid-induced phosphorylations [42]. On the other hand, we have also demonstrated that human PBMC express PLD enzyme(s). Although undetectable in resting and mitogen-stimulated control cells, PLD activity was primed by preincubation of the cells with some fatty acids such as 12-hydroxyeicosatetraenoic [43] or docosahexaenoic acids [44]. Similarly, the activation of lymphocyte PLD by H_2O_2 might also require the presence of fatty acids. Further experiments are needed to determine whether 22:6n-3 primes PLD activity of human PBMC for activation by H_2O_2 and whether increased PA is responsible for PDE activation. Although the particulate PDE activity of control PBMC was quite insensitive to H_2O_2, a

clear PDE-stimulating effect was observed in 22:6n-3-pretreated cells, especially at the highest H_2O_2 concentration used, which could be explained by the above hypothesis.

In most human nutritional studies, the enrichment of cell membranes with n-3 polyunsaturated fatty acids following fish oil intake has been reported to increase GSH-Px activity. It has been assumed that increased GSH-Px activity might be due to increased amounts of cellular lipid hydroperoxides [45]. Some *in vitro* studies have also reported increased GSH-Px activity due to oxidative stress. Thus, the increased GSH-Px activity observed in human platelets after 22:6n-3 treatment has been shown to be suppressed by the antioxidant epicatechin [17]. GSH-Px activation has also been described in erythrocytes after several hours' incubation with acetylphenylhydrazine, known to release superoxides from hemoglobin [46]. However, different patterns are usually observed when cultured cells are submitted to an acute oxidative stress [47]. In the present study, GSH-Px activity of both human PBMC and rat thymocytes was resistant to H_2O_2-induced oxidative stress, a slight 20% decrease being observed whatever the H_2O_2 concentration used in both cell types. A similar resistance of GSH-Px activity to peroxide treatment has been reported for cultured human keratinocytes [47]. It is noteworthy that the GSH-Px specific activity of lymphocytes is about 10- to 100-fold higher than that of keratinocytes [47] and tumor cell lines [48], respectively. Thus, lymphocytes are very likely to contain sufficient levels of GSH-Px activity to rapidly metabolize even high concentrations of H_2O_2. Although GSH-Px activity is known to be directly inhibited by hydroperoxides in an acellular system, their inhibitory effects are more limited and partly reversible when they are added to intact cells [47].

Collectively, these results suggest that lymphocytes are relatively resistant to H_2O_2-induced lipid peroxidation due to their high GSH-Px content, and that low doses of 22:6n-3 are able to prevent some of the H_2O_2-induced alterations such as lipid peroxidation and PDE inhibition. The preservation of these components could be a mechanism through which docosahexaenoic acid might offer some protection against oxidant-induced lymphocyte suppression.

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