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Effects of Fatty Acids on Human Platelet Glutathione Peroxidase: Possible Role of Oxidative Stress

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ABSTRACT. Highly polyunsaturated fatty acids of the n-3 family are known to be inhibitors of platelet functions, but these fatty acids (FA) may alter the platelet antioxidant status, depending on their concentrations. The present study was aimed to investigate the effect of various FA on glutathione-dependent peroxidase (GPx), the required antioxidant enzyme for degrading FA hydroperoxides. Human platelets were enriched *in vitro* with either n-3 (18:3, 20:5, or 22:6), n-6 (18:2 or 18:3) FA, 18:1n-9 or 16:0, and the GPx activity was then measured. It was found that n-3 FA enhanced the GPx activity whereas the others did not affect the enzyme activity. The increased GPx activity was associated with an increased amount of the enzyme measured by Western blotting. The enhanced activity and amount of GPx induced by 22:6n-3, the most potent activator among the n-3 FA, was completely abolished in the presence of cycloheximide at a concentration known to inhibit platelet protein synthesis. Because platelets are devoid of nucleus, which rules out the involvement of transcriptional factors, this suggests that 22:6n-3 might act at a translational level. On the other hand, 22:6n-3 treatment increased the malondialdehyde formation and decreased the vitamin E level in platelets, both events that could be prevented by the antioxidant epicatechin. Because epicatechin also suppressed the enhancement of both the activity and amount of GPx induced by 22:6n-3, we conclude that the increased GPx activity (possibly *via* protein synthesis) might be associated with an oxidative stress induced by 22:6n-3 and/or 20:4n-6 released from the platelet endogenous pool in the course of the 22:6n-3 enrichment. BIOCHEM PHARMACOL 53:4:479–486, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. glutathione peroxidase; docosahexaenoic acid; platelet; vitamin E; malondialdehyde; fatty acids

Since the pioneering epidemiologic studies of Dyerberg *et al.* [1], there has been considerable interest in the potential of fish oil to prevent coronary artery diseases [2, 3]. Fish oil contains high levels of PUFA† of the n-3 family, especially eicosapentaenoic (20:5n-3) and docosahexaenoic (22:6n-3) acids [4]. The ingestion of n-3 family PUFA has been shown to decrease platelet aggregability, which is relevant to the fundamental role played by platelets in hemostasis and thrombosis [3, 5]. In this context, we have previously reported that intake of low doses of purified 20:5n-3 led to a normalization of platelet aggregation in elderly people, and might prevent the relative depletion of platelet vitamin E observed in this population [6]. However, PUFA are susceptible to peroxidation, providing a variety of lipid peroxides and aldehydic breakdown products. Indeed, a higher sensitivity to membrane peroxidation can be expected in animals fed highly polyunsaturated oil [7], and clinical studies using large doses of n-3 PUFA have reported such a peroxidation [8, 9]. Consequently, a higher requirement of vitamin E may be needed in response to high dosages of n-3 PUFA, whereas low concentrations might spare this antioxidant vitamin [6, 10]. Cells also develop other protective systems against lipid peroxidation, such as the selenium-dependent glutathione peroxidase (GPx; EC 1.11.1.9). GPx, one of the major detoxication enzymes [11], catalyzes the degradation of hydroperoxides to the corresponding alcohols using reduced glutathione as a specific hydrogen donor. GPx could then function to protect cells from oxidative damage due to the accumulation of hydroperoxides [11]. In blood platelets, GPx has been functionally associated with the 12-lipoxygenase pathway, where it efficiently converts 12-HPETE, issued from arachidonic acid, into its reduced derivative, 12-HETE [12, 13]. It has been found that GPx activity is especially high in platelets [14]. As such, GPx may regulate the formation of eicosanoids relevant to the platelet function, because this formation depends upon the peroxide tone of the cell [13, 15]. GPx activity results from the expression of multiple isozymes

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† *Abbreviations:* GPx, selenium glutathione-dependent peroxidase; FA, fatty acids; PUFA, polyunsaturated fatty acids; MDA, malondialdehyde; NEFA, non-esterified fatty acids; 12-HPETE, 12-hydroperoxyeicosatetraenoic acid; 12-HETE, 12-hydroxyeicosatetraenoic acid.

[16], and we have previously reported that the main GPx enzyme in platelets has the same molecular characteristics as the classical cellular GPx or GPx-1 [17]. GPx is an homotetramer with a relative molecular mass of 92 kDa, each subunit of 23 kDa containing a seleno-cysteinyl moiety in the active site [18].

Because n-3 PUFA may exhibit pro- or antioxidant activity, depending on the experimental conditions, this prompted us to study the regulation of human platelet GPx by FA. First, we investigated the effects of n-3 FA on human platelet GPx activity as compared to FA of other families. We also examined the action of 22:6n-3, the most potent activator among the n-3 PUFA tested, on the quantity of immunoreactive enzyme. Second, because 22:6n-3 possesses a high degree of unsaturation, we studied its effect on the redox status of the cell in relation to GPx stimulation.

MATERIALS AND METHODS

Materials

FA, fatty acid-free human albumin, NADPH, reduced glutathione, glutathione reductase, *tert*-butyl hydroperoxide, cycloheximide, thiobarbituric acid, and 3,3′-diaminobenzidine were obtained from Sigma-Chimie, L'Isle d'Abeau, France. Epicatechin was obtained from Extrasynthèse, Genay, France. Nitrocellulose membranes were purchased from Schleicher and Schull, Dassel, Germany, and the horseradish conjugated goat antirabbit from Bio-Rad S.A., Ivry-sur-Seine, France.

Platelet Preparation

Human platelet-rich plasma was prepared as previously described [19]. Briefly, blood from healthy donors who had not taken any medication for at least 10 days prior to donation was obtained from the local blood bank. Venous blood was collected into one seventh volume of CPD (89.4 mM sodium citrate, 129 mM glucose, 16.1 mM Na_2HPO_4, 19.6 mM citric acid, pH 5.6) as an anticoagulant. Platelet-rich plasma, obtained by centrifugation of blood at 200 g for 15 min, was acidified to pH 6.4 with citric acid (0.15 M), and centrifuged for 12 min at 900 g. The resulting platelet pellet was resuspended into a Tyrode-HEPES buffer solution (137 mM NaCl, 2.6 mM KCl, 11.9 mM $NaHCO_3$, 0.46 mM NaH_2PO_4, 1 mM $MgCl_2$, 5 mM HEPES, 5.5 mM dextrose, pH 7.35) containing either FA/albumin or albumin alone.

Preparation of the Fatty Acid Solutions

Briefly, the fatty acid solutions were dried and the FA were incubated overnight under nitrogen at 37°C with Tyrode-HEPES buffer containing 3.5 g/L (50 μM) of human albumin. FA/albumin molar ratios of 0.1 to 2.0 (corresponding to a fatty acid concentration of 5 to 100 μM, respectively, precoated on albumin) were used.

Incorporation of Fatty Acids

FA incorporation into platelets was performed as previously described [20]. Platelets isolated from plasma, as stated above, were resuspended into albumin containing FA or FA-free albumin (control) solution to have 3×10^8 platelets/mL, and incubated at 37°C with gentle shaking in the absence or presence of 10 or 100 μM epicatechin or 500 μM cycloheximide. The platelet incubation was limited to 2 hr to keep platelets fully responsive to aggregating agents. After incubation, platelet suspensions were acidified to pH 6.4 and centrifuged for 10 min at 700 g. The platelet pellets were resuspended into Tyrode-HEPES buffer (without albumin) or into 50 mM Tris-HCl buffer (pH 7.6) containing 1 mM dithioerytritol [17].

Fatty Acid Distribution

At the end of the incubation period, the proportion of FA incorporation in platelets and the distribution within the lipid classes were determined. After the 2-hr incubation at 37°C, platelets were centrifuged and the supernatant was kept to evaluate the proportion of fatty acid remaining in this fraction. Then, pellets resuspended in Tyrode-HEPES buffer and cell-free supernatants were extracted twice with ethanol/chloroform (1:2, v/v) containing 50 μM butylated hydroxytroluene as an antioxidant, in the presence of 17:0, tri17:0-triglyceride, di17:0-phosphatidylcholine, and 17:0-cholesterol ester as internal standards. Lipid classes were separated by thin-layer chromatography with a mixture of hexane/diethylether/acetic acid (80:20:1, v/v) into phospholipids, free FA, triglycerides, and cholesterol esters. Lipid classes were scraped off the plate and treated for 90 min with 5% H_2SO_4 in methanol to obtain FA methyl esters [21]. These FA methyl esters were analyzed and quantified by gas-liquid chromatography.

Enzyme Assay

The GPx activity was measured according to the spectrophotometric method of Paglia and Valentine [22] as modified by Chaudière and Gérard [23], with platelets solubilized with lubrol (0.1% final). The assay mixture consisted of 50 mM Tris-HCl, 0.1 mM EDTA, pH 7.6, containing 2 mM GSH, 0.14 mM $NADPH,H^+$ and 0.70 U/mL glutathione reductase. Platelet suspensions were preincubated for 2 min at 37°C in the assay mixture and the reaction was initiated by the addition of 0.2 mM *tert*-butyl-hydroperoxide. Absorbance at 340 nm was recorded in a DU8 spectrophotometer (Beckman, Fullerton, California, USA). Results are expressed in nmol NADPH oxidized per min per mg protein using a molar extinction coefficient of $6.22/cm^2/\mu mol$ for NADPH. Proteins were assayed according to Bradford, using bovine serum albumin as a standard [24].

Western Blotting Procedure

Following the 2-hr incubation, platelets were centrifuged for 10 min at 700 g. The platelet pellets were resuspended

into 50 mM Tris-HCl buffer (pH 7.6) containing 1 mM dithioerythritol, solubilized with lubrol (0.1% final concentration), and frozen until treatment. A rabbit polyclonal antibody was raised against GPx from bovine erythrocytes as previously described [17]. Platelet homogenates were dissolved in a buffer containing 20% (v/v) glycerol, 10% β-mercaptoethanol (v/v), 0.05% (w/v), Bromophenol Blue, 2.5% (w/v) SDS, and 125 mM Tris-HCl, pH 6.8. These homogenates were then heated in a boiling water bath for 10 min before they were electrophoresed in an SDS-15% polyacrylamide gel using the Laemmli system [25]. Following electrophoresis, proteins were electrotransferred to a nitrocellulose membrane in 10 mM CAPS (3-cyclohexylamino-1-propane sulfonic acid) (pH 11) containing 10% methanol. The membrane was then soaked in 50 mM Tris-HCl, 0.15M NaCl, 0.1% Tween, pH 7.6 (buffer A), containing 3% BSA and 1% non-fat dry milk for 2 hr to prevent nonspecific binding. The blocked membrane was washed 3 times in buffer A for 10 min each and incubated with the rabbit polyclonal antiserum (diluted 1/1000 in buffer A containing 1% BSA) for 2 hr at room temperature. After 3 washings in buffer A, the bound antibodies were detected with horseradish-conjugated goat antirabbit Ig G and developed with 3,3′-diaminobenzidine and hydrogen peroxide as substrates. The immunoblot image was monitored using a video camera and the intensity of the bands was quantified by passing the output of the videorecorder through a digital image-processing system (Bioprofil Vilber Lourmat/Fröbel, Lindau, Germany). Results were expressed in μg GPx/mg protein and were determined using human erythrocyte GPx from Sigma (L'Isle d'Abeou, France) as a standard [17].

Malondialdehyde Determination

MDA was determined by RP-HPLC as the MDA-TBA complex according to the method of Therasse and Lemonnier [26]. Briefly, platelet suspensions were immediately frozen until treated with 10 mM thiobarbituric acid (TBA) dissolved in a 0.1 M phosphate buffer (pH 3) and incubated for 1 hr at 95°C 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.

Vitamin E Determination

At the end of the 2-hr incubation period, platelet suspensions were immediately frozen in the presence of ascorbic acid (1%) as an antioxidant, and vitamin E determination was performed according to a previously described method [27]. Briefly, platelet suspensions were mixed with 2 volumes of the mixture ethanol/water (1:1, v/v) containing tocol as an internal standard. After extracting twice with 3 volumes of hexane, the tocopherol isomers were separated by RP-HPLC on a 15-cm column packed with Nucleosil C_{18} (5 μm). The mobile phase methanol/water (95:5, v/v) was pumped at a flow rate of 0.8 mL/min. The α-tocopherol was detected and quantified at 292 nm.

Statistical Analysis

All values were expressed as means ± SEM. Data were analyzed by 1-way ANOVA, followed by the Fisher test. Significance was established at the 0.05 level.

RESULTS

Effects of Different Fatty Acids on GPx Activity

Platelets were incubated for 2 hr in the absence or presence of FA in a Tyrode-HEPES buffer containing 50 μM human FA-free albumin (FA/albumin ratio of 2). After the incubation period, platelets were centrifuged and resuspended in a Tyrode-HEPES buffer without albumin. The GPx activity was measured with *tert*-butyl-hydroperoxide as a substrate with an excess of the cosubstrate GSH in the presence of glutathione reductase and NADPH. Figure 1 shows that incubation with 18:3n-3, 20:5n-3, and 22:6n-3 significantly increased the GPx activity above that of controls

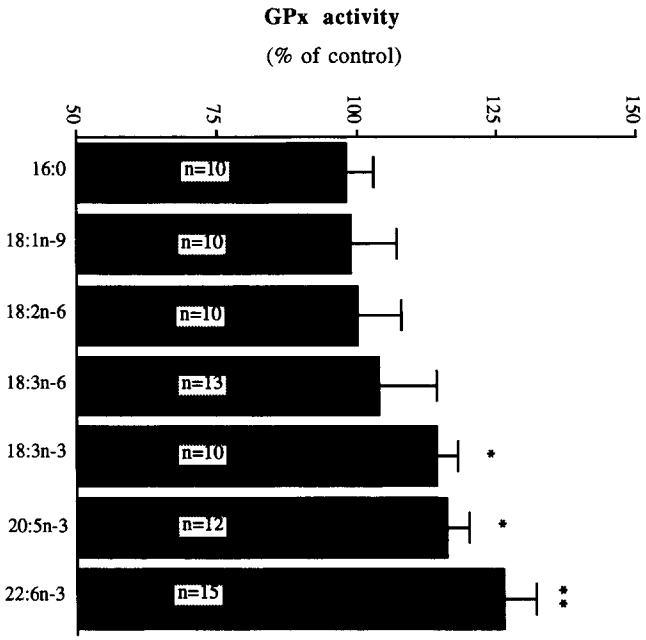

FIG. 1. Effects of different fatty acids on GPx activity. Human platelets were incubated in a Tyrode-HEPES containing human albumin in the absence (control) or presence of fatty acids (palmitic: 16:0, oleic: 18:1n-9, linoleic: 18:2n-6, γ-linolenic: 18:3n-6, α-linolenic: 18:3n-3, eicosapentaenoic: 20:5n-3 or docosahexaenoic: 22:6n-3) for 2 hr at 37°C. Fatty acid/albumin ratios of 2 were used. At the end of the incubation period, the GPx activity was assayed as described in Materials and Methods. Results are expressed in % of control values and are means ± SEM. The control value was 299 ± 14 nmol/min/mg protein. The number of independent experiments is indicated in the Figure; *p < 0.05, **p < 0.01 compared to control.

incubated in the presence of albumin alone. Different FA belonging to the n-6 (18:3, 18:2), n-9 (18:1), or saturated (16:0) families were also tested. Among the FA tested, only 22:6n-3, 20:5n-3, and 18:3n-3 were able to significantly increase the GPx activity; the others were inactive. 22:6n-3 being the most potent, it was used for further experiments.

Effects of 22:6n-3 on GPx

The effects on GPx activity of different concentrations of 22:6n-3 (5 to 100 μM) corresponding to an FA/albumin ratio of 0.1 to 2 were determined. Interestingly, the stimulation observed was already significantly effective with 5 μM 22:6n-3 (FA/albumin ratio of 0.1) (Table 1). However, it appeared that there was an overall progressive increase in GPx activity because the molar ratio was increased from 0.1 to 2 (Table 1). For further experiments, the FA/albumin ratio of 2 was used. Under these conditions (FA/albumin ratio of 2), the proportion of 22:6n-3 incorporated in platelets was 7.85 ± 0.86% (n = 3). The majority was esterified in phospholipids (79.6 ± 5.4%, n = 3) and the remainder was mainly esterified in triglycerides (14.0 ± 5.6%, n = 3) and associated to the nonesterified FA pool (6.3 ± 2.4%, n = 3). In platelet phospholipids, the endogenous proportion of 22:6n-3 was 1.89 ± 0.09 mol% (n = 3) and this was increased to 3.71 ± 0.18 mol% (n = 3) after cell enrichment, representing an approximate 2-fold elevation in the phospholipid content compared to controls. After enrichment, the amount of 22:6n-3 increased by 24.4 ± 12.2 nmol/10^9 platelets (mean ± SD, n = 3) in phospholipids, 2.8 ± 1.3 nmol/10^9 platelets (mean ± SD, n = 3) in triglycerides and 1.6 ± 0.5 nmol/10^9 platelets (mean ± SD, n = 3) in the nonesterified FA pool. 22:6n-3 was not detected in cholesterol esters.

We then measured the protein enzyme by Western blotting in the absence or presence of 22:6n-3. Platelets were resuspended in a Tris buffer instead of Tyrode-HEPES for technical reasons. The GPx activity was then assayed under these conditions and was also found to be significantly higher (+22%) in the 22:6n-3-treated group compared to the control group (Fig. 2). Similarly, the amount of the immunoreactive platelet GPx increased significantly (+26%) in platelets treated with 22:6n-3 compared to control platelets (Fig. 2). Platelets were then incubated with 22:6n-3 in the absence or presence of the protein synthesis inhibitor cycloheximide, and the GPx activity, as well as the immunoreactive platelet, GPx, were measured. The cycloheximide treatment completely abolished the increased GPx activity induced by 22:6n-3 (Fig. 2). Similar results were obtained with measurement of the GPx protein because the increased immunoreactive GPx observed when platelets were treated with 22:6n-3 alone was completely suppressed when platelets were coincubated with cycloheximide (Fig. 2).

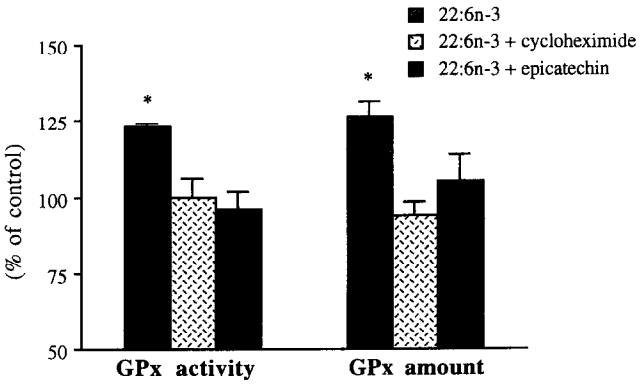

FIG. 2. Effects of 22:6n-3 on the activity and the amount of GPx in the absence or presence of cycloheximide or epicatechin. Platelets were incubated in a Tyrode-HEPES buffer containing human albumin and were supplemented (or not) with 22:6n-3 (fatty acid/albumin ratio of 2), in the absence or presence of cycloheximide (500 μM) or epicatechin (100 μM) for 2 hr at 37°C. Cells were centrifuged and pellets were resuspended in a Tris-HCl buffer (pH 7.6) containing 1 mM dithioerythritol. GPx activity and quantity were determined as described in Materials and Methods. Results are expressed in % of control and are means ± SEM of 6 independent experiments. The control value for the GPx activity was 270 ± 14 nmol/min/mg protein and that for the GPx amount was 6.2 ± 0.5 μg GPx/mg protein. *$p < 0.05$ compared to control.

TABLE 1. Effects of different concentrations of 22:6n-3 on GPx activity

Fatty acid/albumin ratio	GPx activity (% of control)
0.1	109 ± 4*
0.2	114 ± 3*
1	112 ± 3*
2	120 ± 4*

Platelets were incubated for 2 hr at 37°C in a Tyrode-HEPES buffer containing (or not, control) 22:6n-3 precoated on human albumin. Fatty acid/albumin ratios of 0.1 to 2 were employed. Cells were then centrifuged and pellets were resuspended in a Tyrode-HEPES buffer without albumin. GPx activity was assayed as described in Materials and Methods. Results are expressed as % of control values and are means ± SEM of 6 independent experiments. The control value was 301 ± 8 nmol/min/mg protein; *$p < 0.05$ compared to control.

Platelet Antioxidant Status, Lipid Peroxidation and GPx

22:6n-3 being a highly unsaturated fatty acid, it might be peroxidized and/or involved in lipid peroxidation. Its effect on parameters reflecting the platelet antioxidant status was, therefore, evaluated. Then, the effect of 22:6n-3 was assayed on the platelet tocopherol level. The α-tocopherol level was found to be significantly lower in platelets after a 2-hr incubation of 22:6n-3 (Fig. 3). We next evaluated the effect of 22:6n-3 on the overall platelet lipid peroxidation by measuring MDA as the MDA-TBA adduct found in the platelet suspension. The formation of MDA was significantly higher in platelets treated with 22:6n-3 for 2 hr compared to control platelets.

The cotreatment of platelet with the antioxidant phenolic compound epicatechin, a potent antioxidant with regard to lipid peroxidation associated with the polyunsatu-

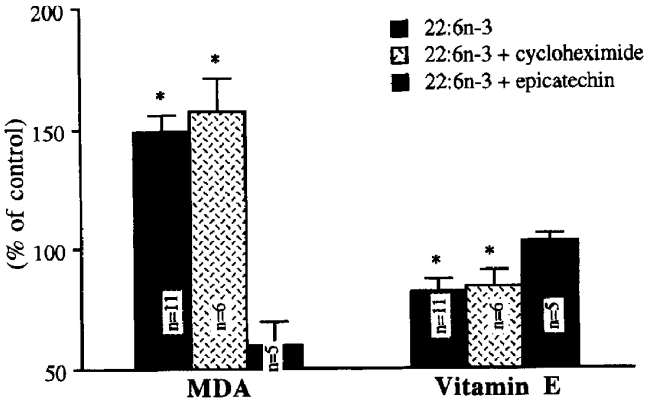

FIG. 3. Influence of 22:6n-3 on MDA and vitamin E in the absence or presence of cycloheximide or epicatechin. Platelets were incubated for 2 hr at 37°C in a Tyrode-HEPES buffer containing (or not) 22:6n-3 precoated on human albumin, in the absence or presence of cycloheximide 500 μM or epicatechin 100 μM. MDA and vitamin E were measured in cell suspension at the end of incubation. Results are expressed as % of control values and are means ± SEM. The control value of MDA was 260 ± 17 pmol/10^9 platelets and that of vitamin E was 1.55 ± 0.12 nmol/10^9 platelets. The number of independent experiments is indicated in the Figure; *p < 0.05 compared to control.

rated fatty acid metabolism in platelets [28], and with 22:6n-3 prevented both a decrease in α-tocopherol and the increased formation of MDA observed after the incubation period with 22:6n-3 alone (Fig. 3). Figure 3 reports on the effect of 100 μM epicatechin, but significant inhibition of the effects of 22:6n-3 treatment could already be seen with 10 μM epicatechin (not shown). Interestingly, such a cotreatment also completely abolished the increased GPx activity previously observed in response to 22:6n-3 alone (Fig. 2). Similarly, the increased amount of the GPx protein induced by 22:6n-3 alone was inhibited by the coincubation with epicatechin. In contrast, the cotreatment of platelets with 22:6n-3 and cycloheximide failed to abolish the oxidative stress induced by platelet treatment with 22:6n-3 alone. MDA and vitamin E levels were unchanged in the presence of cycloheximide compared to platelets treated by 22:6n-3 alone (Fig. 3). Neither epicatechin nor cycloheximide induced any significant change in either the vitamin E or MDA levels in control platelets (results not shown).

Effects of 20:4n-6 on GPx Activity

The incorporation of 22:6n-3 in platelet phospholipids led to an increase of 20:4n-6 in the NEFA pool in platelets. The amount of 20:4n-6 in the NEFA pool was 126 ± 41 pmol/10^9 platelets (mean ± SD, n = 3) in control platelets. After 22:6n-3 enrichment, this amount increased to 203 ± 65 pmol/10^9 platelets (mean ± SD, n = 3). To determine whether this increased 20:4n-6 in the NEFA pool could interfere with GPx activity, experiments with 20:4n-6 were carried out. It is very well known that 20:4n-6 is metabolized by platelet enzymes to form, among others, thromboxane A_2, a powerful aggregating agent. To avoid platelet aggregation, a 20:4n-6/albumin molar ratio of 0.2 (10 μM of 20:4n-6) was used. Interestingly, a significant increase in GPx activity (+19%) occurred in platelets incubated with 20:4n-6 (Fig. 4). This increased GPx activity induced by 20:4n-6 was also abolished by both cycloheximide and epicatechin (Fig. 4), whereas neither epicatechin nor cycloheximide induced any significant change in the GPx activity in control platelets (results not shown).

DISCUSSION

Platelets possess an important system of defense against free radicals generated during the enzymatic peroxidation of arachidonic acid. This antioxidant system mainly consists of vitamin E, which scavenges hydroperoxyl radicals, and GPx, which catalyzes the reduction of hydroperoxides into their hydroxylated derivatives. GPx plays a key role in protection against lipid peroxidation and some studies have reported an increased peroxidation after ingestion of fish oil [8, 9], which is rich in n-3 FA. We report here increased platelet GPx activity after enrichment of platelets with either 22:6n-3, 20:5n-3, or 18:3n-3 when compared with platelets pretreated with albumin alone. Moreover, among the FA tested, the stimulation of the GPx activity was observed with n-3 FA, but not with saturated (16:0), monounsaturated (18:1n-9), or some n-6 PUFA (18:2, 18:3). These results agree with some data observed in *ex vivo* as

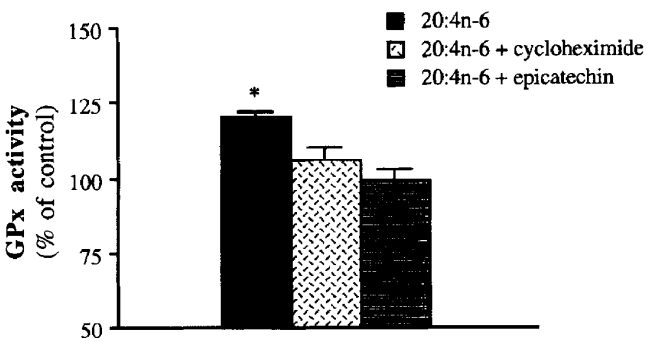

FIG. 4. Effects of 20:4n-6 on GPx activity in the absence or presence of cycloheximide or epicatechin. Platelets were incubated for 2 hr at 37°C in a Tyrode-HEPES buffer containing human albumin and were supplemented (or not) with 20:4n-6 (fatty acid/albumin ratio of 0.2). Platelets were coincubated (or not) with cycloheximide (500 μM) or epicatechin (10 μM). At the end of the incubation period, GPx activity was assayed as described in Materials and Methods. Results are expressed as % of control values and are means ± SEM of 5 independent experiments. The control value was 265 ± 17 nmol/min/mg protein; *p < 0.05 compared to control.

well as *in vitro* models. In using a similar preincubation of peripheral blood mononuclear cells with fatty acids, Joulain *et al.* [29] demonstrated increased GPx activity induced by 20:5n-3 and 22:6n-3. Such a stimulation of GPx activity has also been described *ex vivo* in platelets, as well as in erythrocytes after fish oil supplementations [30, 31]. The GPx activation was stronger in *ex vivo* studies [30, 31] (30 to 40%) than in our *in vitro* model (27% obtained with the most effective fatty acid tested, 22:6n-3). One possible explanation is that the *in vitro* incubation time, limited in the present study to avoid platelet alteration, was not sufficient to induce the maximal effect. Indeed, although our *in vitro* studies reflect the *in vivo* situation after ingestion of fish oil, in terms of changes in platelet fatty acid composition [32, 33], the supplementation periods were not comparable. Moreover, in our *in vitro* studies, the stimulation of GPx activity was lower when 22:6n-3, for example, was incubated for 1 hr instead of 2 (results not shown). The relatively short incubation time could also explain why we did not observe any change in GPx activity induced by 18:2n-6, whereas some reports described decreased GPx activity induced by a diet containing a low amount of 18:2n-6 [34, 35]. It is, however, noteworthy that the increase induced by n-3 FA is similar to the decrease observed in platelets from elderly people [17, 36] or diabetics [37], which makes our observation biologically relevant.

One possible mechanism to explain the 22:6n-3-induced increase in GPx activity is the induction of protein synthesis. Joulain *et al.* [29] have demonstrated that 20:5n-3 was not able to modify the kinetic parameters of the enzyme in peripheral blood mononuclear cells, which favors the hypothesis of increased GPx expression during the incubation period. Despite the absence of nucleus, a *de novo* protein biosynthesis in human platelets has been demonstrated by several authors [38, 39]. Indeed, in our experimental model, the amount of immunoreactive enzyme from platelets incubated in the presence of 22:6n-3 increased significantly, as did the GPx activity, when compared to platelets pretreated with albumin alone. Some studies have demonstrated the incorporation of radiolabeled amino acids by platelet proteins after 1-hr incubation [39] and have shown that it was inhibited by cycloheximide [38, 39]. To test if the GPx synthesis could have occurred in response to 22:6n-3, incubations were carried out in the absence or presence of cycloheximide. Because cycloheximide abolished the increase in GPx activity induced by 22:6n-3, this suggests that *de novo* protein synthesis might be responsible for the increased GPx activity. In agreement, cycloheximide also abolished the increased GPx immunoreactive protein in response to 22:6n-3, as observed with the Western blotting method. Thus, in addition to recent studies showing that n-3 FA may enhance hepatic GPx expression at the transcriptional level [40], our data obtained with anucleated cells such as platelets suggest that these FA may also have a posttranscriptional effect on GPx expression. As such, n-3 PUFA could, for instance, interfere with the rate of mRNA degradation or regulate specific factors controlling the translation. Indeed, a regulation at the translational level has already been established in response to selenium for GPx expression [41], and steady-state levels of GPx mRNA can be lowered by inadequate selenium intake [42].

Whatever the site of the activation, an intriguing question is the chemical form of the n-3 PUFA responsible for such an activation. In the present study, in agreement with previous reports using similar incubation conditions [20, 43], we found that the majority of 22:6n-3 was incorporated in phospholipids (80%), and a small proportion (<10%) of the fatty acid remained in its free form or was converted into monohydroxy derivatives. Although the unesterified form of 22:6n-3 was minor, we cannot exclude that it may activate the enzyme by covalent binding, because such protein modification has been reported in platelets with arachidonate and 20:5n-3 [44].

Another possible mechanism for the stimulation of platelet GPx activity could be an oxidative stress generated by the relatively high concentrations of 22:6n-3 used, as data in the literature have reported that lipid peroxides may lead to an exacerbated GPx activity [45]. To investigate this possibility, we measured platelet MDA and α-tocopherol contents. MDA was higher in 22:6n-3-rich platelets and α-tocopherol was lower, meaning that a moderate, but visible, oxidative stress occurred under our experimental conditions. The increased GPx activity and quantity previously observed in response to 22:6n-3 alone were abolished by cotreatment of platelets with epicatechin. Epicatechin also prevented the alterations of MDA and α-tocopherol levels. Because epicatechin has been proven to be an efficient antioxidant [28, 46], we might consider that at least part of the 22:6n-3-induced increase in GPx activity could be due to the oxidative stress associated with the 22:6n-3 enrichment. However, because the 22:6n-3 enrichment induces a release of 20:4n-6, part of the increased GPx activity could be due to the presence of a higher concentration of this fatty acid in the free FA pool. To test such a possibility, 20:4n-6 at an FA/albumin ratio of 0.2 (10 μM 20:4n-6) was assayed, this low ratio being required to avoid platelet aggregation. Significantly increased GPx activity, although slightly weaker than that observed in response to 22:6n-3, was observed with 20:4n-6, and this increase was also inhibited by both cycloheximide and epicatechin. These data strongly suggest that platelets might respond to an oxidative stress by promoting antioxidant defense as assessed by GPx. This concept has already been pointed out from different experiments done *in vivo* [45]. Our *in vitro* model of anucleated cells suggests that this might also occur at the posttranscriptional level of antioxidant enzyme expression.

In summary, the present findings suggest that 22:6n-3 incorporation into platelets results in increased GPx activity that could be explained by enzyme synthesis. This GPx synthesis could be a response to the oxidative stress generated by the relatively high concentrations of 22:6n-3 used and/or by 20:4n-6, released from the endogenous pool in the course of the experiment.

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