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DIETHYLDITHIOCARBAMATE (DITIOCARB SODIUM) EFFECT ON ARACHIDONIC ACID METABOLISM IN HUMAN MONONUCLEAR CELLS

GLUTATHIONE PEROXIDASE-LIKE ACTIVITY

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Abstract—Diethyldithiocarbamate (DTC), a thiol delivery agent, has been shown to significantly reduce the frequency of primary opportunistic infections in HIV-infected patients. This therapeutic effect has been related to the capacity of DTC to reverse the deleterious effects of the oxidative stress occurring in HIV infection. The influence of DTC on the oxygenated metabolism of arachidonic acid (AA) was investigated in mitogen-stimulated human peripheral blood mononuclear cells (PBMC). Upon incubation with PBMC previously labelled with [^{3}H]AA, Concanavalin A (Con A) markedly increased cyclo-oxygenase and lipoxygenase activities, within 30 min, as judged by thromboxane B_2 (TxB_2) and hydroxyeicosatetraenoic acid (HETE) production. Con A activation of [^{3}H]AA platelets also increased 12-HETE production but did not induce any TxB_2 synthesis. Micromolar concentrations of DTC, added simultaneously with the mitogen, significantly enhanced the synthesis of HETEs above the Con A-induced level while TxB_2-induced synthesis was inhibited but only at DTC concentrations higher than 50 μM. In the presence of nordihydroguaiaretic acid, a lipoxygenase inhibitor, which inhibited the Con A-induced synthesis of HETEs by 78%, DTC no longer stimulated HETE production above the Con A-induced level. Reverse phase HPLC analysis showed that Con A increased the PBMC production of 5-, 12- and 15-HETEs. In the presence of 5 μM DTC, 5-HETE production was entirely suppressed whereas the 15-HETE level was markedly enhanced, 12-HETE production by the contaminating platelets remained unchanged. *In vitro* experiments indicated that DTC alone did not significantly influence 15-hydroperoxyeicosatetraenoic (15-HPETE) production by the soybean 15-lipoxygenase but, in the presence of added reduced glutathione, DTC markedly reduced 15-HPETE into 15-HETE. In addition, DTC was able to substitute for cellular extract in the glutathione peroxidase (GPx) assay system. Taken together, these results indicate that DTC, through its "GPx-like" activity is able to modify the lipoxygenase cascade. Its ability to selectively reduce 15-HPETE known to stimulate immunosuppressive T-cells might help to explain its positive regulatory effect upon the immune system.

Diethyldithiocarbamate (DTC‡) is a thiol delivery agent that has been shown to be safe and efficient in the treatment of HIV-infected patients [1–3]. The mechanism of action of DTC could be related to its antioxidant properties. An increasing amount of data suggest that an oxidative stress and/or a failure of antioxidant defence mechanisms are involved in the pathogenesis of HIV infection [4, 5]. The oxidative stress is due to an excess of reactive oxygen species generated during infection or inflammation. This would result in a decrease of intracellular reduced glutathione (GSH) level and, as a consequence, in the activation of HIV replication [6]. Moreover, since all lymphocyte subsets are sensitive to the oxidative stress [7], this phenomenon could contribute to the progression of HIV-related immunosuppression.

DTC has been shown to protect the cellularity of lymphoid organs in mice exposed to hyperoxic atmosphere. DTC was also reported to protect the mitogenic proliferative response of lymphocytes whereas this response was suppressed in non-treated oxygen-exposed animals [8, 9]. Two models of immune deficiency associated with an oxidative stress have been studied: aging and autoimmune diseases. In both cases, a deficit in the proliferative response of T lymphocytes has been associated with a decrease in serum GSH [10, 11]. Prolonged treatment with DTC prevented the T cell deficiency of old animals [12]. Furthermore, DTC has been shown to prolong the survival of autoimmune MRL-lpr/lpr mice, a strain known to present profound T cell defects accompanying the spontaneous development of a systemic lupus erythematosus-like disease [13].

Some of the biological effects of DTC may be partly explained by the chemical characteristics of the molecule which behaves as a free-radical scavenger [14]. Moreover, DTC is able to spare the

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‡ Abbreviations: AA, arachidonic acid; Con A, concanavalin A; DTC, diethyldithiocarbamate, ditiocarb sodium, Imuthiol®; GSH, reduced glutathione; HETE, hydroxyeicosatetraenoic acid; HPETE, hydroperoxyeicosatetraenoic acid; NDGA, nordihydroguaiaretic acid; PBMC, peripheral blood mononuclear cells; TxB_2, thromboxane B_2.

GSH level of cells exposed to xenobiotics [15]. These effects suggest that the cytoprotective activity of DTC might be related to its antioxidant properties.

Oxygenated eicosanoids from arachidonic acid (AA) and other long chain polyunsaturated fatty acids have been shown to affect the immune response. These immunomodulatory effects are due to both direct and indirect actions on immune cells through the release of platelet activating factor, monokines, lymphokines, eicosanoids and other substances [16, 17]. Upon the addition of various stimuli such as antigens or mitogenic lectins, AA is released from the membrane phospholipid pool of monocytes and lymphocytes, particularly the phosphoinositides [18], and is metabolized via two enzymatic systems which have been clearly demonstrated in monocytes [19]. The cyclooxygenase system produces prostanoids and the lipoxygenase one produces various hydroxyeicosatetraenoic acids (HETEs), leukotrienes and lipoxins. Platelets, which are always contaminating mononuclear cell preparations, also oxygenate AA into prostanoids and 12-HETE [20], and some exchange of the various metabolites may occur during cell–cell interactions [21].

In order to bring further insights into possible mechanisms of DTC immunomodulation, we studied the influence of DTC upon the mitogen-induced metabolism of AA in human peripheral blood mononuclear cells (PBMC) and platelets. Since the preliminary results of this study showed that DTC was able to potentiate the mitogen-induced formation of HETEs, we further investigated whether DTC stimulated the synthesis of all HETEs or a particular one. Finally, we found that the "glutathione-peroxidase like" properties of DTC might be involved in the DTC-stimulated production of HETEs observed during the mitogenic stimulation of PBMC by concanavalin A (Con A).

MATERIALS AND METHODS

Materials. DTC (batch Nos S 1731 and S 1849) was provided by Pasteur-Mérieux (Lyon, France). [^{3}H]AA (200 Ci/mmol) and [^{14}C]AA (52 mCi/mmol) were purchased from CEA (Saclay, France). Concanavalin A, nordihydroguaiaretic acid (NDGA), *t*-butylhydroperoxide, glutathione reductase, NADPH and soybean lipoxygenase were from the Sigma Chemical Co. (St Louis, MO, U.S.A.) and GSH was from Boehringer Mannheim (Meylan, France). Culture medium RPMI 1640 and lymphocyte separation medium were from Flow Laboratories (Labsystems Group, Paris, France).

Preparation of human PBMC. Peripheral blood was obtained from healthy subjects who had not taken any medication for 2 weeks prior to blood donation. Blood was anticoagulated with citrate–phosphate–dextrose buffer, 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 prepared in buffered phosphate saline, pH 7.4 (1% final concentration). The leukocyte-rich supernatant was then removed, layered onto Ficoll-Hypaque and centrifuged at 600 *g* for 15 min at 20°. The resulting mononuclear fraction was washed twice with Hepes-buffered RPMI 1640 medium and resuspended at 4×10^7 cells/mL. Cells were counted in a hematocytometer. Their viability, checked with Trypan blue staining, was routinely higher than 95%.

Preparation of radiolabelled mononuclear cell suspensions. Mononuclear cells were incubated at 37° for 1 hr in the presence of [^{3}H]AA (1.5 μCi/mL) dissolved in ethanol (0.1% final concentration). After incubation, the radiolabelled cells (up to 60% of the total added radioactivity was incorporated) were washed three times in RPMI 1640 medium and resuspended at the initial cellular concentration (4×10^7 cells/mL).

Mitogenic activation of radiolabelled mononuclear cells. Mononuclear suspensions (2 mL) were equilibrated for a few minutes at 37° and increasing concentrations of DTC (diluted onto the special buffered diluent for DTC (batch No. S 1787, Pasteur-Mérieux) were then added with or without addition of 5 μg/10^6 cells Con A. After 30 min at 37°, the incubation was terminated by placing tubes in an ice bath followed by acidification of the medium to pH 3–4, and lipids were extracted with chloroform/ethanol (6:3, v/v) [22]. In experiments performed with NDGA, a lipoxygenase inhibitor, the cell suspensions (2 mL) were pre-incubated with inhibitor solutions (0.1 mL) for 20 min at 37° (NDGA was solubilized in ethanol and then appropriately diluted in water). The mitogen alone or the mitogen plus DTC were then added and the incubation was continued for 30 min. Extraction of total lipids was performed as described above.

Lipid analysis. After recovering the organic phase, it was evaporated to dryness under reduced pressure. Extracts were dissolved in a minimal volume of diethyl ether/methanol (9:1, v/v) and submitted to chromatographic separations [23, 24] on thin-layer plates (silicagel G60, Merck) using two separate migrations with the following solvent (i) hexane/diethyl ether/acetic acid (70:30:1) for separating the various lipid classes [hydroxyheptadecatrienoic acid, HETEs, free AA, triacylglycerols and steryl esters], (ii) diethyl ether/methanol/acetic acid (90:1:2) for the separation of prostanoids, mainly thromboxane B_2 (TxB_2) from total phospholipids. The different phospholipid classes were separated by a third migration with chloroform/methanol/acetic acid/water (50:37.5:3.5:2). The radioactivity of the different lipid spots was detected and quantitated with a radioscanner (Berthold).

In some experiments, HETEs produced by mononuclear cells under mitogenic activation with or without DTC were analysed by reverse phase HPLC on nucleosil C_{18} column. The eluent was methanol/water (75:25) acidified to pH 3 by acetic acid.

DTC interaction with hydroperoxides. The oxidation of DTC by peroxides was monitored spectrophotometrically by scanning the absorption from 320 to 400 nm. Peroxides used were *t*-butylhydroperoxide and 15-hydroperoxyeicosatetraenoic acid (15-HPETE) prepared from AA as a substrate of soybean 15-lipoxygenase [25]. In other experiments, we analysed the product formed

from ^{3}H-radiolabelled 15-HPETE (0.1 mM) when incubated with DTC at 37° for 10 min. The reaction was terminated by extraction with diethyl ether. Extracts were then analysed on TLC plates with the solvent mixture: hexane/diethyl ether/acetic acid (15:85:0.1) at 4° which properly separates 15-HPETE from 15-HETE. The interaction of DTC with the soybean lipoxygenase activity was also investigated as described above.

Glutathione peroxidase-like activity. The glutathione peroxidase-like activity of DTC was measured according to the spectrophotometric method of Chaudière and Gérard [26]. The assay mixture (825 μL) consisted of 50 mM Tris–HCl buffer, pH 7.6, 0.1 mM EDTA, 1 mM GSH, 0.125 mM NADPH, H^+ and 0.325 U/mL glutathione reductase. Absorbance at 340 nm was recorded in a Beckman DU 8 spectrophotometer and then 50 μL DTC (1 μM to 5 mM) was added to estimate the sample blank. Subsequently, hydroperoxide (15-HPETE or *t*-butylhydroperoxide) was added. Appropriate blanks were run in the absence of added DTC and in the presence of hydroperoxide. With no GSH in the assay mixture, there was no absorbance decrease with DTC. The kinetic patterns of the glutathione peroxidase-like activity were investigated as described above using several concentrations of peroxides [*A*] (50–250 μM for *t*-butylhydroperoxide, 50–150 μM for 15-HPETE) and four fixed GSH concentrations [*B*] (0.5, 1, 2, and 4 mM). The overall reaction was assumed to follow the Dalziel model given for a bireactant ping pong mechanism [27] which supposes the following sequence:

$$\mathrm{DTC} \xrightarrow[k_{+1}]{\mathrm{ROOH}[A]} \mathrm{DTC\text{-}ROOH} \xrightarrow[k_{+2}]{\mathrm{ROH}\ \mathrm{GSH}[B]}$$

$$\mathrm{DTC\text{-}SG} \xrightarrow[k_{+3}]{\mathrm{GSH}[B]\ \mathrm{GS\text{-}SG}} \mathrm{DTC}.$$

According to the Dalziel model:

$$\frac{[Eo]}{v} = \frac{\emptyset_1}{[A]} + \frac{\emptyset_2}{[B]}$$

with the following parameters: [*Eo*], DTC concentration; *v*, reaction velocity; [*A*], hydroperoxide concentration; [*B*], GSH concentration; $\emptyset_1 = 1/k_{+1}$ and $\emptyset_2 = 1/k_{+2} + 1/k_{+3}$.

Statistical analysis. Statistical analyses were carried out using the Stat View program (Macintosh). Comparison of more than two groups was performed by ANOVA. A minimal level of significance was fixed at $P < 0.05$.

RESULTS

Effect of mitogenic activation on AA metabolism in ^{3}H-radiolabelled human PBMC

When resting human PBMC were incubated in the presence of nanomolar concentrations of [^{3}H]-AA, the radioactivity was mainly taken up into phospholipids (70% of total incorporated radioactivity), the remainder being associated with neutral lipids (Table 1). In unstimulated cells, the oxygenated metabolism of endogenous AA was rather low since the amount of the main eicosanoids (AA, TxB_2, HETEs) did not exceed 1.13% of the total incorporated radioactivity. Mitogenic activation of PBMC by Con A (5 μg/10^6 cells) significantly stimulated the oxygenated metabolism of endogenous AA (Table 1). TxB_2 synthesis, referred to as a marker of the cyclooxygenase pathway, was substantially increased up to 350% whereas total HETE level, indicative of lipoxygenase activities, was increased by 115%. The overall eicosanoid production (AA, TxB_2, HETEs) was stimulated to the same extent following mitogenic stimulation of PBMC whereas neutral lipids and free AA remained unchanged (Table 1). The radioactivity of the total phospholipids remained unchanged in Con A-activated PBMC as compared to control cells. However, when the different phospholipid classes were further separated by TLC, the radioactivity located in phosphoinositides was found to be significantly decreased ($P < 0.05$) in Con A-treated PBMC whereas the radioactivity of the other classes was not modified as compared to control cells (Fig. 1). This result shows that, under mitogenic activation, AA was released essentially from the phosphoinositide pool.

Influence of DTC on the Con A-stimulated metabolism of AA

In order to search for a possible effect of DTC upon the mitogen-induced oxygenated metabolism of AA, DTC concentrations ranging from 0.5 to 100 μM were added simultaneously with the mitogen during the 30 min of incubation. During this elapsed time, DTC did not induce any cellular mortality even at the highest dose used (100 μM) as estimated by the Trypan blue exclusion test (not shown). DTC did not significantly modify the radioactivity incorporated into total phospholipids, neutral lipids and free AA (Table 1). Although 5 μM DTC tended to increase the mitogen-induced synthesis of TxB_2 (+22% above the Con A-induced level), this effect was not significant. In the presence of higher DTC doses (50 and 100 μM), TxB_2 levels were not different from the control level. This result indicates that high doses of DTC suppress the stimulatory effect of Con A upon TxB_2 synthesis. On the other hand, DTC at the dose of 5 μM significantly increased the amount of total HETEs ($P < 0.05$) produced over the Con A-induced level (Table 1). Thus, the Con A-increased HETE production, which was 115% over the control level, reached 154% in the presence of an additional 5 μM DTC. Thus, DTC could have different effects upon AA metabolism due to its ability to modulate positively or negatively the two main pathways of oxygenated eicosanoid production.

The measurement of the radioactivity associated with the different phospholipid classes indicated that DTC did not modify the mitogen-induced decrease of labelled phosphoinositides in PBMC (not shown).

When PBMC were preincubated for 20 min at 37° in the presence of 10 μM NDGA, a lipoxygenase inhibitor, prior to mitogenic activation, the Con A-induced synthesis of HETEs was reduced by about 78%. In the presence of NDGA, DTC was no longer

Table 1. Effect of DTC on Con A-stimulated AA metabolism in ^{3}H-radiolabelled human PBMC

Conditions	N	Total phospholipids	Triacylglycerols	Steryl esters	AA	TxB_2	HETEs	Total Σ AA, TxB_2 HETEs
Control	15	69.95 ± 3.21	20.16 ± 2.46	6.07 ± 0.89	0.44 ± 0.13	0.21 ± 0.04	0.48 ± 0.06	1.13 ± 0.17
Con A (5 μg/10^6 cells)	20	70.13 ± 3.24	18.89 ± 2.40	5.28 ± 0.78	0.49 ± 0.06	0.95 ± 0.13*	1.03 ± 0.06*	2.48 ± 0.17*
Con A plus DTC (0.5 μM)	3	64.27 ± 9.05	25.40 ± 7.16	6.37 ± 0.90	0.60 ± 0.25	0.73 ± 0.06	0.93 ± 0.07*	2.27 ± 0.22*
Con A plus DTC (5 μM)	15	69.00 ± 4.64	19.50 ± 3.35	5.16 ± 1.08	0.51 ± 0.08	1.16 ± 0.18*	1.22 ± 0.06*†	2.90 ± 0.21*
Con A plus DTC (50 μM)	4	66.86 ± 3.77	22.43 ± 3.70	5.00 ± 0.74	0.70 ± 0.18	0.54 ± 0.05	1.00 ± 0.11*	2.25 ± 0.22*
Con A plus DTC (100 μM)	3	67.85 ± 6.35	22.83 ± 5.67	5.10 ± 0.45	0.77 ± 0.29	0.38 ± 0.10	0.87 ± 0.03*	2.03 ± 0.36

PBMC suspensions (2 mL, 80 × 10^6 cells) labelled with [^{3}H]AA were incubated at 37° for 30 min either without (control) or with 5 μg Con A/10^6 cells in the absence or presence of increasing DTC concentrations. Lipid extracts were processed as described in Materials and Methods. Results are expressed as percentages of the total incorporated radioactivity and are means ± SEM. N indicates the number of separate experiments.

Statistical analysis by ANOVA is represented:

* Different from the control level, $P < 0.05$ by the Fisher test.

† Different from the Con A-induced level, $P < 0.05$ by the Fisher test.

Since minor lipids are not considered in the table, total does not come to 100% in each row.

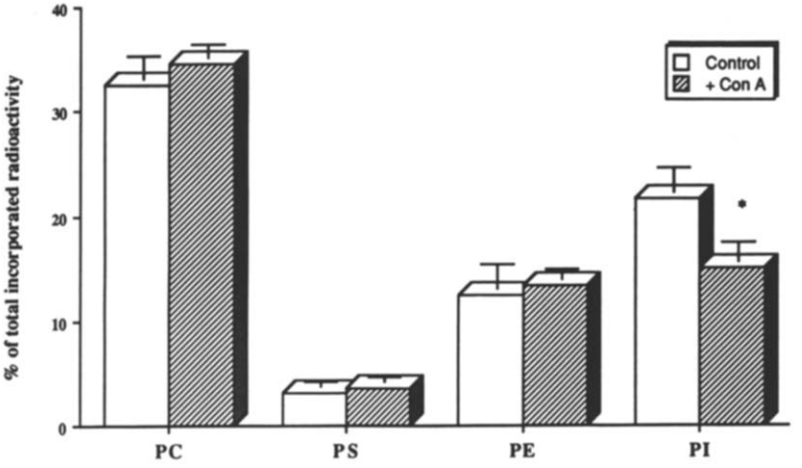

Fig. 1. Effect of mitogenic activation of ^{3}H-radiolabelled human PBMC on the radioactivity of the different phospholipid classes. Incubation conditions were the same as in Table 1 and lipid extracts were processed as described in Materials and Methods. Results are expressed as percentages of the total incorporated radioactivity and are means ± SEM of 11 experiments. Statistical analysis by the unpaired Student's *t*-test is represented: *$P < 0.05$. Abbreviations: PC, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine; PI, phosphatidylinositol.

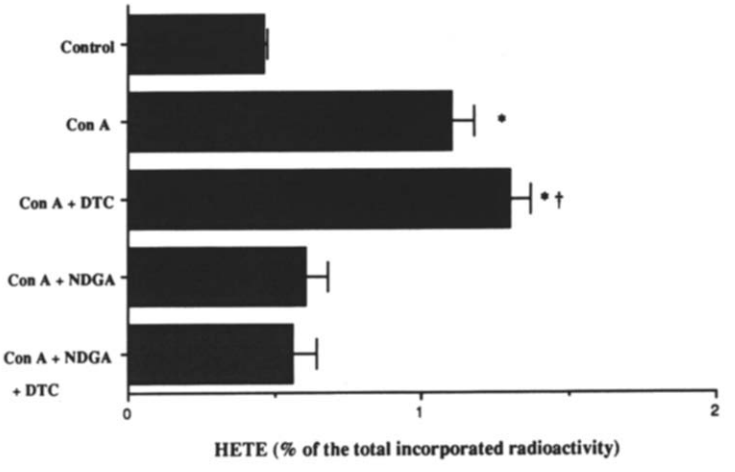

Fig. 2. Inhibition of human PBMC lipoxygenase activities by NDGA. PBMC were incubated for 20 min with 10 μM NDGA and then activated by Con A for 30 min at 37° in the absence or presence of 5 μM DTC. Results are expressed as percentages of the total incorporated radioactivity and are means ± SEM of six experiments. *Different from the control level, $P < 0.05$; †different from the Con A-induced level, $P < 0.05$.

able to stimulate the production of HETEs above the Con A-induced level (Fig. 2).

Identification of the HETEs selectively increased by DTC during the mitogenic activation of PBMC

TLC analyses of eicosanoids synthesized during the mitogenic activation of PBMC indicated that total HETEs were further increased by DTC simultaneously added with the mitogen. As a first approach to the identification of HETEs, we studied the influence of DTC on the Con A-stimulated metabolism of AA in human platelets known to produce only 12-HETE. In Con A-stimulated platelets (2×10^8 platelets, previously radiolabelled with [^{3}H]AA for 60 min at room temperature per assay; 2.5 μg Con A per 10^6 platelets), the production of 12-HETE was only slightly and not significantly increased (+46 ± 18%, N = 5) over the basal level. In marked contrast with PBMC, Con A was not able to induce any TxB_2 synthesis. Furthermore, 5 μM DTC, a concentration which proved to be effective in PBMC, did not significantly stimulate 12-HETE synthesis further (+17 ± 15%, N = 5, above the Con A-induced level) in Con A-treated platelets (not shown).

Table 2. Influence of DTC on HETEs produced during the mitogenic activation of ^{3}H-radiolabelled human PBMC

Assays	N	15-HETE (nmol/4 × 10^7 cells)	12-HETE (nmol/4 × 10^7 cells)	5-HETE (nmol/4 × 10^7 cells)	Total HETEs (nmol/4 × 10^7 cells)
Control	7	0.02 ± 0.01	0.02 ± 0.01	ND	0.04 ± 0.04
Con A	7	0.29 ± 0.10	0.72 ± 0.14*	0.28 ± 0.14*	1.29 ± 0.20*
Con A + DTC (5 μM)	7	0.74 ± 0.14*†	0.77 ± 0.15*	0†	1.52 ± 0.15*

Incubation conditions were the same as in Table 1. After lipid extraction, 5-HETE, 12-HETE and 15-HETE were separated by reverse phase HPLC as described in Materials and Methods. Results are expressed as nmol HETE ± SEM per 4 × 10^7 cells. N indicates the number of separate experiments.

Statistical analysis by ANOVA is represented:

* Different from the control level, $P < 0.05$ by the Fisher test.

† Different from the Con A-induced level, $P < 0.05$ by the Fisher test.

ND, not detected.

Further reverse phase HPLC separation of the total HETEs produced in Con A-activated PBMC pointed out the presence of 5-, 12- and 15-HETEs in the following per cent ratio: 22:56:22. Unexpectedly, when simultaneously added with Con A to PBMC, DTC totally suppressed 5-HETE synthesis, significantly stimulated (+155% above the Con A-induced level) 15-HETE formation and did not modify 12-HETE level, the net result of these changes being a rise in total HETEs above the Con A-stimulated level (Table 2).

In vitro *interactions of DTC with hydroperoxides*

DTC could be oxidized by various hydroperoxides which resulted in a rise of its absorbance over the 320–400 nm range [28]. As shown in Fig. 3, the incubation of 1 mM DTC in the presence of 100 μM 15-HPETE markedly increased DTC absorbance. Since 15-HPETE absorbance was negligible at this concentration, this result suggests either the oxidation of DTC to disulfiram or the formation of a 15-HPETE–DTC complex. The addition of 2 mM GSH to the 15-HPETE–DTC mixture almost entirely prevented the increase of DTC absorbance, suggesting therefore that GSH could prevent DTC oxidation. Interestingly, a decrease of DTC absorbance was also observed when GSH was added to the mixture after 20 min of 15-HPETE–DTC interaction, suggesting that GSH could also reduce oxidized DTC (not shown).

When ^{3}H-radiolabelled 15-HPETE was incubated at 37°, in borate buffer, less than 20% underwent a spontaneous oxidation to 15-HETE within a 10 min incubation period (Fig. 4). DTC alone or GSH alone did not increase HPETE reduction above the spontaneous one measured in control assays. However, in the presence of 2 mM GSH, DTC markedly increased 15-HETE formation which reached 40 and 45% with 5 and 10 μM DTC, respectively (Fig. 4). Similar results were obtained in experiments designed to study the influence of DTC on soybean 15-lipoxygenase activity. DTC did not reduce 15-HPETE produced by the soybean lipoxygenase nor influence the enzyme activity. In contrast, when GSH was added to the incubation mixture, DTC effectively reduced the major part of 15-HPETE produced from AA by the soybean lipoxygenase (not shown). Taken together these results suggest that DTC exhibits a "glutathione peroxidase-like" activity *per se*, at concentrations shown to increase HETE production.

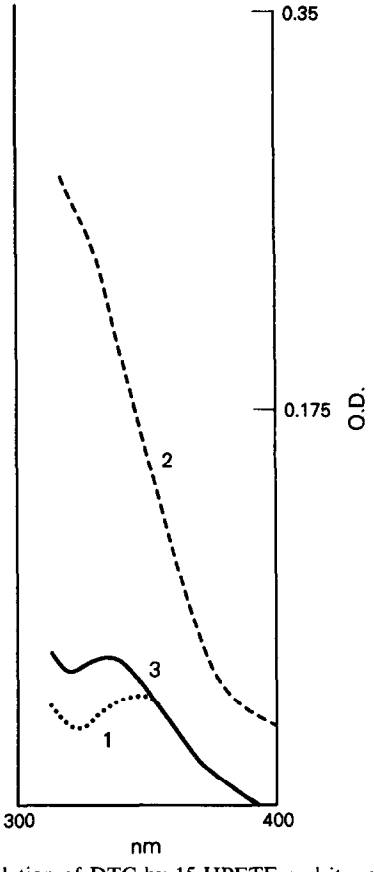

Fig. 3. Oxidation of DTC by 15-HPETE and its reversal by GSH. DTC (1 mM) was incubated in the presence of 100 μM 15-HPETE or 100 μM 15-HPETE plus 2 mM GSH. (1) DTC; (2) DTC plus 15-HPETE; (3) DTC plus 15-HPETE plus GSH.

Glutathione peroxidase-like activity of DTC, in vitro

The ability of DTC to reduce hydroperoxides in

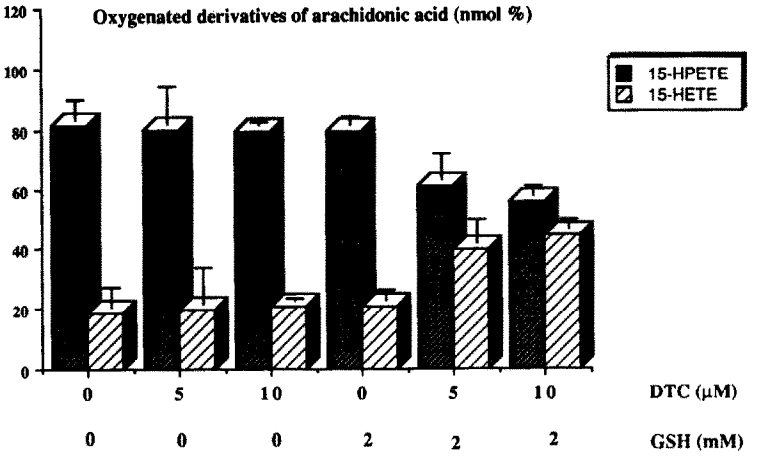

Fig. 4. Reduction of ^{3}H-radiolabelled 15-HPETE by DTC in the presence of GSH. ^{3}H-Radiolabelled 15-HPETE (0.1 mM) was incubated in 1 mL (100 nmol 15-HPETE per assay) borate buffer, pH 9.0, for 10 min at 37°, in the following conditions: without addition, with 5 or 10 μM DTC alone, with 5 or 10 μM DTC plus 2 mM GSH, with 2 mM GSH alone. Reaction was terminated by extraction with diethyl ether. Results are expressed in nmol % and are means ± SD of three separate experiments.

the presence of GSH was further investigated by the coupled test system using NADPH/glutathione reductase as an indicator. Tracks showing the decrease of absorbance after correcting for the blank with only the hydroperoxide present (Δ A) as a function of time, indicate that DTC reduced 15-HPETE at a higher rate than *t*-butylhydroperoxide (Fig. 5). Furthermore there was a linear dependence on the concentration of DTC until 1 and 2 mM with 15-HPETE and *t*-butylhydroperoxide substrates, respectively. In marked contrast with the cellular glutathione peroxidase enzyme which is known to exhibit a linear response with increasing GSH concentrations [29], DTC showed a saturation of its glutathione peroxidase-like activity beyond 1 mM (Fig. 6). The kinetic behaviour of the glutathione peroxidase-like activity of 100 μM DTC was further investigated using the Dalziel equation as described in Materials and Methods. Double reciprocal plots established for several GSH concentrations clearly converged (not shown) whereas Dalziel plots of the cellular enzyme were demonstrated to be parallel [29]. In marked contrast with the kinetic patterns of the cellular enzyme, K_m^Aapp and V_{max}^Aapp. for DTC were not directly proportional to GSH concentrations (Table 3). Whereas K_m^A app. was usually 3–4-fold higher for 15-HPETE as the substrate than for *t*-butylhydroperoxide, the velocity constant of the reaction of DTC with peroxide was one order of magnitude higher for 15-HPETE than for *t*-butylhydroperoxide.

DISCUSSION

Several works related to DTC biological activity have reported paradoxical effects of this compound, particularly concerning its role in preventing cell oxidation injury. However, the DTC concentrations used in these studies varied from nanomolar to millimolar range. Most of the DTC effects, peroxidative in nature, such as increase of lipid peroxidation, intracellular copper accumulation [30] or superoxide dismutase inhibition [9] have been observed at millimolar concentrations. DTC, known to chelate metal ions, is able to bind Cu^{2+} to form a highly lipophilic complex which facilitates copper accumulation within the cell [31]. Increased cellular copper has been shown to enhance lipid peroxidation [32] through the oxidation of essential thiol groups, thus effecting an imbalance of the cellular redox system [33]. In the present work, we used micromolar DTC concentrations which were demonstrated by others to inhibit *in vitro* lipid peroxidation and to prevent both the destruction of microsomal polyunsaturated fatty acids and malondialdehyde formation [34, 35]. Interestingly, DTC concentration reached 5 μM in the plasma of treated patients after administration of a 10 mg/kg dose which is the recommended one for the treatment of HIV-infected patients.

We have examined the effect of DTC on the mitogenic-induced metabolism of endogenous AA in human PBMC. Firstly, under mitogenic activation, AA was released from membrane phospholipids, mainly the phosphoinositide pool, and was metabolized via two oxidative pathways. We have demonstrated that the synthesis of cyclooxygenase (TxB_2) and lipoxygenase (HETEs) products was significantly increased in mitogen-activated mononuclear cells. However, the experimental conditions used in the present study (cell labelling with trace amount of [^{3}H]AA, short incubation period in the presence of the mitogen) did not allow us to detect any prostaglandin formation. This result is in good agreement with a previous report from Parker *et al.* [36] showing that no prostaglandin synthesis was detectable till 120 min of mitogenic activation by phytohemagglutinin. Micromolar DTC concen-

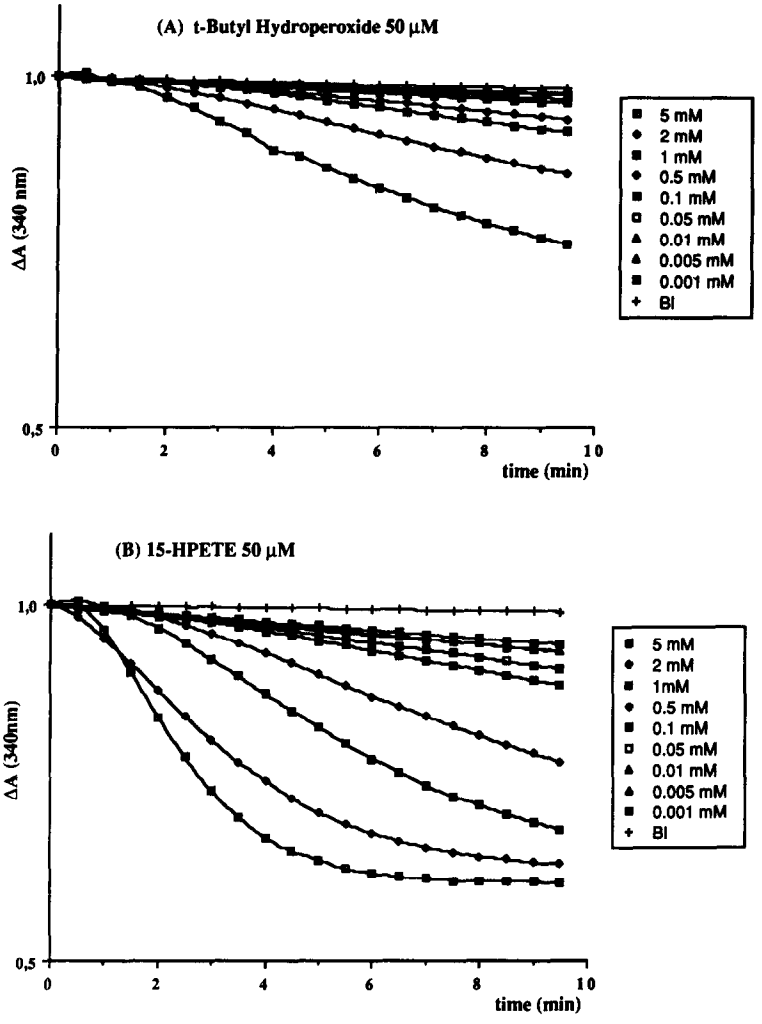

Fig. 5. Glutathione peroxidase-like activity of DTC. The decrease of absorbance at 340 nm in the assay carried out as described in Materials and Methods was followed as a function of time. The reaction was started upon addition of 50 μM *t*-butylhydroperoxide (A) or 50 μM 15-HPETE (B). Δ *A* was corrected for blanks run in the absence of added DTC and in the presence of hydroperoxide. GSH concentration was 1 mM.

trations, added to mitogen-activated cells, enhanced the production of HETEs whereas only higher doses decreased the mitogen-induced TxB_2 formation. DTC seems to exert opposite effects on the cylooxygenase and lipoxygenase systems depending on the concentration used, the former being negatively and the latter positively modulated. The potentiation by DTC of the mitogen-induced HETE production is of interest since several lines of evidence have implicated the lipoxygenase pathway in lymphocyte activation [37]. Thus, lipoxygenase inhibitors have been reported to depress murine thymocyte proliferation and IL-2 synthesis [38], and to suppress the cytotoxic activity and proliferation of cytotoxic T lymphocytes [39]. As previously pointed out by others [16, 36, 37, 40], Con A-stimulated PBMC, which contain accessory cells and platelets, synthesize various hydroxy derivatives of AA including 5-, 12- and 15-HETE. It is noteworthy that, in our hands, purified platelets failed to respond to Con A activation as far as TxB_2 or 12-HETE synthesis is concerned. Similar results concerning a lack of platelet response to phytohemagglutinin have been reported by Parker *et al.* [36]. Reverse phase HPLC analysis of HETEs synthesized by Con A-activated cells in the presence of DTC showed that DTC increases 15-HETE synthesis while inhibiting 5-HETE, 12-HETE production remaining unchanged. The opposite variations of 5- and 15-HETE levels is in agreement with previous results of Vanderhoek *et al.* [40] showing that the preincubation of polymorphonuclear leukocytes with 15-HETE strongly inhibited the 5-lipoxygenase pathway.

Furthermore, *in vitro* studies showed that 5 and 10 μM DTC was able to reduce 15-HPETE to 15-HETE, but only in the presence of GSH. This result suggests that DTC exhibits a "glutathione peroxidase-like" activity *per se*, as demonstrated in our work

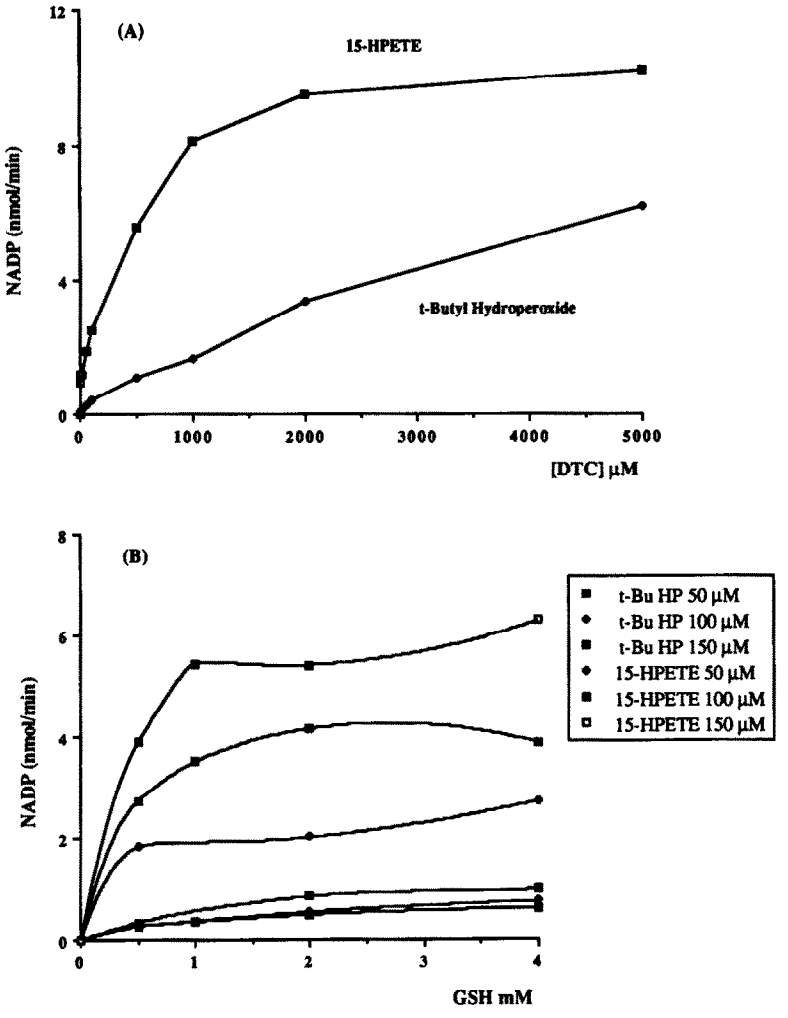

Fig. 6. Dependence of glutathione peroxidase-like activity of DTC on DTC concentration (A) and on GSH concentration (B). In (A), the glutathione peroxidase-like activity was measured for various DTC concentrations in the 1 μM to 5 mM range. The reaction was started upon addition of 50 μM hydroperoxide. Assays were performed with 1 mM GSH. In (B), DTC concentration was 100 μM. Plots are given for hydroperoxide concentrations of 50, 100 and 150 μM.

Table 3. Experimental conditions of the kinetic experiments and corresponding kinetic data of the glutathione peroxidase-like activity of DTC

Peroxide	GSH (mM)	DTC (μM)	K_m^A app. (μM)	Eo/V_{max}^A app. (10^{-4} sec)	$Ø_1$ (10^{-8} M sec)	k_{+1} (10^6 M^{-1} sec^{-1})	$Ø_2$ (10^{-6} M sec)
t-BuHP	4	100	88.5	30.6	27.1	3.69	12.23
t-BuHP	2	100	73.4	40.1	29.5	3.39	8.03
t-BuHP	1	100	60.9	70.1	42.7	2.34	7.01
t-BuHP	0.5	100	52.4	101.3	53.1	1.88	5.07
15-HPETE	4	100	189.9	4.1	7.8	12.82	1.64
15-HPETE	2	100	382.1	2.5	9.7	10.31	0.51
15-HPETE	1	100	273.9	3.4	9.4	10.64	0.34
15-HPETE	0.5	100	159.6	6.7	10.7	9.35	0.33

The kinetic parameters were determined graphically by plotting $[Eo]/v$ as a function of $1/[A]$ according to the Dalziel equation (Materials and Methods): K_m^A app., reciprocal of the abscissa intercept; Eo/V_{max}^A app, ordinate intercept; $Ø_1$, slope; k_{+1}, reciprocal of $Ø_1$; $Ø_2$, ordinate intercept × $[B]$. Curves were fitted by linear regression analysis. For each GSH concentration, the following peroxide concentrations were used: *t*-butylhydroperoxide (*t*-BuHP): 50, 65, 100, 150 and 250 μM; 15-HPETE: 50, 65, 100 and 150 μM.

and in the previous work of Kumar *et al.* [28]. These authors have previously shown that among several sulfhydryl compounds tested including 2-mercaptoethane-sulfonic acid, cysteamine, *S*-(2-aminoethyl) isothiouronium Br-HBr and *N*-(2 mercaptopropionyl) glycine, only 2-(3-aminopropylamino) ethanethiol dihydrochloride (WR-1065), dithiothreitol and DTC were able to reduce NADPH, H^+ in a glutathione peroxidase assay system, using H_2O_2 as the peroxide source, in the absence of any cellular extract [28]. Among the active compounds, DTC was found 2.5-fold more effective than WR-1065 on an equimolar basis whereas dithiothreitol exhibited only a weak activity. More recently Müller *et al.* [41] and Wendel *et al.* [42] have described a similar glutathione peroxidase-like activity for the seleno organic compound Ebselen. As we have observed for DTC, Ebselen also showed a saturation of its peroxidase-like activity for GSH concentrations higher than 2 mM. In marked contrast, the velocity of the rat liver microsomal enzyme [41] or the purified bovine blood enzyme [29] at infinite concentration of peroxide was a linear function of the concentration of GSH. Results from Müller *et al.* [41] and Wendel *et al.* [42], as well as the results of the present study, clearly indicate that organic compounds which possess a peroxidase-like activity exhibit kinetic patterns very different from those of the cellular enzyme. In analogy to the mechanism known for the seleno enzyme, Wendel *et al.* [42] have supposed that Ebselen could give a mixed selenylsulphide with GSH followed by oxidation of the selenium and its reduction by a second GSH. A similar mechanism involving the formation of a mixed DTC–GSH sulfide followed by its reduction by a second GSH might be speculated. However, it is noteworthy that the sulfur analog of Ebselen proved to be devoid of glutathione peroxidase-like activity [41].

In Con A-activated PBMC, DTC stimulates the lipoxygenase pathway. Cellular lipoxygenase catalyses the oxygenation of AA into HPETEs which are further reduced into HETE by the selenoenzyme glutathione peroxidase [43]. Since DTC no longer stimulates HETE production when lipoxygenases are inhibited by NDGA, this result suggests that DTC, at micromolar concentrations, does not behave as an oxidant agent able to favour the chemical oxidation of AA into hydroperoxides. All the results seem to confirm a direct effect of DTC on hydroperoxides of AA. DTC seems to facilitate hydroperoxide reduction but not to stimulate its formation. At concentrations of DTC higher than 5 μM, no effect on HETE production was demonstrated, a result that suggests a narrow range of active DTC concentration on the lipoxygenase cascade. At this stage of the study, the mechanism by which DTC increases 15-HETE and decreases 5-HETE production remains unclear.

On the other hand, DTC neither interferes with phosphoinositide breakdown under mitogenic activation nor is able to induce this breakdown when added alone (unpublished data). DTC appears then to be able to act on AA oxygenated metabolism which plays a regulatory role in the control of immune processes but not on events directly involved in the triggering of the T-cell receptor, such as phospholipase C-induced phosphatidylinositol hydrolysis.

In conclusion, DTC, known to stimulate T-lymphocyte functions, is also able to modulate lipoxygenase activity of mitogen-activated PBMC. However, DTC mechanism of action on the lipoxygenase system appears rather complex since it both increases 15-HETE and decreases 5-HETE formation. Its ability to selectively reduce 15-HPETE, previously demonstrated to stimulate immunosuppressive $CD8^+$ positive T-cells while 15-HETE did not exhibit such properties [44], might contribute to explain its positive regulatory effects upon the immune system.

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