Biochemical Pharmacology, Vol. 53, pp. 927–935, 1997.

ISSN 0006-2952/97/$17.00 + 0.00
PII S0006-2952(96)00832-5

Correlation Between Arachidonic Acid Oxygenation and Luminol-Induced Chemiluminescence in Neutrophils: Inhibition by Diethyldithiocarbamate

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ABSTRACT. Neutrophils from allergic subjects were hypersensitive to stimulation by low calcium ionophore concentration (0.15 μM), resulting in an increased formation of leukotriene B_4 (LTB_4), 5S-hydroxy-6,8,11,14-(E,Z,Z,Z)-eicosatetraenoic acid (5-HETE), and other arachidonic acid metabolites through the 5-lipoxygenase pathway. In parallel, luminol-dependent chemiluminescence was also higher in neutrophils from allergic patients at the basal state and after stimulation by calcium ionophore, revealing an enhancement of radical oxygen species and peroxide production. The activity of glutathione peroxidase, the main enzyme responsible for hydroperoxide reduction, was lowered in these cells. Diethyl-dithiocarbamate (DTC) induced a concentration-dependent decrease in chemiluminescence and arachidonic acid metabolism after neutrophil stimulation. These data show that the elevation of arachidonic acid metabolism in neutrophils from allergic patients is strongly correlated with oxidative status. This elevation may be the consequence of an increased cellular hydroperoxide known to activate 5-lipoxygenase (5-LOX) activity and/or an increased arachidonic acid availability, due either to phospholipase A_2 (PLA_2) activation or inhibition of arachidonate reesterification into phospholipids. Lowering this oxidative status was associated with a concomitant decrease of this metabolism. Our results suggest that the effect of DTC may be the consequence of an inhibition of peroxyl radical and cellular lipid hydroperoxide production. Thus, DTC may modulate arachidonic acid metabolism in neutrophils by modulating the cellular hydroperoxide level. BIOCHEM PHARMACOL 53;7:927–935, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. neutrophils; allergic patients; arachidonic acid and oxidative metabolism inhibition; chemiluminescence; diethyldithiocarbamate

Leukotriene B_4 or 5S, 12R-dihydroxy-6,8,10,14-(Z,E,E,Z)-eicosatetraenoic acid (LTB_4)¶ is an endogenous mediator, especially relevant in human pathological states such as hypersensitivity reactions and inflammatory diseases [1–3]. Moreover, numerous observations have indicated that LTB_4 may play a significant role in the modulation of certain immunological reactions [4, 5]. Therefore, the study of its biosynthesis in normal and pathological states has been undertaken by many investigators. The starting point of LTB_4 production is the action of 5-lipoxygenase (EC 1.13.11.34) (5-LOX) on arachidonic acid released from phospholipids in stimulated cells, particularly neutrophils [6]. 5-Lipoxygenase has to be activated in order to oxygenate arachidonic acid, and the transfer of the enzyme from the cytosol to the membrane [7] as well as its binding to plasma membrane via the specific protein, the five lipoxygenase activating protein (FLAP) [8] represent two events in this activation. Moreover, human 5-LOX requires fatty acid hydroperoxides as stimulating or activating factors. This has been demonstrated with homogenates from neutrophils [9–11] or intact cells [12, 13], providing evidence that 5-LOX activity and LTB_4 formation in neutrophils depend on a critical threshold level of fatty acid hydroperoxides. After stimulation by activating agents such as the calcium ionophore A23187 or the *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP), neutrophils also produce

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¶ *Abbreviations:* AA, arachidonic acid; 5-LOX, 5-lipoxygenase; PLA_2, phospholipase A_2; FLAP, five lipoxygenase activating protein; 5-HPETE, 5S-hydroperoxy-6,8,11,14-(E,Z,Z,Z)-eicosatetraenoic acid; LTA_4, 5S,6S-oxido-7,9,11,14-(E,E,Z,Z)-eicosatetraenoic acid; LTB_4, leukotriene B_4, 5S,12R-dihydroxy-6,8,10,14-(Z,E,E,Z)-eicosatetraenoic acid; Δ6-*trans*-LTB_4, 5S,12R-dihydroxy-6,8,10,14-(E,E,E,Z)-eicosatetraenoic acid; 12epi-Δ6-*trans*-LTB_4, 5S,12S-dihydroxy-6,8,10,14-(E,E,E,Z)-eicosatetraenoic acid; 20-OH-LTB_4, 5S,12R,20-trihydroxy-6,8,10,14-(Z,E,E,Z)-eicosatetraenoic acid; 5-HETE, 5S-hydroxy-6,8,11,14-(E,Z,Z,Z)-eicosatetraenoic acid; PGB_2, prostaglandin B_2; DTC, diethyldithiocarbamate, dithiocarb sodium, Imuthiol®; fMLP, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; GMCSF, granulocyte macrophage colony stimulating factor; NDGA, nordihydroguaiaretic acid; RP-HPLC, reversed phase-high performance liquid chromatography; IC_{50}, inhibitor concentration leading to 50% inhibition.

Received 19 April 1996; accepted 8 October 1996.

reactive oxygen derivatives (respiratory burst) commonly characterized by luminol- or lucigenin-induced chemiluminescence [14]. Diethyldithiocarbamate (DTC) is a thiol-containing compound that has been shown to be an immunoprotective agent under conditions of oxidative stress [15–18] and to exhibit a glutathione peroxidase-like activity [19]. It has also been shown to possess some properties in the treatment of HIV-infected patients [20–22]. In the present work, we have investigated the effect of DTC on LTB_4 generation and luminol-induced chemiluminescence in neutrophils from allergic and healthy subjects.

MATERIALS AND METHODS

Materials

DTC was provided by Pasteur-Mérieux (Marcy l'Etoile, France). LTB_4, 5S-hydroxy-6,8,11,14-(E,Z,Z,Z)-eicosatetraenoic acid (5-HETE), 5S,12R-dihydroxy-6,8,10,14-(E,E,E,Z)-eicosatetraenoic acid (Δ6-*trans*-LTB_4), 5S,12S-dihydroxy-6,8,10,14-(E,E,E,Z)-eicosatetraenoic acid (12-epi-Δ6-*trans*-LTB_4), 5S,12R,20-trihydroxy-6,8,10,14-(Z,E,E,Z)-eicosatetraenoic acid (20-OH-LTB_4), and prostaglandin B_2 (PGB_2) were obtained from Cayman Chemical Co. (Ann Arbor, MI, USA). *Tert*-butylhydroperoxide, arachidonic acid, and the calcium ionophore A23187 were from Sigma Chemical Co. (St Louis, MO, USA). All solvents used were of analytical grade. Methanol was purchased from Carlo Erba Farmitalia (Romilly sur Andelle, France), and acetonitrile ("far UV" for HPLC) from Fisons (Loughborough, UK). All other products used were of analytical grade or better.

Subjects

Thirty-three atopic subjects, 15 females and 18 males (age range: 19 to 60 years), were entered in this study. Informed consent was obtained from all patients, who had a positive atopic allergic disease (allergic rhinitis or asthma, or both). They were symptomatic and were examined at the hospital during the critical season of polinization in our region. They all had positive allergic skin tests, elevated IgE, and specific IgE levels. Eleven healthy volunteers (three females and eight males, age range 18 to 50 years) with negative personal or family history of atopy and no clinical evidence of atopic or other diseases, served as controls. All subjects claimed not to have taken any drugs for at least 2 weeks before the blood punction.

Methods

NEUTROPHIL PREPARATION. All subjects were fasted for at least 12 h before venipuncture. Venous blood was collected into polypropylen tubes containing 5 mL of 3.8% sodium citrate solution and immediately centrifuged at 120 × *g* for 20 min at room temperature. Neutrophils were purified from the lower phase by sedimentation on dextran followed by Ficoll-Paque centrifugation as previously described [23]. The purified neutrophils were suspended into Tyrode solution, without calcium and magnesium, and supplemented with 1 mM HEPES (pH 7.35) and 5 mM glucose. The purity of neutrophil preparations was more than 95%, as evaluated by May-Gruenwald-Giemsa staining. The trypan blue exclusion assay showed that more than 99% of the cells were viable.

CELL STIMULATION. In a volume of 200 μL, neutrophils (4×10^6 cells) were preincubated for 5 min at 37°C alone or with increasing concentrations of DTC, dissolved in the buffer provided by the manufacturer (6.4 mM NaH_2PO_4, $2H_2O$, 10.3 mM Na_2HPO_4, $12H_2O$, 136.7 mM NaCl, and 1.5 mM EDTA, Na salt) in the presence of 1.6 mM Ca^{2+} and 0.4 mM Mg^{2+}. Then 100 μL calcium ionophore solution (final concentration 0.15, 0.5, or 1.5 μM) in Tyrode-HEPES buffer and 100 μL Ca^{2+} and Mg^{2+} solution in the same buffer, to adjust the cation concentrations to 1.6 and 0.4 mM, respectively, were successively and quickly added, and the mixture (0.4 mL) incubated for another 5 min at 37°C. The reaction was terminated by the addition of 0.5 mL of methanol containing 100 pmol of PGB_2, as an internal standard, and by cooling at −20°C until the analysis. The final concentrations of DTC were 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9} M, these concentrations being doubled during preincubation with the neutrophils. The calcium ionophore was diluted in the Tyrode-HEPES buffer at the appropriate concentration from a 3 mM stock solution in DMSO, and used immediately. In some experiments, neutrophil activation was carried out in the presence of 10 μM exogenous arachidonic acid added before the calcium ionophore.

PURIFICATION AND ASSAY OF ARACHIDONATE METABOLISM. The methanolic homogenate was centrifuged at 1500 × *g* for 10 min at 0°C. Routinely, 100 μL of supernatant were analyzed directly without prior extraction or fractionation. Neutrophil arachidonic acid metabolites were purified and assayed by reversed phase-high performance liquid chromatography (RP-HPLC) as described [22] with a Li-Chrospher 100 RP-18 column (length: 22 cm; internal diameter: 4 mm; particle size: 5 μm) (Merck, Darmstadt, Germany). For the separation of LTB_4, Δ6-*trans*-LTB_4, 12-epi-Δ6-*trans*-LTB_4, and 20-OH-LTB_4, the column was isocratically eluted with the mixture methanol/acetonitrile/water/acetic acid (250:300:400:4, v/v) (flow rate: 0.7 mL/min), and the effluent monitored at 270 nm. For the purification of 5-HETE, the mixture methanol/acetonitrile/water/acetic acid (300:450:200:4, v/v) was used as the mobile phase (flow rate: 0.4 mL/min), and the effluent monitored at 235 nm. The pH of both mobile phases was adjusted at 5.6 with 28% NH_4OH. Quantitation of metabolites was performed by comparing peak areas with those of standards chromatographed under the same conditions.

CHEMILUMINESCENCE ASSAY. Dark chemiluminescence was measured in a LUMAC®/3M, M 2010 Biocounter lu-

minometer (Lumac BV, Schaesberg, The Netherlands). One hundred microliters neutrophil suspension (2×10^5 cells), or 100 μL vehicle, was preincubated for 5 min with 100 μL DTC solution in polystyrene cuvettes set in the cuvette compartment of the luminometer thermostated at 37°C. Then, 200 μL luminol (Lumac), the chemiluminescence probe, (10^{-5} M in Tyrode-HEPES buffer supplemented with 2 mM Ca^{2+} and 0.5 mM Mg^{2+}, pH 7.4) was added followed by 10 μL calcium ionophore A23187 at the appropriate concentration 30 sec later (time zero). The cuvette content was gently mixed, the sample conveyed to the measurement position and the light emission recorded at 30-sec intervals within a 6-min period.

NEUTROPHIL GLUTATHIONE PEROXIDASE ACTIVITY DETERMINATION. Glutathione peroxidase activity was determined according to the method of Paglia and Valentine [24]. Neutrophils were homogenized by sonication (5 cycles of 10 sec). In a spectrophotometer cuvette, 100 μL homogenate was mixed with 1.5 mL of 50 mM Tris-HCl buffer (pH 7.6) containing 0.1 mM EDTA, 0.14 mM NADPH, 1 mM GSH, and 1 unit of glutathione reductase. After 2 min preincubation at 37°C, the substrate *tert*-butylhydroperoxide (0.3 mmol) was added. The NADPH oxidation was followed by recording the decreased absorbance at 340 nm for 5 min after addition of substrate. The glutathione peroxidase activity was defined as the number of nanomol NADPH oxidized per min per 10^6 neutrophils and calculated on the basis of a NADPH molar absorbance of 6.22×10^6 at 340 nm.

DATA AND STATISTICAL ANALYSIS. LTB_4 levels are presented as pmol (mean ± SEM for separate experiments) generated after 5 min stimulation of 4×10^6 neutrophils. The chemiluminescence response is expressed in the integrated form, as the total accumulated photons per minute vs. the time after stimulus addition. Thus, data represent the chemiluminescence velocity at various time points relative to the stimulus addition. The average of the velocity (± SEM) for separate experiments is given. Comparison of the data obtained with neutrophils from healthy and allergic subjects was carried out using the Wilcoxon's nonparametric rank sum test. When data groups could be paired (i.e. effect of DTC vs. control), the Wilcoxon's paired *t*-test was used. Probabilities corresponding to $P < 0.05$ were considered to be significant. The significance of the correlation coefficient has been determined with the Spearman's rank correlation test. The difference between the glutathione peroxidase activity in neutrophils from allergic and healthy subjects was tested using the Wilcoxon's nonparametric rank sum test.

RESULTS

Effect of DTC on Arachidonic Acid Metabolism in Neutrophils From Allergic Patients and Healthy Subjects

The level of LTB_4, 5-HETE, and other metabolites (20-OH-LTB_4, Δ6-*trans*-LTB_4, 12-epi-Δ6-*trans*-LTB_4) biosynthesized from arachidonic acid through the 5-lipoxygenase pathway can be easily determined by reversed phase-high performance liquid chromatography. The amount of these metabolites produced after 5 min stimulation at 37°C by 0.15 μM calcium ionophore of 4×10^6 neutrophils are presented in Table 1. The percentage of inhibition vs. the control values was ca. 90% when neutrophils were preincubated with 10^{-5} M DTC. Levels of arachidonic acid metabolites produced were significantly different from control values in the presence of 10^{-8} to 10^{-5} M DTC. With 10^{-9} M DTC, these levels were lowered further (ca. 16% inhibition for LTB_4 and more than 20% for the other metabolites), but not significantly. The decrease in 20-OH-LTB_4 paralleled that of LTB_4, and the LTB_4/20-OH-LTB_4 ratio

TABLE 1. Effect of DTC on arachidonic acid metabolism in neutrophils from allergic patients

	Diethyldithiocarbamate concentrations (M)					
5-LOX-metabolites	**0 (11)†**	**10^{-9} (9)**	**10^{-8} (8)**	**10^{-7} (11)**	**10^{-6} (11)**	**10^{-5} (6)**
LTB_4	238.5 ± 57.4§	200.9 ± 69.1 (−15.7%)‡	155.1 ± 62.8* (−34.9%)	130.7 ± 50.5* (−45.2%)	94.7 ± 35.3* (−60.3%)	23.1 ± 16.9* (−90.3%)
20-OH-LTB_4	72.8 ± 18.7	55.4 ± 18.1 (−23.9%)	43.1 ± 17.7* (−40.8%)	40.3 ± 14.7* (−44.6%)	31.2 ± 11.8* (−57.1%)	6.6 ± 4.4* (−90.9%)
Δ6-*trans*-LTB_4	49.7 ± 12.5	37.1 ± 10.4 (−25.4%)	25.4 ± 11.2* (−48.9%)	9.8 ± 5.4* (−80.4%)	1.8 ± 1.7* (−96.3%)	2.4 ± 2.3* (−95.3%)
12-epi-Δ6-*trans*-LTB_4	38.4 ± 8.5	25.8 ± 8.8 (−32.8%)	16.6 ± 8.2* (−56.9%)	7.3 ± 4.9* (−80.9%)	1.6 ± 1.5* (−95.8%)	1.3 ± 1.2* (−96.6%)
Total LTB_4-related metabolites	407.1 ± 95.3	319.2 ± 103.8 (−21.6%)	240.1 ± 105.3* (−41.1%)	188.2 ± 72.2* (−53.8%)	129.4 ± 47.2* (−68.2%)	34.1 ± 25.4* (−91.6%)
5-HETE	246.2 ± 38.5	165.3 ± 42.2 (−32.8%)	101.6 ± 33.7* (−58.3%)	49.1 ± 25.3* (−80.1%)	44.9 ± 24.9* (−81.8%)	32.1 ± 21.7* (−86.9%)

Neutrophils were activated by the calcium ionophore (0.15 μM), for 5 min at 37°C, in the presence of Ca^{2+} (1.6 mM) and Mg^{2+} (0.4 mM), with increasing concentrations of DTC. Arachidonate metabolites were separated and assayed by HPLC. The amount of each metabolite is expressed in pmol after 5 min stimulation of 4×10^6 neutrophils (mean ± SEM).

† Number of separate experiments.

‡ Percentage inhibition vs. controls.

* Significantly different from the corresponding control level ($P < 0.05$).

remained constant and equal to approximately 3.4. The production of the two LTB_4 isomers, Δ6-*trans*-LTB_4 and 12-epi-Δ6-*trans*-LTB_4, formed by nonenzymatic hydrolysis of residual 5S,6S-oxido-7,9,11,14-(E,E,Z,Z)-eicosatetraenoic acid (LTA_4), was also decreased in parallel to LTB_4 production. However, the percentage inhibition of these two isomers was higher than that of LTB_4 at each DTC concentration. It may also be noticed that the 5-HETE production appeared more sensitive to the action of DTC than the LTB_4 biosynthesis. Indeed, the DTC concentration leading to 50% inhibition (IC_{50}) of 5-HETE formation was approximately 20-fold lower than that of LTB_4 (about 5 nM and 100 nM, respectively). These data show that the biosynthesis of all these metabolites was decreased in a concentration-dependent manner when neutrophils were preincubated with DTC for 5 min before ionophore stimulation. In addition, a positive correlation between the DTC-induced inhibition of LTB_4 synthesis and that of the luminol-dependent chemiluminescence in neutrophils from allergic patients stimulated by 0.15 μM calcium ionophore A23187 could be demonstrated with the Spearman's rank correlation test. For example, in the presence of 10^{-6} M DTC, the regression coefficient is equal to 0.847 (P = 0.038) (not shown).

Figure 1 shows that in line with our earlier reports [25], the amount of LTB_4 produced was higher in neutrophils from allergic patients activated in the presence of 10 μM arachidonic acid than in neutrophils activated alone. Figure 1 also shows that DTC (10^{-6} M) did not alter the LTB_4 production in neutrophils activated in the presence of arachidonic acid. Similar results were obtained concerning the other arachidonic acid metabolites produced (not shown). Thus, the inhibitory effect of DTC on arachidonic acid metabolism through the 5-lipoxygenase pathway in neutrophils from allergic patients could be overcome when these cells were activated by the calcium ionophore (0.15 μM) in the presence of exogenous arachidonic acid. Taken together, these results show that the target step for DTC action is likely to be the formation of 5S-hydroperoxy-6,8,11,14-(E,Z,Z,Z)-eicosatetraenoic acid (5-HPETE), the precursor of 5-HETE and LTA_4, and/or the release of arachidonic acid in response to the calcium ionophore.

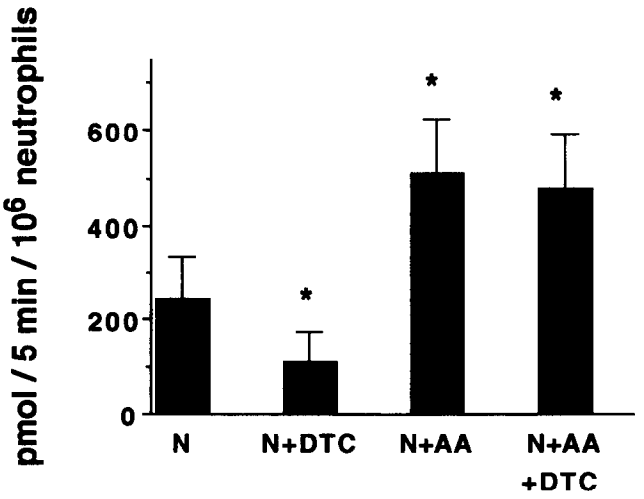

FIG. 1. Effect of exogenous arachidonic acid on the inhibition of LTB_4 production by DTC in neutrophils from allergic subjects. Neutrophils were activated by the calcium ionophore (0.15 μM) for 5 min at 37°C, alone (N) or in the presence of DTC (10^{-6} M) (N+DTC), arachidonic acid (AA) (10 μM) (N+AA), or both (N+AA+DTC). The amount of LTB_4 is expressed in pmol after 5 min activation of 4 × 10^6 neutrophils (mean ± SEM of four determinations). *Significantly different from neutrophils activated in the absence of DTC and AA (P < 0.05).

The results obtained with neutrophils from healthy subjects are summarized in Table 2. The formation of LTB_4 and 5-HETE was not detectable in neutrophils stimulated by 0.15 μM ionophore A23187. This formation was only detectable after cell activation by 0.5 or 1.5 μM ionophore. DTC significantly lowered the production of LTB_4 and 5-HETE at the concentrations of 10^{-6} and 10^{-9} M only after cell stimulation by 0.5 μM ionophore. The percentage inhibition was higher in the case of 5-HETE (100% at 10^{-6} M and 65% at 10^{-9} M) than in the case of LTB_4 (89% at 10^{-6} and 53% at 10^{-9} M) formation. The DTC-induced inhibition of arachidonic acid metabolism in neutrophils from control subjects may also be overcome by the addition of exogenous fatty acid (not shown).

Luminol-Induced Chemiluminescence of Neutrophils from Allergic and Healthy Subjects

Neutrophils from allergic and healthy subjects exhibited luminol-induced chemiluminescence during the incubation of neutrophils alone at 37°C, in the absence of external stimulus (basal level) (Fig. 2A) and after stimulation by 0.15 μM calcium ionophore A23187 (Fig. 2B). In the absence of external stimulus, the weak but detectable chemiluminescence observed reflects the degree of activation in resting neutrophils (Fig. 2A). No detectable signal above the background was measured in the absence of luminol, indicating that the chemiluminescence measured resulted from the oxidation of this probe. Heat treatment of granulocytes for 5 min in a boiling water bath totally inhibited the chemiluminescence (data not shown).

Also as depicted in Fig. 2, the luminol-dependent chemiluminescence was higher in neutrophils from allergic patients (upper traces) than in those from healthy subjects (lower traces) at the basal level (Fig. 2A) as well as after stimulation by the calcium ionophore (Fig. 2B). After stimulation by 0.15 μM calcium ionophore, the observed luminol-dependent chemiluminescence plateaued from 4 min after the ionophore addition, both in neutrophils from allergic and healthy subjects. The highest values were three to four times greater in neutrophils from allergic patients than in those from healthy donors (Fig. 2B). Five minutes after luminol addition, the basal level of chemiluminescence was also three to five times higher in neutrophils from allergic patients than in those from healthy subjects (Fig. 2A).

TABLE 2. Effect of DTC on LTB_4 and 5-HETE production in neutrophils from healthy subjects

Ionophore (μM)	5-LOX metabolites	Diethyldithiocarbamate concentration (M)		
		0 (3)†	10^{-9} (3)	10^{-6} (3)
0.15	LTB_4	ND¶	ND	ND
	5-HETE	ND	ND	ND
0.5	LTB_4	154.4 ± 10.5§	71.8 ± 57.5	15.6 ± 15.6* (−89.9%)‡
	5-HETE	79.2 ± 30.4	27.4 ± 20.1* (−65.4%)	ND
1.5	LTB_4	301.7 ± 15.9	278.7 ± 6.9	275.2 ± 1.9
	5-HETE	544.1 ± 208.6	525.4 ± 193.5	506.4 ± 195.4

Neutrophils were activated by the calcium ionophore at three different concentrations, for 5 min at 37°C, in the presence of Ca^{2+} (1.6 mM) and Mg^{2+} (0.4 mM), with increasing concentrations of DTC. Arachidonate metabolites were separated and assayed by HPLC. The amount of each metabolite is expressed in pmol after 5 min stimulation of 4×10^6 neutrophils (mean ± SEM).

† Number of separate experiments.

‡ Percentage inhibition vs. the control. ND = nondetectable.

* Significantly different from the corresponding control ($P < 0.05$).

Effect of Diethyl Dithiocarbamate Upon Luminol-dependent Chemiluminescence in Neutrophils from Allergic Patients

Figure 3 shows that DTC inhibited in a dose-dependent fashion the luminol-dependent chemiluminescence resulting from the stimulation of neutrophils by 0.15 μM calcium-ionophore A23187. For clarity, the figure shows only data obtained in control assays and after neutrophil preincubation with 10^{-5}, 10^{-6}, and 10^{-8} M DTC. This figure also shows that the level of chemiluminescence 5 min after the ionophore addition was lowered by ca. 60% in the presence of 10^{-6} M DTC (Fig. 3, lower trace vs. middle trace). Inhibition was not increased with 10^{-8} M DTC. In neutrophils from healthy subjects, the basal or ionophore-induced luminol-dependent chemiluminescence in the presence of 10^{-5} and 10^{-6} M DTC was not different from the background level (data not shown).

Lowered Glutathione Peroxidase Activity in Neutrophils From Allergic Patients

Figure 4 shows that the glutathione peroxidase activity in neutrophils from allergic patients (1.3 ± 0.2 nmol of NADPH oxidized per min per 10^6 neutrophils; $n = 22$), was significantly lower ($P < 0.05$) than in neutrophils from healthy subjects (2.9 ± 0.9 nmol of NADPH oxidized per min per 10^6 neutrophils; $n = 8$).

DISCUSSION

The results presented in this report show that DTC inhibits both the production of LTB_4 and luminol-dependent chemiluminescence in neutrophils from allergic patients stimulated by 0.15 μM calcium ionophore A23187. A positive correlation could also be found between these two indices of inhibition. In neutrophils from healthy subjects stimulated by 0.15 μM ionophore, LTB_4 production was not detectable by HPLC, consistent with our earlier results [25], and the DTC effect could not be assessed by this approach. However, LTB_4 production was measured in neutrophils from healthy subjects stimulated with 0.5 μM ionophore and was also reduced in the presence of DTC. The production of Δ6-*trans*-LTB_4 and 12-epi-Δ6-*trans*-LTB_4, the two LTB_4 isomers formed by nonenzymatic hydrolysis of the residual LTA_4, as well as that of 20-OH-LTB_4 and 5-HETE were also decreased by DTC in parallel to LTB_4. These results suggest that the target step for DTC action may be upstream to 5-HPETE, the substrate for LTA_4 and 5-HETE production, i.e. at the step of the 5-lipoxygenase, the enzyme that converts arachidonic acid into 5-HPETE and/or at the level of phospholipase A_2 (PLA_2), the enzyme that releases arachidonic acid from phospholipids. Interestingly, DTC appeared more active in neutrophils from allergic patients than in those from healthy subjects. This suggests that it may be principally active in slowing down an exacerbated process.

Billah *et al.* [26] have reported that the ionophore A23187 induces a concentration-dependent release of arachidonic acid from phospholipids and the subsequent LTB_4 formation in neutrophils from healthy subjects, beginning at a concentration higher than 0.3 μM and plateauing from 1 to 1.5 μM. The results presented in this report are in agreement with these data. The production of LTB_4 in neutrophils from healthy subjects was observed only after cell activation by 0.5 μM ionophore, not after stimulation by 0.15 μM. In neutrophils from allergic patients, the curve representing the amount of arachidonic acid released and LTB_4 produced vs. the ionophore concentration is shifted to the left (Chabannes, unpublished observations). Arachidonic acid and LTB_4 are already released upon neutrophil stimulation by 0.15 μM ionophore [25] and their amounts were maximal in response to 1.5 μM ionophore. The elevation of arachidonic acid metabolism in neutrophils from allergic subjects in response to the activation by the cal-

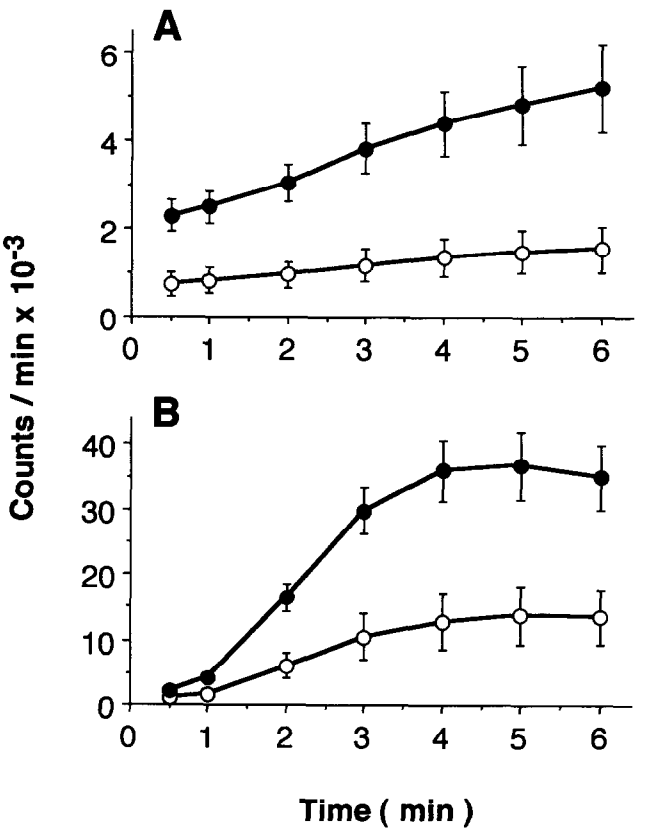

FIG. 2. **Luminol-dependent chemiluminescence of neutrophils from allergic and healthy subjects. Neutrophils in Tyrode-HEPES buffer supplemented with 1.6 mM Ca^{2+} and 0.4 mM Mg^{2+} were preincubated for 5 min in polystyrene cuvettes in a luminometer thermostated at 37°C. Then luminol (10^{-5} M) was added followed, 30 sec later (time zero), by calcium ionophore (0.15 μM) (induced chemiluminescence; B) or vehicle (basal chemiluminescence; A). The light emission was recorded at 30 sec intervals within a 6-min period. Data are expressed in counts x 10^{-3} per min and are the mean ± SEM of 10 (neutrophils from healthy subjects; ○) or 13 (neutrophils from allergic patients; ●) experiments.**

cium ionophore A23187 was discussed in our earlier report [25]. It might be the consequence of an intrinsic abnormal hyperactivity of 5-LOX [27] or PLA_2 [28] and/or of an extrinsic neutrophil activation ("priming") by various cytokines, such as the granulocyte macrophage colony stimulating factor (GMCSF) [29, 30], in allergic diseases. The inhibitory effect of DTC on the LTB_4 production was observed after stimulation by 0.15 μM ionophore in neutrophils from allergic patients and by 0.5 μM ionophore in neutrophils from healthy subjects. This inhibition was no longer observed when neutrophils from both healthy and allergic subjects were activated by 1.5 μM ionophore alone, or when neutrophils from allergic patients were activated by 0.15 μM in the presence of exogenous arachidonic acid. Because the amount of arachidonic acid released is near its maximum in neutrophils activated by 1.5 μM ionophore, the DTC-induced inhibition of LTB_4 production appears to be closely linked to the amount of arachidonic acid released

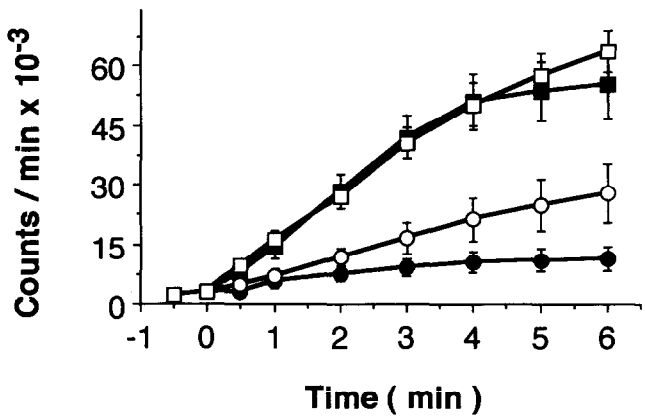

FIG. 3. **Effect of DTC on the luminol-dependent chemiluminescence in neutrophils from allergic subjects. Neutrophils were activated by calcium ionophore (0.15 μM) at 37°C, in the presence of luminol (10^{-5} M) and 0 (□), 10^{-5}M (●), 10^{-6} M (○) or 10^{-8} M (■) of DTC. The light emission was recorded at 30-sec intervals after addition of ionophore, over a 6-min period. Data are expressed in counts x 10^{-3} per min and are the mean ± SEM of 4–10 determinations.**

upon cell activation and is prevented by exogenous arachidonic acid supplementation. Therefore, the target step for DTC action could be at the PLA_2 activity releasing arachidonic acid from phospholipids.

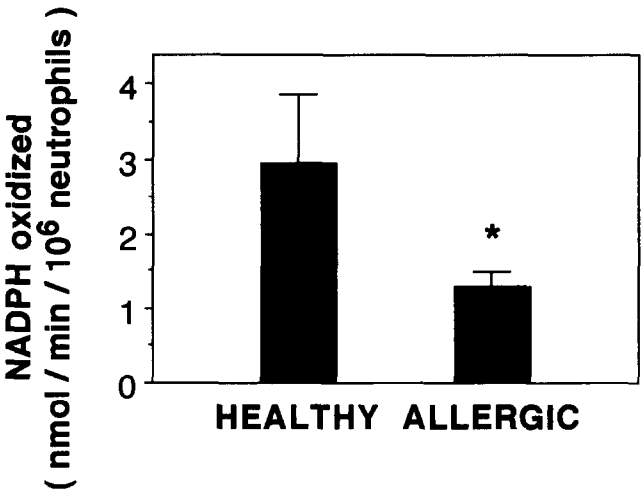

FIG. 4. **Glutathione-dependent peroxidase activity in neutrophils from allergic and healthy subjects. Neutrophil homogenates were incubated for 2 min at 37°C in 50 nM Tris-HCl buffer (pH 7.6), with 0.1 mM EDTA, 0.14 mM NADPH, 1 mM reduced glutathione, and 1 unit of glutathione reductase. Then, the reaction was started by the addition of 0.3 mM tertio-buthylhydroperoxide. The NADPH oxidation was followed by recording the decrease in absorbance at 340 nm for 5 min after addition of subsatrate. The glutathione peroxidase activity is expressed as nmol of NADPH oxidized per min and per 10^6 neutrophils. Values as nmol of NADPH oxidized per ;min and per 10^6 neutrophils. Values are the means ± SEM of 22 (neutrophils from allergic patients) and 8 (neutrophils from healthy subjects) assays. *Significantly different from healthy subjects ($P < 0.05$).**

After stimulation by activating agents such as calcium ionophore A23187 or fMLP, neutrophils produce reactive oxygen derivatives and oxygen-generated free radicals ("respiratory burst"). It is well known that double bonds of polyunsaturated fatty acids in cellular phospholipids exhibit marked sensitivity to peroxidative attacks induced by free radical processes, leading to the formation of fatty acid hydroperoxides. The chemiluminescence measured was higher with neutrophils from allergic patients than with cells from healthy subjects, both at the basal state and after ionophore activation, reflecting a higher capacity of cells from allergics to produce active oxygen metabolites. It has been reported that the elevated neutrophil "respiratory burst" observed in allergic diseases could be the consequence of the cell "priming" by cytokines [31]. Luminol-dependent chemiluminescence has been used as an index of hydroperoxide formation in the cell [32]. Thus, the high luminol-dependent chemiluminescence observed in activated neutrophils from allergic patients might be related to an increased production of fatty acid hydroperoxides. The inhibitory effect of DTC on luminol-dependent chemiluminescence has already been demonstrated in zymosan-stimulated mouse phagocytes [14] and rat neutrophils [33]. It has also been reported that DTC inhibits the lipid peroxidation promoted by different systems [34, 35]. The inhibitory effect of DTC on lipid peroxidation may be based on its action as cosubstrate for glutathione-dependent peroxidase reaction, as Hosni *et al.* [19] have found that DTC, in the presence of glutathione, exhibits a peroxidase-like activity.

The formation of peroxidized fatty acids in membrane phospholipids is also known to activate endogenous phospholipase A_2 (PLA_2) to liberate preferentially the peroxidized acids, which are then reduced by the catalytic activity of glutathione peroxidase [36, 37]. It has been reported that *t*-butylhydroperoxide can increase PLA_2 activity and stimulate the release of arachidonic acid from pulmonary arterial endothelial cells [38], and that reactive oxygen species are involved in the activation of cellular PLA_2 [39]. Thus, it is possible that DTC, by slowing down the release of arachidonic acid in A23187-stimulated neutrophils, could inhibit the synthesis of 5-LOX products. The decrease in arachidonic acid release could be the consequence of a direct action of DTC on PLA_2 activity, but it is also conceivable that DTC might act on PLA_2 by reducing the hydroperoxide tone of the cell.

The effect of lipid hydroperoxides on the induction of 5-lipoxygenase activity in neutrophils has been well documented [9–11, 13]. This phenomenon can be easily explained by the fact that 5-lipoxygenase contains a nonhaem iron atom at the catalytic site, under the ferric state in the active enzyme, the lipid hydroperoxides maintaining the iron atom in this oxidized ferric form [40]. The addition of glutathione peroxidase to a neutrophil homogenate causes a rapid arrest of 5-lipoxygenase activity [10, 13], and this inhibition is also reversed by lipid hydroperoxides [13] or by systems promoting lipid peroxidation [10]. Finally, other reducing agents without SH groups are potent inhibitors of 5-lipoxygenase by reducing the enzyme active site iron to the inactive ferrous state [41]. Other substances have been reported to inhibit the 5-lipoxygenase pathway and LTB_4 production. They include antioxidants such as nordihydroguaiaretic acid (NDGA) [42], vitamin E [43, 44] and various reducing agents [41]. Dietary vitamin E elicits *in vivo* a dose-dependent reduction of the 5-lipoxygenase product formation in rat neutrophils [43], an effect likely mediated by the inhibition of arachidonate release from endogenous phospholipids [45]. Vitamin E is also known to reduce the levels of intracellular fatty acid hydroperoxides, activators of 5-lipoxygenase, which are elevated in vitamin E deficiency [46]. NDGA, a lipid soluble antioxidant, also reduces LTB_4 production, likely by means of an indirect inhibition of phospholipase A_2, by trapping free radicals and by preventing the oxidative modification of the substrate [47]. The low level of glutathione-dependent peroxidase activity in these cells may contribute to maintain the high cellular peroxide status.

Thus, the inhibition of arachidonic acid metabolism in neutrophils activated in the presence of DTC could be the consequence of an inhibition of the 5-lipoxygenase enzyme through the DTC-induced decrease of hydroperoxide tone. This mechanism may occur in concert with the inhibition of PLA_2 activity.

In summary, neutrophils from allergic subjects were highly sensitive to stimulation by low calcium ionophore concentration, resulting in an increase in arachidonic acid metabolism through the 5-lipoxygenase pathway in parallel to an increase in luminol-dependent chemiluminescence, reflecting an augmentation of cellular lipid peroxidation. Hydroperoxides may increase arachidonic acid availability by activation of phospholipase A_2 and/or inhibition of arachidonate reesterification into phospholipids, in addition to increasing 5-lipoxygenase activity. DTC induced a concentration-dependent decrease in chemiluminescence correlated to an inhibition of arachidonic acid-derived eicosanoid production. DTC may control arachidonic acid metabolism in human neutrophils by modulating the level of cellular hydroperoxides.

This work was supported by INSERM and a grant from Pasteur-Mérieux Sérums et Vaccins.

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