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Effects of 1-Chloro-2,4,6-Trinitrobenzene on 5-Lipoxygenase Activity and Cellular Leukotriene Synthesis

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ABSTRACT. 5-Lipoxygenase (EC 1.13.11.34) is the key enzyme in the regulation of leukotriene synthesis. Here, the effects of various substituted nitrobenzene compounds on 5-lipoxygenase activity and the formation of leukotriene B_4 (LTB_4) were studied in polymorphonuclear leukocytes (PMNL), B lymphocytes, and human whole blood. 1-Chloro-2,4,6-trinitrobenzene (TNCB) was found to inhibit calcium ionophore A23187-induced leukotriene synthesis in PMNL in a biphasic manner. Thus, 1.0 μM TNCB caused 50% inhibition of LTB_4 formation, but only 16% inhibition was found at 10 times higher concentration. In contrast, this higher concentration of TNCB activated the synthesis of LTB_4 when PMNL were stimulated with arachidonic acid alone, demonstrating that TNCB can exert both stimulatory and inhibitory effects on leukotriene synthesis depending on the experimental conditions. The inhibitory effect of 1.0 μM TNCB on ionophore A23187-induced leukotriene synthesis could be circumvented by addition of exogenous arachidonic acid. At high concentrations of TNCB (25–100 μM), the drug blocked ionophore A23187-induced leukotriene synthesis. TNCB also inhibited LTB_4 formation in B lymphocytes, as well as in human whole blood. The activity of recombinant 5-lipoxygenase was inhibited by TNCB, and reduced glutathione or β-mercaptoethanol counteracted this inhibition. This suggests that TNCB might inhibit 5-lipoxygenase by alkylating thiol groups. TNCB possessed a high specificity for 5-lipoxygenase with only modest inhibitory effects on 12-lipoxygenase (EC 1.13.11.31), 15-lipoxygenase (EC 1.13.11.12), and phospholipase A_2 (EC 3.1.1.4) activities. Taken together, these results show that TNCB can both specifically inhibit and stimulate leukotriene formation and might be useful in further studies on the regulation of 5-lipoxygenase. BIOCHEM PHARMACOL **55**;6:863–871, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. arachidonic acid; leukotriene; 5-lipoxygenase; phospholipase A_2; 1-chloro-2,4,6-trinitrobenzene; 5-lipoxygenase activating protein

Leukotrienes (LT)† constitute a class of potent biological lipid mediators derived from arachidonic acid [1]. The key enzyme in leukotriene synthesis, 5-lipoxygenase (EC 1.13.11.34), is expressed in polymorphonuclear leukocytes (PMNL), monocytes/macrophages, and B lymphocytes [1, 2–4]. 5-Lipoxygenase possesses two catalytic activities, the conversion of arachidonic acid to 5-hydroperoxy-eicosatetraenoic acid (5-HPETE) and the subsequent formation of LTA_4. This compound can be further metabolised to LTC_4 or LTB_4, reactions catalysed by LTC_4 synthase and LTA_4 hydrolase (EC 3.3.2.6), respectively. Alternatively, LTA_4 can be degraded nonenzymatically to 6-trans-LTB_4 and 6-trans-12-epi-LTB_4. FLAP (5-lipoxygenase activating protein) acts as an arachidonic acid transfer protein, necessary for cellular synthesis of leukotrienes from the endogenous pool of arachidonic acid [5]. Leukotrienes are thought to be involved in several common diseases including asthma [6] and inflammatory bowel disease [7, 8]. LTB_4 also stimulates immunoglobulin secretion and proliferation of human B lymphocytes as well as cytokine release from T cells and monocytes, suggesting an immunomodulatory role of this compound [9].

Two therapeutic strategies have been developed to inhibit the synthesis of 5-lipoxygenase products: direct 5-lipoxygenase inhibitors and indirect inhibitors which interfere with FLAP [5]. A variety of redox-active compounds have been identified as inhibitors of 5-lipoxygenase. 5-Li-

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† *Abbreviations:* ATP, adenosine 5′-triphosphate; diamide, azodicarboxylic acid bis(dimethylamide); DNCB, 1-chloro-2,4-dinitrobenzene; DNFB, 1-fluoro-2,4-dinitrobenzene; 5-HETE, 5(*S*)-hydroxy-6,8,11,14-eicosatetraenoic acid; 5-HEPE, 5(*S*)-hydroperoxy-6,8,11,14-eicosatetraenoic acid; FLAP, 5-lipoxygenase activating protein; 4-VP, 4-vinylpyridine; LT, leukotriene; LTA_4, LTB_4 and LTC_4, leukotriene A_4, B_4 and C_4; NEM, *N*-ethylmaleimide; PC, phospahtidylcholine; PE, phospahtidylethanolamine; PLA_2, phospholipase A_2; PMNL, polymorphonuclear leukocyte; RP-HPLC, reverse-phase high pressure liquid chromatography; TNCB, 1-chloro-2,4,6-trinitrobenzene.

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poxygenase contains a nonheme iron, and several inhibitors reduce the iron from the active ferric form to the inactive ferrous form. *N*-alkylhydroxyamic acids and selenide inhibit 5-lipoxygenase by reducing the ferric iron [10, 11] as well as acting as competitive inhibitors [10]. In contrast, nonredox-based inhibitors of 5-lipoxygenase, based on a thiopyrano[2,3,4-c,d]indole structure, are known to inhibit 5-lipoxygenase by forming a dead-end complex [12]. In addition, certain quinoline and indole-based structures, [5, 13] bind to FLAP, thereby indirectly inhibiting cellular leukotriene synthesis [14–16].

Several thiol-reactive compounds, such as 1-chloro-2,4-dinitrobenzene (DNCB), azodicarboxylic acid bis (dimethylamide) (diamide), and *N*-ethylmaleimide (NEM) have been shown to induce the formation of 5-lipoxygenase products in granulocytes, stimulated only with arachidonic acid [17, 18]. Furthermore, these compounds stimulate the ionophore A23187-induced metabolism of exogenous arachidonic acid to LTB_4 in B lymphocytes [4, 19]. The intracellular mechanism of action of some of these compounds may be mediated by a lowering of the glutathione levels, resulting in higher intracellular levels of fatty acid peroxides [17]. However, another mechanism of action of NEM is to interfere with protein sulfhydryl groups [20].

This report describes the effect of certain thiol-reactive compounds, particularly TNCB, on 5-lipoxygenase activity and cellular leukotriene synthesis in human granulocytes and B lymphocytes.

MATERIALS AND METHODS

Materials

Cell culture medium was purchased from GIBCO and HPLC solvents from Rathburn Chemicals. Arachidonic acid, 5(S)-HETE and LTB_4 were obtained from Biomol. Monoflow™ 2 scintillation liquid was obtained from National Diagnostics. The calcium ionophore A23187 was acquired from Calbiochem. Diamide, NEM, DNCB and 4-vinylpyridine (4-VP) were obtained from Sigma. DNFB, 2,5-dichloro-nitrobenzene and 3,4-dichloronitro-benzene were produced by Aldrich Chem. Co. and TNCB by Tokey Kasei, Tokyo, Japan. [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid (211.8 Ci/mmol), 1-palmitoyl-2-[1-^{14}C]arachidonyl phosphatidylcholine (57 mCi/mmol) and 1-palmitoyl-2-[1-^{14}C]arachidonyl phosphatidylethanolamine (57 mCi/mmol) were purchased from DuPont New England Nuclear.

Cultivation and Isolation of Cells

The B cell line BL-41 E95-A [21] was cultivated in RPMI 1640 supplemented with 10% fetal calf serum, 100 μU/mL penicillin and 100 μg/mL streptomycin and grown in a humidified atmosphere with 5% CO_2 [19]. The cultures were seeded at a cell density of 0.2×10^6 cells/mL and harvested at approximately 1×10^6 cells/mL. The cells were washed twice in Dulbecco's phosphate-buffered saline (PBS) immediately before experiments. Human PMNL were isolated from concentrates of leukocytes, obtained from healthy donors at the Karolinska Hospital, by dextran sedimentation and density gradient centrifugation using Lymphoprep (Nycomed Pharma. Co.) as previously described [3].

Incubation of Cells

Cells (10^7) were suspended in PBS (1 mL) and incubated for 10 min at 37° with or without inhibitor. Thereafter, the cells were stimulated with calcium ionophore A23187 (2.5 μM) in the absence or presence of arachidonic acid (20 μM). The samples were then incubated for 10 min at 37° and the reactions terminated by the addition of 1 mL methanol.

Incubation of Whole Blood

Blood from healthy donors was collected utilizing heparinized vacutainer tubes. Aliquots of 1 mL were incubated at 37° with TNCB for 10 min prior to the addition of A23187 (30 μM) and subsequently incubated for 20 min. The reaction was terminated by the addition of 1 mL PBS supplemented with 5 mM EDTA. Then, the samples were centrifuged immediately at 650 *g* for 10 min and plasma was collected, mixed with 1 mL methanol, and centrifuged at 650 *g* for 10 min to remove precipitated proteins. The resulting supernatant was analysed for eicosanoid content.

Radiolabelling and Assessment of Arachidonic Acid Release

PMNL (2×10^6/mL) were suspended in RPMI 1640 containing 0.2 μCi ^{3}H-arachidonic acid/mL and incubated for 90 min at 37°. Then, the cell suspension was centrifuged at 150 *g* for 10 min and washed twice with PBS to remove unincorporated arachidonic acid. Labelled cells (10^7) were resuspended in 1mL PBS containing 2 mg/mL fatty acid-free albumin. The samples were incubated with or without TNCB for 10 min at 37° prior to the addition of 2.5 μM A23187. Then, the cell suspensions were incubated for another period of 5 min. The reaction was terminated with 1 volume of methanol, containing 0.5% acetic acid and 40 μM stearic acid.

Lipoxygenase Activity Assay of Recombinant 5-Lipoxygenase

Recombinant human 5-lipoxygenase was expressed in *E. coli* and purified as described previously [22]. The enzyme was preincubated with TNCB, at various concentrations, for 10 min at 20° in 60 μL of a buffer comprised of 50 mM Tris-HCl (pH 7.5) and 1 mM EDTA. The enzyme reaction was initiated by the addition of 40 μL of the substrate cocktail containing 100 μM arachidonic acid, 10 μM 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid (13-HPOD), 1.9 mM $CaCl_2$, 0.025 mg/mL phosphatidylcho-

line, 5 mM adenosine 5′-triphosphate (ATP), 1.2 mM EDTA, and 78 mM Tris-HCl pH 7.5. The 5-lipoxygenase protein concentration in the assay mixture was approximately 1 μg/mL. After incubation at 20° for 5 min, the reactions were terminated by additions of 300 μL of MeCN/H_2O/HOAc (2:1:0.008) with 1.1 nmol of 17(*S*)-hydroxy-7,10,13 (*Z*),15(*E*)-docosatetraenoioc acid (17-OH-22:4) as an internal standard. Protein precipitate was removed by centrifugation. An aliquot of the supernatant was injected into a Nova-Pac C18 reverse-phase HPLC column column (Waters Associated) and eluted with MeCN/H_2O/HOAc (11:9:0.04) at 1.2 mL/min. The amounts of 5(*S*)-hydroxy- and 5(*S*)-hydroperoxy-6(*E*),8,11,14(*Z*)-eicosatetraenoic acid (5-HETE and 5-HPETE, respectively) were quantified by the UV absorbance of 234 nm relative to the internal standard.

Analysis of Soybean 15-Lipoxygenase and Platelet 12-Lipoxygenase Activities

Soybean 15-lipoxygenase (EC 1.13.11.12) (Fluka AG) was dissolved in 0.1 M sodium borate buffer, pH 8.5, at 4 μg/mL. TNCB was added to the enzyme solution for 5 min at 20° prior to the addition of 150 μM arachidonic acid. The incubations were allowed to proceed for 5 min before termination by the addition of 1 mL methanol. For analysis of 12-lipoxygenase (EC 1.13.11.31) activity, human washed platelets were suspended in Ca^{2+}-free PBS (1 mL) supplemented with 1 mM EDTA and sonicated three times for five sec on ice. TNCB was added prior to the addition of ATP (1 mM), $CaCl_2$ (2 mM) and arachidonic acid (20 μM). The samples were incubated for 10 min at 37°. Subsequently, the reactions were terminated by addition of 1 mL methanol.

PLA_2 Assay

PMNL (4×10^7) were resuspended in a buffer containing 20 mM Tris-HCl (pH 7.5), 2 mM EGTA, 1 mM EDTA, 1 mM phenylmethylsulfonyl flouride (PMSF), 20 μg/mL soybean trypsin inhibitor, 0.1 mg/mL bacitracin, 0.5 mM benzamidin, 0.02 mM leupeptin and 10% glycerol and homogenized twice by sonication for 5 sec at 4°. After centrifugation at 100,000 *g* for 60 min, the supernatant was collected and PLA_2 activity was assayed with a 1:1 mixture of 1-palmitoyl-2-[1-^{14}C]arachidonyl phosphatidylcholine and 1-palmitoyl-2-[1-^{14}C]arachidonyl phosphatidylethanolamine as standard substrates. The phospholipids were dried under nitrogen and resuspended in assay buffer [80 mM glycine (pH 9.0), 5 mM $CaCl_2$, 1 mg/mL albumin in 10% glycerol] to yield a final concentration of 2 μM phosphatidylcholine:phosphatidylethanolamine (PC:PE) 1:1. This preparation was sonicated in a water bath for 10 min at 4°. The reaction was initiated by adding 100 μL supernatant and allowed to proceed for 30 min at 37° in a shaking water bath. The reaction was terminated with 2 volumes of methanol containing 0.5% acetic acid and 40 μM stearic acid followed by vigorous vortexing.

HPLC Analysis of Leukotrienes, Monohydroxy Acids and Arachidonic Acid

Prostaglandin B_1 (171 pmol) was added to samples, as internal standard, prior to centrifugation at 650 *g* for 10 min to remove precipitated proteins and cell fragments. The resulting supernatants were applied to disposable octadecyl reverse-phase columns (Chromabond, C18, 100 mg, Macherey–Nagel) for solid phase extraction of lipids. Extracts were analysed in a reverse-phase (RP) HPLC system equipped with a Radial-Pak cartridge (5 × 100 mM) packed with 4 μM Novapak C18 material, guarded by a Novapak C18 column (Waters Associated). The mobile phase was methanol:water:trifluoroacetic acid 70:30:0.007 (by vol) for leukotrienes and monohydroxy acids and 85:15:0.007 (by vol) for arachidonic acid. The flow rate was 1.2 mL/min and ultraviolet monotoring was performed at 270 nM and 235 nM for leukotrienes and monohydroxy acids, respectively. Qualitative analysis was performed by comparison with retention times of synthetic standards. Quantitative determination was performed by peak area integration. Radioactivity was detected with a β-RAM HPLC flow-through monitoring system (Inus System Inc.) coupled on-line to a UV spectrophotometer.

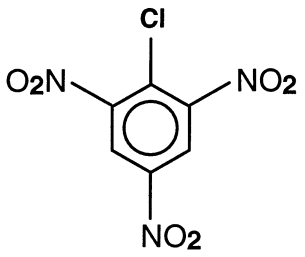
Cell Viability, Protein Determination and Statistical Analysis

Cellular viability was >90% as examined by trypan blue dye exclusion. Protein content was measured with a protein assay kit (Bio-Rad) according to Bradford's method with bovine serum albumin as the standard protein [23]. Statistical analyses were performed with Student's paired *t*-test.

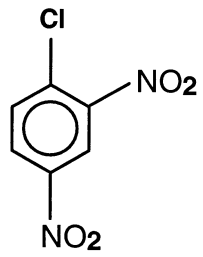
RESULTS

Effects of Thiol-Reactive Compounds on Cellular Leukotriene Synthesis

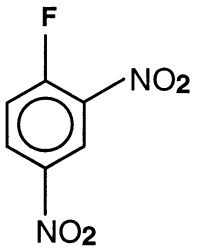
The effect of various nitro-substituted benzene derivatives (Fig. 1) on cellular leukotriene synthesis in human PMNL was investigated. The cells were stimulated with calcium ionophore A23187 and arachidonic acid in the presence of the drugs (100 μM, Table 1). All the nitro-substituted benzene compounds inhibited the synthesis of LTB_4, 6-trans-LTB_4 and 6-trans-12-epi-LTB_4 and 5-HETE, with TNCB as the most potent inhibitor. For comparison, the effects of the structurally related 4-vinylpyridine (4-VP) as well as diamide and NEM on leukotriene formation were also examined. In contrast to the nitro-substituted benzene compounds, these agents stimulated the formation of LTB_4 induced by ionophore A23187 and arachidonic acid (Table 1). Figure 2 shows the dose-response curve of the effect of TNCB on leukotriene synthesis in PMNL. The cells were preincubated with various amounts of TNCB for 10 min



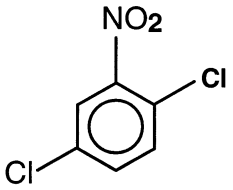
1-chloro-2,4,6-trinitrobenzene (TNCB)



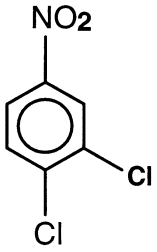
1-chloro-2,4-dinitrobenzene (DNCB)



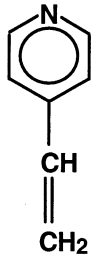
1-fluoro-2,4-dinitrobenzene (DNFB)



1-nitro-2,5-dichlorobenzene (2,5-DCNB)



1-nitro-3,4-dichlorobenzene (3,4-DCNB)



4-vinylpyridin (4-VP)

FIG. 1. Structure of certain thiol-reactive compounds.

and thereafter stimulated with 2.5 μM A23187 for another period of 10 min. TNCB exhibited a biphasic inhibitory effect on the formation of LTB_4. At a concentration of 0.5 μM and 1.0 μM TNCB, the levels of LTB_4 were 52.8 ± 6.6% and 45.0 ± 10.2% of control, respectively (mean value ± SD, $P < 0.001$ vs. control, $n = 5$). However, at a concentration of 10 μM TNCB, the level of LTB_4 was higher (73.6 ± 8.6% of control; mean value ± SD, $P < 0.001$ vs. 1.0 μM TNCB, $n = 5$) compared to the amount produced in the presence of 1.0 μM TNCB. At higher concentrations of TNCB (>25 μM), the formation of LTB_4 was abrogated (Fig. 2). A similar pattern was observed for the effect of TNCB on the formation of 5-HETE and the nonenzymatic derivates of LTA_4: 6-trans-LTB_4 and 6-trans-12-epi-LTB_4 (data not shown). TNCB also inhibited the formation of LTB_4 and 5-HETE in the monoclonal B cell line BL41-E95A after stimulation with 100 μM NEM plus 2.5 μM A23187 and 20 μM arachidonic acid (data not shown).

The cytosolic 85 kDa PLA_2 has been demonstrated to be susceptible to thiol-active compounds [24, 25]. Therefore, we raised the question as to whether the inhibitory effect of TNCB on leukotriene synthesis in PMNL could be due to inhibition of the release of arachidonic acid and thus overcome with exogenous arachidonic acid. In fact, when PMNL were stimulated with 2.5 μM A23187 in the presence of 20 μM exogenous arachidonic acid, no inhibition on leukotriene synthesis was observed with 1 μM TNCB (Fig. 3). Furthermore, a tendency to increased level of LTB_4 was observed at 5 and 10 μM TNCB, in compar-

TABLE 1. Effects of thiol-reactive compounds on the formation of 5-lipoxygenase metabolites in PMNL

Compound (100 μM)	LTB_4 (% activity)	6-*trans*-LTB_4 + 6-*trans*-12-epi-LTB_4 (% activity)	5-HETE (% activity)
EtOH	100 ± 6.8	100 ± 4.5	100 ± 8.8
DNCB	78.2 ± 1.9	30.8 ± 1.6	31.6 ± 20.2
TNCB	0 ± 0	0 ± 0	0.37 ± 0.4
DNFB	21.1 ± 4.9	3.1 ± 0.9	6.0 ± 3.5
2,5-DCNB	52.4 ± 10.0	20.8 ± 1.5	48.0 ± 28.2
3,4-DCNB	73.5 ± 10.8	34.6 ± 1.1	51.3 ± 30.3
4-VP	146.3 ± 14.3	147.3 ± 6.3	79.3 ± 44.4
NEM	198.5 ± 12.2	53.6 ± 12.4	36.4 ± 33.6
Diamide	133.3 ± 12.9	108.6 ± 8.5	88.9 ± 50.8

PMNL were incubated with the indicated compound (100 μM) for 10 min and subsequently stimulated with 2.5 μM A23187 and 20 μM arachidonic acid for another 10 min. The results are expressed as percentages of LTB_4, 6-*trans*-LTB_4 and 6-*trans*-12-epi-LTB_4 and 5-HETE levels, respectively, in vehicle (ethanol)-treated control samples. Data show the mean ± SD. (n = 4). In control incubations, the amounts of LTB_4, 6-*trans*-LTB_4 plus 6-*trans*-12-epi-LTB_4, and 5-HETE were 46.8 ± 5.0, 115.0 ± 14.4 and 280.2 ± 32.7 pmol/10^6 cells, respectively.

ison with the amount produced in the presence of 1 μM TNCB (Fig. 3). The inhibitory effect of higher doses of TNCB on leukotriene synthesis was not affected by the addition of arachidonic acid.

To investigate if the inhibition of LTB_4 synthesis by low-dose TNCB was due to inhibition of phospholipase activity, the effect of TNCB on the release of endogenous arachidonic acid from PMNL was investigated. The cells were labelled with ^{3}H-arachidonic acid for 90 min prior to the addition of TNCB for 10 min and thereafter stimulated with 2.5 μM A23187 for 5 min. The release of ^{3}H-labelled arachidonic acid was analysed by RP-HPLC with on-line radioactivity determination. The release of arachidonic acid in non-stimulated cells was below the detection limit (Fig. 4a). Cells activated with 2.5 μM A23187 released

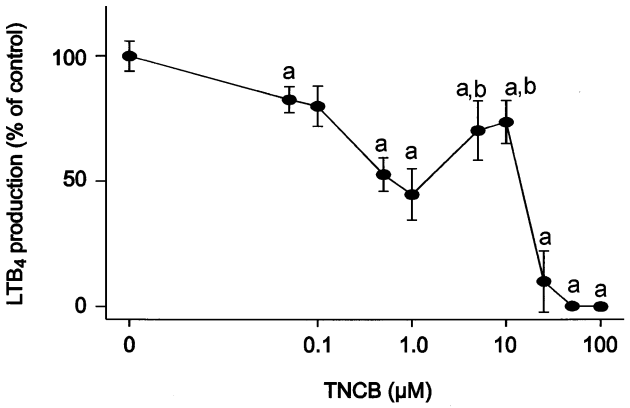


FIG. 2. Effects of TNCB on A23187-induced LTB_4 formation in PMNL. PMNL (10^7) were incubated with TNCB for 10 min at 37° and subsequently stimulated with 2.5 μM A23187 for 10 min. The results are expressed as percentage of LTB_4 formation in vehicle (ethanol)-treated control samples. Data show the mean ± SD, n = 5 (a: $P < 0.001$ vs. control and b: $P < 0.001$ vs. 1 μM TNCB). The amount of LTB_4 in control incubations was 35.7 ± 9.3 pmol/10^6 cells.

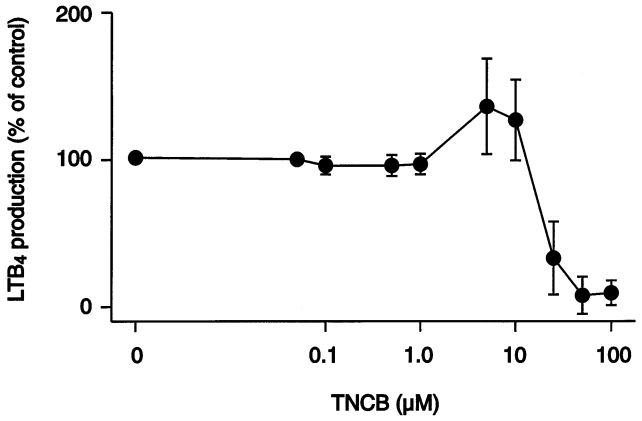


FIG. 3. Effects of TNCB on A23187-induced LTB_4 formation in PMNL in the presence of exogenous arachidonic acid. PMNL (10^7) were incubated with TNCB for 10 min and subsequently stimulated with 2.5 μM A23187 and 20 μM arachidonic acid for 10 min. The results are expressed as percentage of LTB_4 formation in vehicle (ethanol)-treated control samples. Data show the mean ± SD, n = 4. The amount of LTB_4 in control samples was 46.8 ± 5.0 pmol/10^6 cells.

radiolabelled LTB_4 (1561 cpm), LTC_4 (1139 cpm), 5-HETE (2465 cpm) and arachidonic acid (1252 cpm) (Fig. 4b). In addition, radioactivity was found in the front peak, probably due to formation of ω-oxidized metabolites of LTB_4. In the presence of 0.5 μM TNCB, the amounts of ^{3}H-labelled 5-lipoxygenase products decreased, while the amount of free ^{3}H-arachidonic acid increased (Fig. 4c). However, incubation with 5.0 μM TNCB led to increased amounts of ^{3}H-labelled 5-lipoxygenase products and decreased amounts of arachidonic acid in comparison to the amounts released in the presence of 0.5 μM TNCB (Figs. 4c and d). These results are in agreement with the dose-response curve of the effect of TNCB on leukotriene formation in unlabelled PMNL (Fig. 2).

Since it has previously been reported that DNCB can stimulate the formation of 5-HETE and LTB_4 in human PMNL after stimulation with arachidonic acid alone [17], we investigated whether TNCB could also stimulate LTB_4 formation under these experimental conditions. Accordingly, PMNL were incubated with TNCB (Fig. 5) and then stimulated with 20 μM arachidonic acid. PMNL incubated with only arachidonic acid produced 5.5 ± 2.2 pmol LTB_4/10^6 cells. Interestingly, arachidonic acid-inducible formation of LTB_4 increased in the presence of 5–10 μM TNCB (Fig. 5). LTB_4 formation increased 3.8-fold at 10 μM, but no stimulation was observed at higher TNCB concentrations. These results are also consistent with the biphasic curve obtained with TNCB on leukotriene synthesis (Fig. 2).

Effects of TNCB on Recombinant 5-Lipoxygenase

Incubation of purified recombinant 5-lipoxygenase with 100 μM arachidonic acid in the presence of various concentrations of TNCB demonstrated that this compound

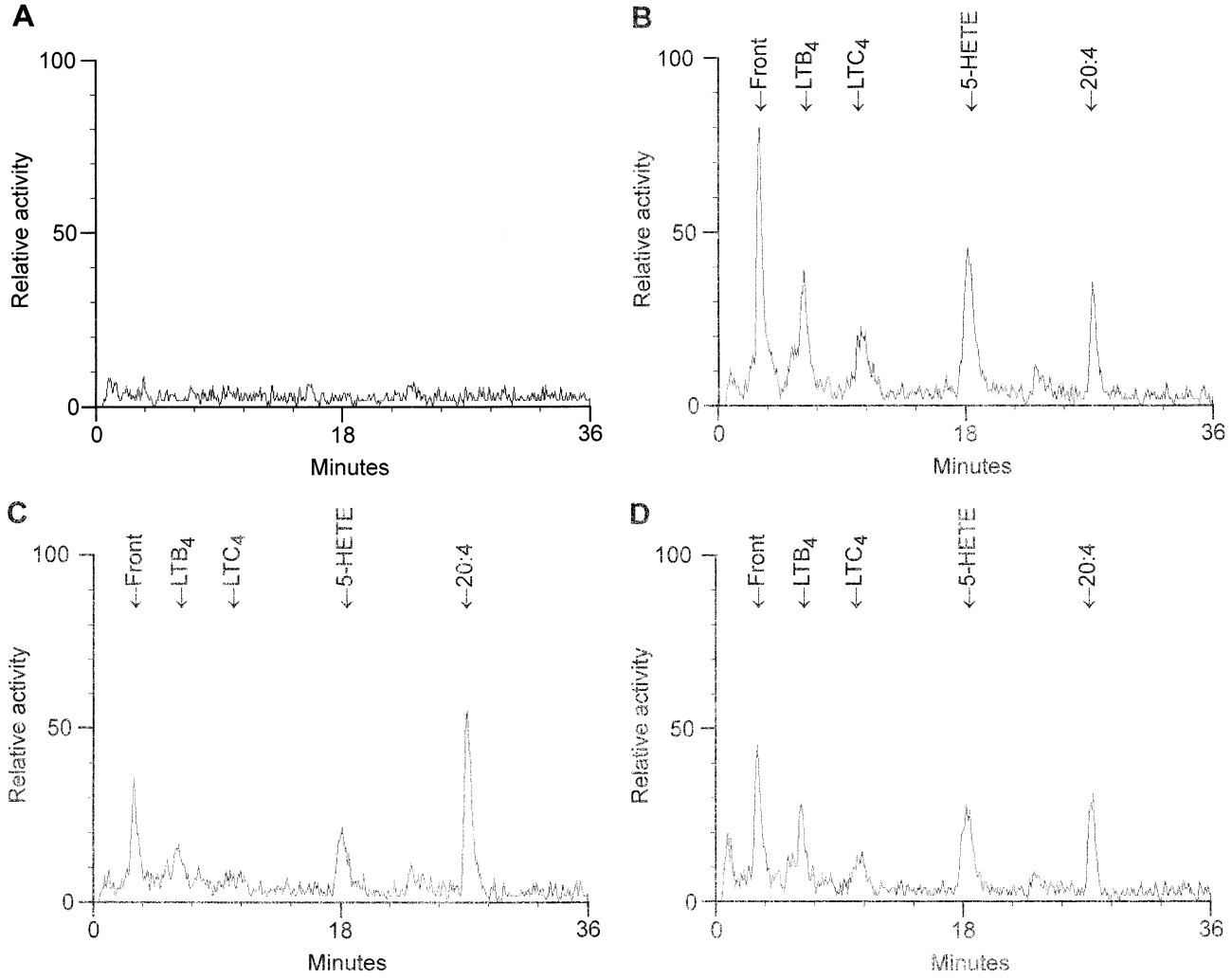


FIG. 4. Effects of TNCB on the release of ^{3}H-labelled arachidonic acid and ^{3}H-labelled 5-lipoxygenase metabolites by PMNL after A23187 stimulation. PMNL (10^7) were treated with ^{3}H-labelled arachidonic acid and subsequently incubated with or without TNCB (0.5 or 5.0 μM) for 10 min and thereafter stimulated with 2.5 μM A23187 for 5 min in the presence of albumin (1 mg/mL). The chromatograms show elusion of radioactivity eluting from the reverse-phase column. Compounds eluting from the reverse-phase column in a 70:30:0.007 to 85:15:0.007 (methanol:H_2O:trifluoroacetic acid) gradient. Arrows indicate the retention times of synthetic standards. A) Nonactivated PMNL; B) PMNL stimulated with ionophore A23187; C) PMNL preincubated with 0.5 μM TNCB for 10 min and subsequently stimulated with ionophore A23187; D) PMNL preincubated with TNCB (5 μM) for 10 min and subsequently stimulated with A23187. Data shown are from one representative experiment out of three.

dose-dependently inhibited 5-lipoxygenase activity (Fig. 6). This inhibition could be partially counteracted by reduced glutathione (10 mM). Thus, 100 μM TNCB provoked almost 100% and 18.8 ± 1.4% inhibition of LTB_4 synthesis in the absence and presence of glutathione, respectively. Similar results were also obtained with β-mercaptoethanol (data not shown).

Effects of TNCB on A23187-Induced LTB_4 Formation in Human Whole Blood

Incubation of human whole blood with 30 μM A23187 for 20 min led to the formation of 94.1 ± 3.6 pmol LTB_4/mL blood (Fig. 7). TNCB concentration-dependently inhibited A23187-induced formation of LTB_4 in human whole blood. At a concentration of 5.0 μM TNCB, the LTB_4 formation was reduced to 54.9 ± 6.8% (mean value ± range, $n = 2$) of that found in control incubations.

Effects of TNCB on 12-Lipoxygenase, 15-Lipoxygenase and Phospholipase A_2 Activities

To investigate the specificity of the inhibitory action of TNCB on 5-lipoxygenase activity, the effects of TNCB on soybean 15-lipoxygenase, human platelet 12-lipoxygenase and human PLA_2 activities were investigated. 12-Lipoxygenase activity was measured in human platelet homogenate, 15-lipoxygenase activity in solutions of soybean lipoxygenase, and phospholipase A_2 activity in the 100,000 *g* supernatant of homogenates of PMNL. TNCB inhibited 12-lipoxygenase, 15-lipoxygenase and PLA_2 activities only slightly and at 100 μM, the activities were 70.7 ± 10.7,

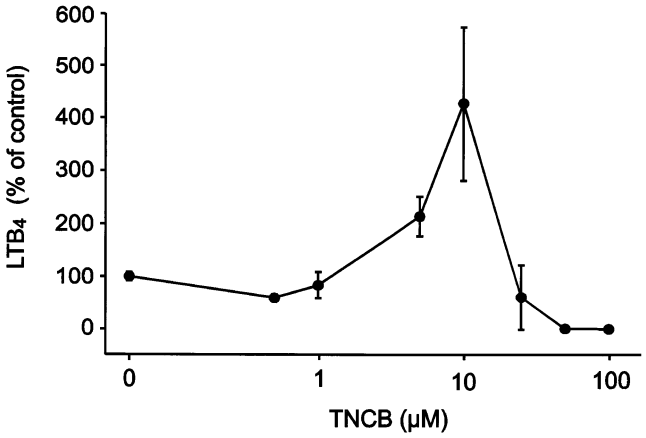


FIG. 5. Effects of TNCB on arachidonic acid-induced LTB_4 formation in PMNL. PMNL (10^7) were incubated with TNCB for 10 min and subsequently stimulated with 20 μM arachidonic acid for 10 min. The results are expressed as percentage of LTB_4 formation in vehicle (ethanol)-treated control samples. Data show the mean values ± range of two separate experiments performed in duplicate. The amount of LTB_4 in control samples was 5.5 ± 2.2 pmol/10^6 cells.

85.9 ± 16.0 and 75.0 ± 11.2% (mean ± range from two separate experiments performed in duplicate), respectively, of ethanol-treated control samples. Furthermore, the LTA_4 hydrolase (EC 3.3.2.6) activity was unaffected by 100 μM TNCB, as measured as the conversion of LTA_4 (3.8 μM) to LTB_4 in homogenates of PMNL (data not shown).

DISCUSSION

Previous studies have shown that thiol-active compounds stimulate leukotriene synthesis in human leukocytes [4, 17, 19]. In this study, we have further characterised the effects of several thiol-active derivatives on the formation of leukotrienes in PMNL and B lymphocytes. A number of different nitro-substituted benzene compounds were found to inhibit the formation of leukotrienes in PMNL (Table 1). In this respect, TNCB was the most potent inhibitor of leukotriene formation. In contrast to TNCB, 4-VP, NEM and diamide increased the formation of LTB_4 (Table 1). NEM possibly stimulated LTA_4 hydrolase as indicated by decreased formation of the nonenzymatic products of LTA_4 and 5-HETE (Table 1).

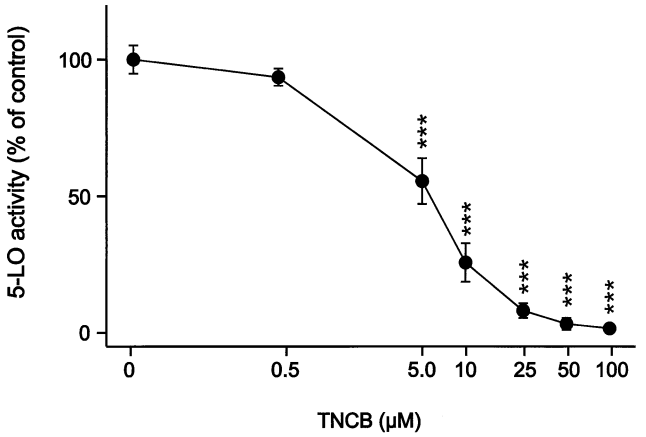


FIG. 6. Effects of TNCB on recombinant 5-lipoxygenase activity. Purified recombinant human 5-lipoxygenase was analysed for activity in the presence of TNCB. The formation of 5-HPETE and 5-HETE was measured by reverse-phase HPLC. The results are expressed as percentage of 5-HPETE plus 5-HETE formation in vehicle (ethanol)-treated control samples. The specific activity in control samples was 11.9 ± 10.5 μmol 5-HPETE and 5-HETE/mg protein. Data show the mean values ± SD, n = 3. ***$P < 0.001$.

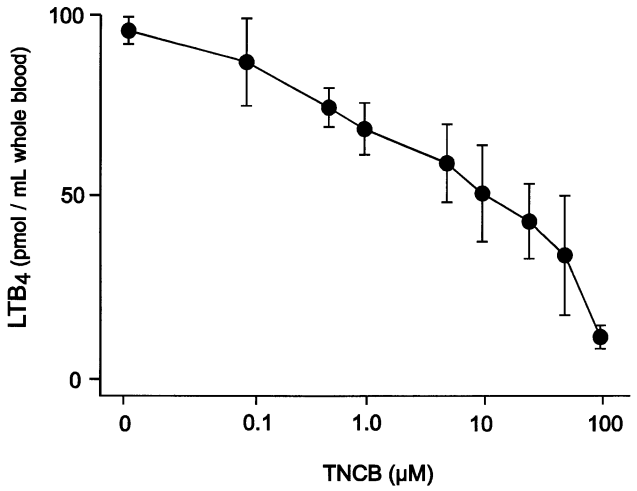


FIG. 7. Effects of TNCB on LTB_4 formation in whole blood. Venous blood was collected in heparinized vacutainer tubes. Aliqouts (1 mL) were incubated for 10 min with TNCB at indicated concentrations and subsequently stimulated with 30 μM A23187 for an additional 20 min. After dilution with buffer and centrifugation at 650 g, the resulting plasma was analysed for LTB_4 content. To allow separation of LTB_4 and 5S, 12S-diHETE on HPLC, LTB_4 content was analysed on a Nova-Pac C18 column eluted with acetonitrile:methanol:water: acetic acid (vol/vol, 30:25:45:0.5). The results are expressed as percentage of LTB_4 level in vehicle (ethanol)-treated control samples. The amount of LTB_4 in control samples was 94.1 ± 2.1 pmol/mL whole blood. Data show the mean values ± range from two separate experiments performed in duplicate.

TNCB both inhibited and stimulated leukotriene synthesis in PMNL (Figs. 2, 3, and 5). The inhibition of leukotriene synthesis by low concentrations of TNCB could be overcome by exogenous arachidonic acid. There are several possible explanations for this observation. First, the inhibition of leukotriene formation could be due to a suppression of arachidonic acid release (Figs. 2 and 3), possibly due to an effect of TNCB on cellular phospholipases. Cytosolic phospholipase A_2 has been demonstrated to be susceptible to thiol-reactive agents such as iodoacetamide [24, 25], and it was demonstrated that Cys-331 of $cPLA_2$ is involved in this reaction [20, 25]. However, there was no inhibitory effect of TNCB (0.5 μM) on the release of arachidonic acid from PMNL (Fig. 4). Furthermore, TNCB did not inhibit phospholipase A_2 activity in homogenates of PMNL. TNCB might also disturb the interaction between FLAP and 5-lipoxygenase, another possible mech-

anism of action of TNCB. The observation that arachidonic acid circumvented the effect of low doses of TNCB supports this possibility, since it was previously shown that exogenous arachidonic acid can counteract the inhibitory effect of the FLAP inhibitor MK-886 [26, 27]. Nonactivated PMNL produce very low levels of leukotrienes after incubation with exogenous arachidonic acid alone. However, this synthesis was markedly stimulated by the addition of 10 μM TNCB (Fig. 5). The mechanism of action of TNCB in this respect is not known. One explanation might be that TNCB causes an increase in intracellular Ca^{2+}. However, it has been demonstrated that DNCB has no effect on intracellular Ca^{2+} concentration [17], and it is therefore unlikely that TNCB should have any effects on intracellular Ca^{2+} levels. Changes in the intracellular redox potential might be an alternative explanation to the effects of TNCB on whole cell leukotriene synthesis. Intracellular redox changes might, directly or indirectly, lead to changes in enzymatic activity. Tyrosin phosphorylation or an increased amount of disulfide-linked proteins affects enzyme activity, and both are known to be affected by the intracellular redox potential [28, 29]. Similar mechanisms might account for the observed effects of TNCB on PMNL and leukotriene synthesis. In any event, this result indicates that TNCB possesses both an inhibitory and stimulatory effect on leukotriene synthesis in PMNL. The stimulatory properties of TNCB can counteract the inhibitory effect of TNCB on leukotriene synthesis at 10 μM. These results might explain the observation that 10 μM TNCB, in contrast to 1 μM TNCB, only slightly inhibited ionophore A23187-induced LTB_4 synthesis (Fig. 2).

TNCB also inhibited the activity of recombinant 5-lipoxygenase (Fig. 6). The inhibitory effect of TNCB could be prevented by the addition of β-mercaptoethanol or reduced glutathione to the assay buffer, indicating that TNCB inhibition might be mediated through interaction with thiol groups. The three nitro groups in TNCB might act as electron-scavenging residues, thus rendering the benzene ring vulnerable to nucleophilic attack. The extra nitro group in TNCB relative to DNCB renders this compound a better substrate for attacking nucleophiles, thus promoting nucleophile aromatic substitution reactions. The reactive propensity is directly related to the electrophilic nature of carbon 1 of the benzene ring. The most likely nucleophilic protein groups are NH_2, OH and SH residues of lysine, serine and cysteine, respectively [30]. It is possible that TNCB alkylates a nucleophilic group in 5-lipoxygenase and thereby abrogates the activity of the enzyme. It was previously found that ebselen inhibits mammalian 5- and 15-lipoxygenases, and it was suggested that this was due to a reaction with a critical SH group [31]. TNCB displayed selectivity for 5-lipoxygenase with only modest inhibitions of platelet 12-lipoxygenase, soybean 15-lipoxygenase, and PMNL PLA_2 at 100 μM. This relative specificity might enhance the applicability of TNCB to further investigations of leukotriene biosynthesis.

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