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Inhibition by troglitazone of the antigen-induced production of leukotrienes in immunoglobulin E-sensitized RBL-2H3 cells

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1 The effect of troglitazone, an anti-diabetic drug with insulin-sensitizing action, on antigen-induced production of leukotriene (LT) B_4, C_4 and E_4 and prostaglandin D_2 (PGD_2) was examined in dinitrophenol (DNP)-specific immunoglobulin E (IgE)-sensitized RBL-2H3 mast cells following stimulation by the antigen, DNP-conjugated human serum albumin. Levels of LTB_4, C_4 and E_4 and PGD_2 in the conditioned medium were enzyme-immunoassayed.

2 Troglitazone inhibited the antigen-induced production of LTB_4, C_4 and E_4 and the potency of the inhibition was comparable to that of zileuton, a specific inhibitor of 5-lipoxygenase (5-LOX) and a clinically used anti-asthmatic drug. Neither troglitazone nor zileuton affected antigen-induced production of PGD_2, arachidonic acid release from membrane phospholipids and degranulation.

3 Troglitazone inhibited LTB_4 production by the supernatant fraction of RBL-2H3 cell lysate with similar potency to zileuton, suggesting that troglitazone inhibits LT production by direct inhibition of 5-LOX activity.

4 Furthermore, it was shown that troglitazone as well as zileuton inhibited LTB_4 production in A23187-stimulated rat peritoneal neutrophils.

5 These findings suggest that troglitazone inhibits antigen-induced LT production in the IgE-sensitized RBL-2H3 cells and A23187-stimulated rat peritoneal neutrophils by direct inhibition of 5-LOX activity.

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Abbreviations: BSA, bovine serum albumin; DNP, dinitrophenol; DNP-HSA, DNP-conjugated human serum albumin; EIA, enzyme-immunoassay; EMEM, Eagle's minimum essential medium; FBS, foetal bovine serum; FLAP, 5-lipoxygenase activating protein; IgE, immunoglobulin E; LOX, lipoxygenase; LT, leukotriene; PG, prostaglandin; PIPES, piperazine-N,N′-bis(2-ethanesulphonic acid); PPAR, peroxisome proliferator-activated receptor

Introduction

Troglitazone ((±)-5-[4-(6-hydroxy-2,5,7,8-tetramethylchroman-2-ylmethoxy)benzyl]-2,4-thiazolidinedione) is an anti-diabetic reagent which is capable of improving insulin resistance in diabetic patients (Suter *et al*., 1992; Nolan *et al*., 1994). The chemical structure of troglitazone is composed of two components, a thiazolidinedione ring and a chroman ring. Thiazolidinediones that contain the thiazolidine ring have recently been demonstrated to be ligands of peroxisome proliferator-activated receptor (PPAR)-γ, one of the isoforms of a group of transcription factors that regulates gene expression of enzymes associated with lipid homeostasis (Lehmann *et al*., 1995; Willson *et al*., 1996). PPAR-γ ligands have recently been demonstrated to inhibit production of inflammatory cytokines in human monocytes (Jiang *et al*., 1998) and activation of mouse macrophages (Ricote *et al*., 1998). On the other hand, a chroman ring is a component of vitamin E that shows anti-oxidant activity. Vitamin E also inhibits 5-lipoxygenase (5-LOX) activity, with the IC_{50} of 5 μM (Reddanna *et al*., 1985).

RBL-2H3 cells, a cell line from rat basophilic leukaemia cells having mucosal mast cell characteristics, produce leukotriene (LT) C_4 and prostaglandin (PG) D_2 by antigen stimulation after sensitization with immunoglobulin E (IgE) (Lin *et al*., 1991; Westcott *et al*., 1996; Hamasaki *et al*., 1999). Using IgE-sensitized RBL-2H3 cells, the effect of troglitazone on antigen-induced production of LTB_4, C_4 and E_4 and PGD_2 were examined and compared with those of zileuton, an inhibitor of 5-LOX and clinically used for the treatment of asthma (Dahlen *et al*., 1998). The effects of troglitazone and zileuton on antigen-induced production of PGD_2 and degranulation in IgE-sensitized RBL-2H3 cells were also examined. Furthermore, to analyse the mechanism of action of troglitazone, the effect of troglitazone on LTB_4 production in a cell-free system of RBL-2H3 cells and on A23187-induced LTB_4 production in rat peritoneal neutrophils were also examined.

Methods

RBL-2H3 cell culture and treatment with drugs

RBL-2H3 cells (American Type Culture Collection, Manassas, VA, U.S.A.) were suspended at a concentration of 5×10^5 cells ml^{-1} in Eagle's minimum essential medium (EMEM, Nissui

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Seiyaku, Tokyo, Japan) containing 10% (v v^{-1}) foetal bovine serum (FBS, Dainippon Pharmaceutical Co., Osaka, Japan), penicillin G potassium (18 μg ml^{-1}), streptomycin sulfate (50 μg ml^{-1}) (Meiji Seika, Tokyo, Japan) and 0.1% (v v^{-1}) conditioned medium of dinitrophenol (DNP)-specific IgE-producing hybridoma (kindly supplied from Dr Kazutaka Maeyama at Ehime University, Ehime, Japan) and 1.0 ml of the cell suspension was poured into each well of a 12-well cluster dish (Costar, Cambridge, MA, U.S.A.).

To examine the effects of drugs, the cells were preincubated with troglitazone (Sankyo Co., Tokyo, Japan) or zileuton (kindly supplied from Abbott Laboratories, Abbott Park, IL, U.S.A.) for 2 h before the antigen challenge. The cells were then washed and incubated for the periods indicated in piperazine-N,N′-bis(2-ethanesulphonic acid) (PIPES) buffer (in mM: PIPES 6.25, NaCl 30, KCl 1.25, glucose 5.6, $MgCl_2$ 1.4, NaOH 40, and 0.5 g ml^{-1} bovine serum albumin (BSA), pH 7.2) containing DNP-conjugated human serum albumin (DNP-HSA, Sigma Chemical Co., St. Louis, MI, U.S.A.) (Hirasawa *et al*., 1995) and with or without various concentrations of troglitazone or zileuton. Drugs were dissolved in dimethylsulphoxide and added to the medium. Final concentration of the vehicle was adjusted to 0.001% (v v^{-1}).

After treatment with drugs, the viability of the cells were examined by the ability of mitochondrial succinate dehydrogenase to cleave 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide to the blue compound formazan (Kobayashi *et al*., 1994); no cytotoxic effect was observed (data not shown).

Determination of levels of LTB_4, C_4 and E_4 and PGD_2 in the conditioned medium

After incubation, the conditioned medium was collected, centrifuged at $1500 \times g$ and 4°C for 5 min, and the supernatant was used for the determination of LTB_4, C_4 and E_4 and PGD_2. Levels of LTB_4, C_4 and E_4 and PGD_2 in the conditioned medium were determined using each enzyme-immunoassay (EIA) kit (Cayman Chemical, Ann Arbor, MI, U.S.A.) (Pradelles *et al*., 1990). Accuracy tests and linearity tests using each EIA kit revealed that levels of each arachidonate metabolite in the conditioned medium could be determined without prior extraction and purification. The assay limit for each arachidonate metabolite is as follows; 5.5 pg ml^{-1} for LTB_4, 8.0 pg ml^{-1} for LTC_4, 5.6 pg ml^{-1} for LTE_4, and 7.0 pg ml^{-1} for PGD_2. The cross-reactivity of each antibody to other arachidonate metabolites was less than 0.001%. Troglitazone or zileuton at the concentrations examined in the experiments did not affect the determination of levels of each arachidonate metabolite.

Determination of the release of radioactivity from [^{3}H]-arachidonic acid-labelled RBL-2H3 cells

RBL-2H3 cells (2.5×10^5 cells) were incubated for 18 h at 37°C in 0.5 ml medium containing DNP-specific IgE and [^{3}H]-arachidonic acid (2.7 kBq, specific radioactivity 2.5 TBq $mmol^{-1}$, NEN Life Science Products, Boston, MA, U.S.A.). Then, 50 μl of medium containing drugs was added to each well, and further incubated for 2 h at 37°C. Cells were then washed and incubated for 15 min at 37°C in 0.5 ml of PIPES buffer containing DNP-HSA (50 ng ml^{-1}) and the corresponding concentrations of drugs. Before the antigen challenge, the cells in each well of one group were lysed with 0.5 ml of 0.1% (v v^{-1}) solution of Triton X-100 in EMEM and total radioactivity incorporated in the cells was determined. Released radioactivity in the conditioned medium was determined and expressed as per cent of total radioactivity incorporated in the cells.

Determination of the degranulation of RBL-2H3 cells

Degranulation of RBL-2H3 cells was determined as hexosaminidase release into the conditioned medium (Hirasawa *et al*., 1995). Ten microliters of 1 mM of *p*-nitrophenyl N-acetyl β-D-glucosamide (Sigma Chemical Co.) solution in 0.1 M citrate buffer (0.1 M sodium citrate, 0.1 M citric acid, pH 4.5) was added to 10 μl of the conditioned medium and incubated at 37°C for 1 h. Then, 250 μl of 0.1 M sodium carbonate buffer (0.1 M $NaHCO_3$, 0.1 M Na_2CO_3, pH 10.0) was added to stop the reaction and the absorbance at 405 nm was measured. Before the antigen challenge, the cells in each well of one group were lysed with 1.0 ml of 0.1% (v v^{-1}) solution of Triton X-100 in water, and total amount of hexosaminidase in the cells before the antigen challenge was determined. Degranulation of the cells was expressed as follows; degranulation (%) = (total amount of hexosaminidase in the conditioned medium after the antigen challenge) × (total amount of hexosaminidase in the cells before the antigen challenge)$^{-1}$ × 100.

Determination of LTB_4 production by 20,000 × g supernatant of RBL-2H3 cells

RBL-2H3 cells (2.5×10^7 cells) were harvested by trypsinization, suspended in 500 μl of buffer I (in mM: BES 10, PIPES 10 and EDTA 1, pH 6.8) and lysed by sonication (Carter *et al*., 1991). The lysate was centrifuged at $20{,}000 \times g$ and 4°C for 20 min. A 12.5 μl aliquot of the supernatant fraction (6.9 μg protein) was preincubated for 2 h at 37°C with 12.5 μl of buffer I containing troglitazone or zileuton. Then, 50 μl of assay buffer (in mM: BES 10, PIPES 10, EDTA 1, $CaCl_2$ 0.7, and NaCl 100, pH 6.8) containing troglitazone or zileuton was added and reactions were initiated by addition of 3 μl of aqueous NH_4OH (0.028%, v v^{-1}) containing 1 μmol of arachidonic acid (Sigma Chemical Co.). Reactions were terminated after 30 min by acidification with HCl to pH 3.5. PGE_2 (Sigma Chemical Co.) (40 pg in 100 μl) was added for the correction of recovery. The supernatant was mixed with 675 μl of water-glacial acetic acid (98.3 : 1.7, v v^{-1}) and applied on a Sep-Pak C_{18} cartridge (Waters, Milford, MA, U.S.A.). The columns were then sequentially washed with 5 ml each of the following mixtures of methanol:water:glacial acetic acid (33 : 66 : 1, 70 : 30 : 0.1, and 100 : 0 : 0, v v^{-1}) and the eluant of 70% methanol was collected into the siliconized glass tube. The eluant was evaporated under the vacuum condition, reconstituted in 1.25 ml of PIPES buffer, and concentrations of LTB_4 were determined using EIA kit (Cayman Chemical). The recovery during the extraction procedure was corrected by the amount of PGE_2 in the final fraction determined by radioimmunoassay (Ohuchi *et al*., 1985).

Polymorphonuclear leucocyte culture and treatment with drugs

Male Sprague-Dawley strain rats, specific pathogen-free (Charles River Japan, Kanagawa, Japan), were used. The rats were treated in accordance with procedures approved by the Animal Ethics Committee of the Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai, Japan. Rat peritoneal neutrophils were harvested 16 h after intraperitoneal injection of 40 ml of Ca^{2+}-free Krebs-Ringer solution containing 1% (w v^{-1}) casein (casein from milk, vitamin-free,

Wako Pure Chemicals, Osaka, Japan) which had been sterilized by autoclaving at 121°C for 15 min (Edamatsu *et al*., 1997). The peritoneal cells were washed twice with Ca^{2+}-free phosphate-buffered saline (PBS, pH 7.4) and finally suspended in RPMI 1640 medium (Nissui Seiyaku) containing 0.25% (w v^{-1}) BSA (Sigma Chemical Co.) at 5×10^5 cells ml^{-1}. The purity of neutrophils was more than 97% as assessed by May-Grünwald-Giemsa staining.

Rat peritoneal neutrophils (1×10^6 cells) were preincubated for 2 h at 37°C in 2 ml of EMEM containing 10% (v v^{-1}) FBS and various concentrations of troglitazone or zileuton (final concentrations of each drug were 0, 0.01, 0.1 and 1 μM). After three washes, the cells were incubated for 30 min at 37°C in 2 ml of PIPES buffer containing A23187 (1 μM) and the corresponding concentrations of troglitazone or zileuton. Concentrations of LTB_4 in the conditioned medium were determined using EIA kit (Cayman Chemical).

After treatment with drugs, the viability of the cells were examined (Kobayashi *et al*., 1994); no cytotoxic effect was observed (data not shown).

Statistical analysis

Results were analysed for statistical significance by one-way ANOVA followed by Dunnett's test for multiple comparisons and Student's *t*-test for unpaired observations.

Results

Stimulation by the antigen of eicosanoid production in RBL-2H3 cells

The IgE-sensitized RBL-2H3 cells were incubated at 37°C for 15 min in the presence of the various concentrations of the antigen DNP-HSA, and levels of LTB_4, C_4 and E_4 and PGD_2 in the conditioned medium were determined. Without antigen stimulation, cells did not produce detectable amount of each eicosanoid (Figure 1a). When the cells were incubated in medium containing the antigen at concentrations of 50 ng ml^{-1} or greater, levels of each eicosanoid in the conditioned medium at 15 min were increased. The effect reached a maximum at a concentration of 100 ng ml^{-1} (Figure 1a). In subsequent experiments, cells were stimulated with DNP-HSA at a concentration of 50 ng ml^{-1}. The non-sentizied RBL-2H3 cells with IgE did not produce detectable amount of each eicosanoid by the antigen (50 – 200 ng ml^{-1}) at 30 min (data not shown).

Time course of the antigen-induced eicosanoid production in RBL-2H3 cells

When the IgE-sensitized RBL-2H3 cells were incubated in medium containing 50 ng ml^{-1} of DNP-HSA, levels of LTB_4 and C_4 in the conditioned medium were increased time-dependently until 30 min after the antigen challenge, and decreased thereafter (Figure 1b). In contrast, levels of LTE_4 and PGD_2 were increased until 60 min (Figure 1b). In subsequent experiments, levels of each eicosanoid were determined 30 min after antigen challenge.

Effects of preincubation time with troglitazone on the antigen-induced production of LTC_4 in RBL-2H3 cells

The IgE-sensitized RBL-2H3 cells were preincubated at 37°C for 0, 15, 30, 60, 120 and 240 min in the presence of 1 μM troglitazone, washed and incubated at 37°C for 30 min in the presence of the antigen DNP-HSA (50 ng ml^{-1}) and 1 μM of troglitazone. As shown in Figure 2, LTC_4 production was significantly inhibited following 15 min preincubation, and the inhibitory effect of troglitazone was increased time-dependently till 2 h, at which time maximum inhibition was noted (Figure 2). Therefore, in the following experiments, IgE-sensitized RBL-2H3 cells were preincubated for 2 h in the presence of drugs, washed, and incubated for 30 min in medium containing the antigen DNP-HSA (50 ng ml^{-1}) and corresponding concentrations of drugs.

As shown in Table 1, troglitazone inhibited the antigen-induced production of LTB_4, C_4 and E_4 in a concentration-dependent manner. Zileuton, a 5-LOX inhibitor, also inhibited the antigen-induced production of LTB_4, C_4 and E_4 concentration-dependently (Table 1). IC_{50} values of troglitazone for the

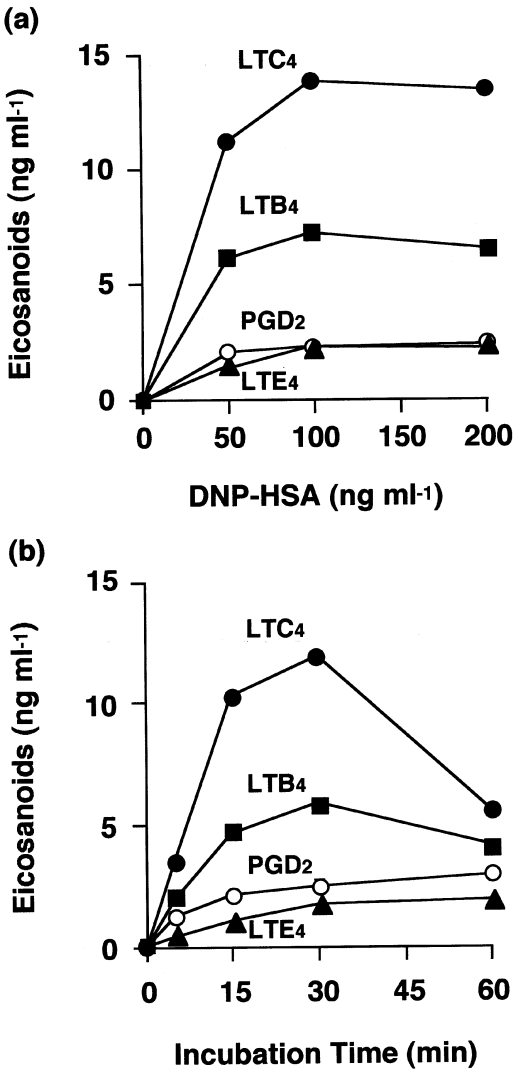

Figure 1 Effects of the antigen DNP-HSA on the production of LTB_4, C_4, E_4 and PGD_2 by RBL-2H3 cells. (a) RBL-2H3 cells (5×10^5 cells) were incubated for 15 min at 37°C in 1 ml of PIPES buffer containing the indicated concentrations of DNP-HSA. (b) RBL-2H3 cells (5×10^5 cells) were incubated for 0, 5, 15, 30 and 60 min at 37°C in 1 ml of PIPES buffer containing DNP-HSA (50 ng ml^{-1}). Values are the means ± s.e.mean from four wells; each s.e.mean is within the symbol. Without the antigen stimulation, levels of each eicosanoid in the conditioned medium were less than the detectable amount.

production of LTB_4, C_4 and E_4 were 0.41 ± 0.05, 0.38 ± 0.06 and 0.32 ± 0.01 μM, and those of zileuton were 0.36 ± 0.06, 0.35 ± 0.09 and 0.34 ± 0.05 μM, respectively (means ± s.e.mean from four independent determinations).

Both troglitazone and zileuton did not inhibit antigen-induced PGD_2 production (Table 1).

Effects of troglitazone on the antigen-induced arachidonic acid release in RBL-2H3 cells

The release of radioactivity from [^{3}H]-arachidonic acid-labelled RBL-2H3 cells at 15 min was significantly increased by the antigen challenge (Table 2). Treatment with troglitazone at 1 μM did not affect the antigen-induced release of radioactivity (Table 2). Zileuton at 1 μM also did not affect antigen-induced release of radioactivity (Table 2). The release of radioactivity from non-sensitized RBL-2H3 cells with IgE was not increased significantly by the antigen (50 ng ml^{-1}) at 15 min (1.63 ± 0.55, mean ± s.e.mean from four wells).

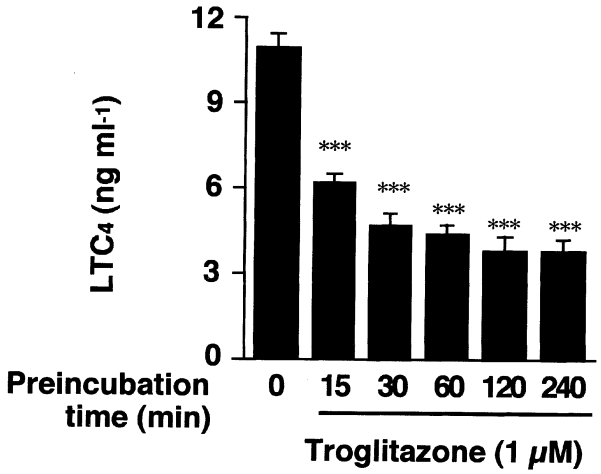

Figure 2 Effects of preincubation time with troglitazone on the antigen-induced LTC_4 production in RBL-2H3 cells. RBL-2H3 cells (5×10^5 cells) were preincubated for the periods indicated at 37°C in 1 ml of medium containing 1 μM of troglitazone. After three washes, the cells were further incubated for 30 min at 37°C in 1 ml of PIPES buffer containing DNP-HSA (50 ng ml^{-1}) and 1 μM of troglitazone. Values are the means ± s.e.mean from four wells. Statistical significance: ***$P < 0.001$ vs the group without preincubation (0 min).

Effects of troglitazone on the antigen-induced degranulation of RBL-2H3 cells

The antigen challenge increased the release of hexosaminidase from the cells when determined 15 min after antigen challenge (Table 3). In the presence of troglitazone or zileuton at 0.01 to 1 μM, antigen-induced degranulation was not inhibited (Table 3). The release of hexosaminidase from non-sensitized RBL-2H3 cells with IgE was not increased significantly by the antigen (50 ng ml^{-1}) at 15 min (0.27 ± 0.04, mean ± s.e.mean from four wells).

Effects of troglitazone on LTB_4 production by 20,000 × g supernatant of RBL-2H3 cells

To avoid the influence of the 5-LOX activating protein (FLAP), effects of troglitazone on the LTB_4 production by the cytosol fraction of the intact RBL-2H3 cells were examined. As shown in Figure 3, troglitazone inhibited LTB_4 production by the supernatant fraction of the cell lysate in a concentration-dependent manner. The IC_{50} was calculated to be 0.38 ± 0.04 μM (mean ± s.e.mean from four independent determinations). Zileuton, a 5-LOX inhibitor, also inhibited LTB_4 production in a concentration-dependent manner, and the IC_{50} was calculated to be 0.34 ± 0.05 μM (mean ± s.e.mean from four independent determinations). These findings indicate that troglitazone directly inhibits 5-LOX enzyme as zileuton does.

Table 2 Effects of troglitazone and zileuton on the antigen-induced eicosanoids release from RBL-2H3 cells

Treatment	*Released radioactivity* (%)
None	1.51 ± 0.49***
DNP-HSA	6.49 ± 0.49
DNP-HSA + Troglitazone 1 μM	6.00 ± 0.62
DNP-HSA + Zileuton 1 μM	6.49 ± 0.90

[^{3}H]-Arachidonic acid-labelled RBL-2H3 cells (2.5×10^5 cells) were incubated for 15 min at 37°C in 0.5 ml of PIPES buffer with or without DNP-HSA (50 ng ml^{-1}) and 1 μM of troglitazone or zileuton. Released radioactivity is expressed as per cent of total radioactivity incorporated in the cells. Values are the means ± s.e.mean from four wells. Statistical significance: ***$P < 0.001$ vs the group treated with DNP-HSA alone.

Table 1 Effects of troglitazone and zileuton on the antigen-induced production of eicosanoids by RBL-2H3 cells

Treatment		*Eicosanoid Production* (ng ml^{-1}) LTB_4	LTC_4	LTE_4	PGD_2
None		N.D.	N.D.	N.D.	N.D.
DNP-HSA		5.89 ± 0.18	11.93 ± 0.39	1.65 ± 0.13	2.37 ± 0.26
DNP-HSA + Troglitazone	0.01 μM	5.62 ± 0.21	11.50 ± 0.82	1.57 ± 0.08	2.25 ± 0.05
	0.1 μM	5.48 ± 0.07*	9.21 ± 0.59*	1.33 ± 0.06*	2.28 ± 0.30
	1 μM	1.55 ± 0.18***	3.84 ± 0.26***	0.53 ± 0.08***	2.51 ± 0.08
DNP-HSA + Zileuton	0.01 μM	5.48 ± 0.11	10.58 ± 0.95	1.69 ± 0.07	2.12 ± 0.21
	0.1 μM	4.58 ± 0.07***	7.14 ± 0.26***	1.30 ± 0.01*	2.30 ± 0.11
	1 μM	1.88 ± 0.14***	3.75 ± 0.33***	0.65 ± 0.10***	2.43 ± 0.27

RBL-2H3 cells (5×10^5 cells) were incubated for 30 min at 37°C in 1 ml of PIPES buffer with or without DNP-HSA (50 ng ml^{-1}) and the indicated concentrations of troglitazone or zileuton. Values are the means ± s.e.mean from four wells. N.D. means not detectable. Statistical significance: *$P < 0.05$, ***$P < 0.001$ vs the group treated DNP-HSA alone.

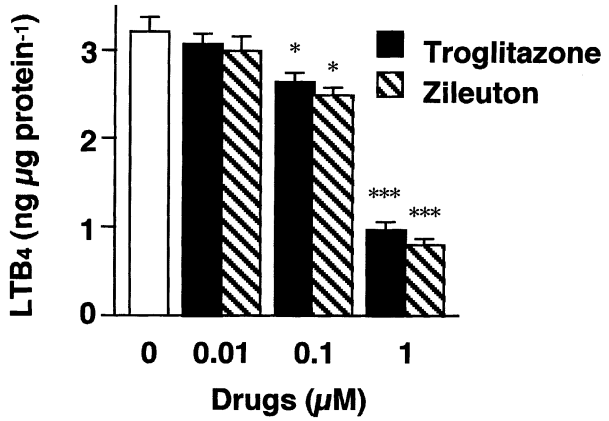

Figure 3 Effects of troglitazone and zileuton on the production of LTB_4 by $20,000 \times g$ supernatant fraction of intact RBL-2H3 cells. RBL-2H3 cells (2.5×10^7 cells) were lysed by sonication, and the lysate was centrifuged at $20,000 \times g$ and 4°C for 20 min. An aliquot of the supernatant fraction was preincubated for 2 h at 37°C in the presence of the indicated concentrations of troglitazone or zileuton, and further incubated for 30 min at 37°C in the presence of arachidonic acid (1 μM). LTB_4 produced is expressed as ng LTB_4 per 1 μg protein. Values are the means ± s.e.mean from four samples. Statistical significance: $^*P<0.01$, $^{***}P<0.001$ vs the control.

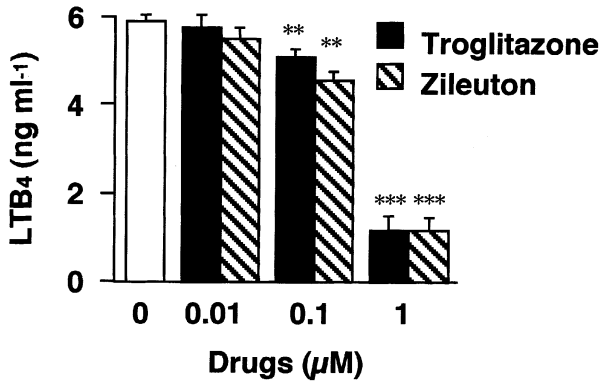

Figure 4 Effects of troglitazone and zileuton on LTB_4 production in rat peritoneal neutrophils. Rat peritoneal neutrophils (1×10^6 cells) were preincubated for 2 h at 37°C in 1 ml of medium containing troglitazone or zileuton. After three washes, the cells were incubated for 30 min at 37°C in 1 ml of PIPES buffer containing A23187 (1 μM) and the corresponding concentrations of troglitazone or zileuton. Values are the means ± s.e.mean from four wells. Statistical significance: $^{**}P<0.01$, $^{***}P<0.001$ vs the control.

Effects of troglitazone on A23187-induced LTB_4 production by rat peritoneal neutrophils

To show that the effect of troglitazone is not specific to antigen-induced LT production in RBL-2H3 cells, effects of troglitazone on A23187-induced LTB_4 production by rat peritoneal neutrophils were examined. As shown in Figure 4, LTB_4 production by rat peritoneal neutrophils was increased by 1 μM A23187, and troglitazone inhibited A23187-induced LTB_4 production in a concentration-dependent manner. The IC_{50} was calculated to be 0.35 ± 0.05 μM (mean ± s.e.mean from four determinations). Zileuton also inhibited A23187-induced LTB_4 production in a concentration-dependent manner, and the IC_{50} was calculated to be 0.30 ± 0.04 μM (mean ± s.e.mean from four determinations). These findings indicate that troglitazone inhibits LT production in neutrophils as well as in RBL-2H3 cells.

Table 3 Effects of troglitazone and zileuton on the antigen-induced hexosaminidase release in RBL-2H3 cells

Treatment		*Hexosaminidase release* (%)
None		$0.25 \pm 0.05^{***}$
DNP-HSA		43.87 ± 0.50
DNP-HSA + Troglitazone	0.01 μM	44.12 ± 0.50
	0.1 μM	44.12 ± 0.50
	1 μM	43.36 ± 1.51
DNP-HSA + Zileuton	0.01 μM	44.87 ± 0.50
	0.1 μM	46.13 ± 0.50
	1 μM	44.12 ± 0.17

RBL-2H3 cells (5×10^5 cells) were incubated for 15 min at 37°C in 1 ml of PIPES buffer with or without DNP-HSA (50 ng ml^{-1}) and the indicated concentrations of troglitazone or zileuton. Released hexosaminidase is expressed as per cent of total hexosaminidase in the cells before the antigen challenge. Values are the means ± s.e.mean from four wells. Statistical significance: $^{***}P<0.001$ vs the group treated with DNP-HSA alone.

Discussion

In this study, we have determined the profile of antigen-induced LT production in IgE-sensitized RBL-2H3 cells. The antigen DNP-HSA (50 ng ml^{-1}) prominently increased the production of LTB_4, C_4 and E_4 and PGD_2 (Figure 1), the release of [^{3}H]-arachidonic acid (Table 2), and cell degranulation (Table 3). Over the period from 30 to 60 min after antigen stimulation, concentrations of LTB_4 were decreased probably due to ω-oxidation (Shak & Goldstein, 1984), because the antibody to LTB_4 used in our experiment does not cross-react with 20-carboxy LTB_4 (less than 0.001%). However, at present, there is no report suggesting that RBL-2H3 cells express the specific cytochrome P-450 responsible for ω-oxidation of LTB_4. Concentrations of LTC_4 were also decreased during this period but concentrations of LTE_4 were increased slightly, suggesting that LTC_4 was metabolized to LTD_4 and LTE_4 by γ-glutamyl transpeptidase and γ-glutamyl transdipeptidase, respectively (Andersen *et al.*, 1982; Orning *et al.*, 1982). In the present study, we could not determine concentrations of LTD_4, because a LTD_4-specific antibody is not available. The decrease in LTC_4 levels in the conditioned medium during the incubation period has also been reported in mouse bone marrow-derived mast cells; c-*kit* ligand-induced increase in LTC_4 levels in the conditioned medium reached a maximum at 10 min and declined thereafter (Murakami *et al.*, 1995).

We have demonstrated that troglitazone inhibits the antigen-induced increase in the production of LTB_4, C_4 and E_4 in IgE-sensitized RBL-2H3 cells (Figure 2, Tables 1 and 2). The potency of the inhibitory effect of troglitazone was comparable to that of zileuton, an anti-asthmatic drug and a specific 5-LOX inhibitor (Carter *et al.*, 1991; Dahlen *et al.*, 1998; Tan, 1998).

Because troglitazone did not affect antigen-induced PGD_2 production (Table 1) and the release of [^{3}H]-arachidonic acid from membrane phospholipids (Table 2), we suggest that troglitazone does not inhibit cyclo-oxygenase and phospholipase A_2, respectively.

An inhibitory effect of troglitazone and zileuton on LTB_4 production was also observed in the $20,000 \times g$ supernatant fraction of RBL-2H3 cells (Figure 3), suggesting that troglitazone inhibits 5-LOX directly. However, we cannot exclude the possibility that troglitazone inhibits LTA_4

hydrolase (Gut *et al*., 1987) or glutathione-*S*-transferase which metabolizes LTA_4 into LTC_4 (Bach *et al*., 1984). As we used the $20,000 \times g$ supernatant fraction of RBL-2H3 cells, an effect of troglitazone on FLAP, the activity of which is observed in the nuclear fraction (Woods *et al*., 1993), can be excluded. Our observation that the IC_{50} for LTB_4 synthesis resembles that for inhibition of LTC_4 synthesis suggests that troglitazone inhibits 5-LOX, a common upstream enzyme for LTB_4 and LTC_4 synthesis. Troglitazone also inhibited A23187-stimulated LTB_4 production in rat peritoneal neutrophils (Figure 4). This finding indicated that inhibition of LT production is not specific to antigen-stimulated RBL-2H3 cells.

The chemical structure of troglitazone is composed of a thiazolidinedione ring and a chroman ring. PPAR-γ ligands, including troglitazone, inhibit nitric oxide (NO) production and induction of NO synthase in macrophage-like cell lines (Ricote *et al*., 1998), and the IC_{50} of troglitazone for NO production is greater than 10 μM. PPAR-γ ligands also inhibit the tumour promoter-induced production of TNF-α in human monocytes, and the IC_{50} of troglitazone for TNF-α production is 10 μM (Jiang *et al*., 1998). PPAR-γ is expressed predominantly in adipose tissue and in the immune system in adult rats (Braissant *et al*., 1996), but it remains to be clarified whether PPAR-γ is expressed in RBL-2H3 cells. Because IC_{50}s for the inhibition of NO production and TNF-α production are higher than those for the inhibition of the antigen-induced LT production in RBL-2H3 cells, it is possible that the inhibition of the antigen-induced LT production by troglitazone is not expressed through PPAR-γ, but is due to a direct effect on 5-LOX. Staels *et al*. (1998) demonstrated that BRL49653, a PPAR-γ-specific thiazolidinedione, inhibited neither IL-1-induced 6-keto-$PGF_{1\alpha}$ production nor cyclo-oxygenase-2 protein induction in human aortic smooth muscle cells. No effect of the PPAR-γ agonist BRL49653 on IL-1-induced 6-keto-$PGF_{1\alpha}$ production (Staels *et al*., 1998) was noted similar to our finding that troglitazone showed no effect on antigen-induced PGD_2 production (Table 1).

The lack of effect of both troglitazone and zileuton on antigen-induced degranulation of IgE-sensitized RBL-2H3 cells (Table 3) suggests that the concomitantly produced LTs do not play significant roles in mast cell degranulation.

Loi *et al*. (1997) reported that after oral administration of troglitazone 400 mg every morning for 15 days to patients with type II diabetes, mean steady-state plasma C_{max} of troglitazone reached 1.54 μg ml^{-1}. This concentration of troglitazone is equivalent to 3.49 μM, and is sufficient to inhibit LT production in RBL-2H3 cells.

In conclusion, troglitazone inhibited antigen-induced LT production in IgE-sensitized RBL-2H3 cells and A23187-stimulated rat peritoneal neutrophils as potently as zileuton, an anti-asthmatic drug and a specific inhibitor of 5-LOX.

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