Itraconazole-mediated inhibition of calcium entry into platelet-activating factor-stimulated human neutrophils is due to interference with production of leukotriene B_4

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Summary

The primary objective of this study was to probe the involvement of leukotriene B_4 (LTB_4) in itraconazole (0·1–5 μM)-mediated inhibition of Ca^{2+} uptake by chemoattractant-activated human neutrophils. Following exposure of the cells to platelet-activating factor (PAF, 200 nM), LTB_4 was measured by immunoassay, while neutrophil cytosolic Ca^{2+} concentrations were determined by a fura-2/AM-based spectrofluorimetric procedure. Activation of neutrophils was accompanied by an abrupt and sustained (for about 1 min) elevation in cytosolic Ca^{2+} which was associated with increased generation of LTB_4, both of which were attenuated significantly by itraconazole at 0·5 μM and higher. The inhibitory effect of the anti-mycotic on Ca^{2+} uptake by PAF-activated cells was mimicked by an LTB_4 antibody, as well as by LY255283 (1 μM) and MK886 (0·5 μM), an antagonist of LTB_4 receptors and an inhibitor of 5'-lipoxygenase-activating protein, respectively, while addition of itraconazole to purified 5'-lipoxygenase resulted in inhibition of enzyme activity. A mechanistic relationship between itraconazole-mediated inhibition of LTB_4 production and Ca^{2+} influx was also supported by the observation that pulsed addition of purified LTB_4 to PAF-activated neutrophils caused substantial restoration of Ca^{2+} uptake by cells treated with the anti-mycotic. Taken together, these observations suggest that the potentially beneficial anti-inflammatory interactions of itraconazole with activated neutrophils result from interference with production of LTB_4, with consequent attenuation of a secondary LTB_4-mediated wave of Ca^{2+} uptake by the cells.

Introduction

Itraconazole, an imidazole anti-mycotic, antagonizes the uptake of Ca^{2+} by activated human neutrophils, as well as by other types of mammalian cell [1–5]. In the case of neutrophils, itraconazole-mediated inhibition of Ca^{2+} influx not only interferes with the Ca^{2+}-dependent proinflammatory activities of the cells, but also prevents their reactivation by preventing store-operated refilling of intracellular Ca^{2+} stores [1,2,5]. These inhibitory effects of the anti-mycotic on Ca^{2+} influx have been observed not only in chemoattractant-activated neutrophils, but also following the addition of Ca^{2+} to neutrophils with depleted intracellular Ca^{2+} stores suspended in Ca^{2+} free medium [5]. Although potentially beneficial, the molecular/biochemical basis of these secondary, anti-inflammatory interactions of itraconazole with neutrophils have not been established. Possible mechanisms include inhibition of cytochrome P450 (CYP450) enzyme systems, specifically CYP450 epoxygenase, which converts arachidonate to epoxyeicosatrienoic acid, a putative Ca^{2+} influx factor [1,2,6,7], as well as direct antagonism of Ca^{2+} channels [8,9].

Interference with the production of leukotriene B_4 (LTB_4) represents an alternative, albeit unexplored, mechanism of itraconazole-mediated interference with store-operated uptake of Ca^{2+} by activated neutrophils. In this respect it is noteworthy that itraconazole has been reported to inhibit the production of LTB_4 by intact neutrophils activated with the Ca^{2+} ionophore, A23187 [10], while endogenous-generated LTB_4 has been reported to amplify the uptake of Ca^{2+} following addition of the chemoattractant, platelet-activating factor (PAF), to neutrophils [11].

In the current study we have investigated the effects of itraconazole on the production of LTB_4 by PAF-activated neutrophils and its relationship to alterations in Ca^{2+} influx.

Materials and methods

Chemicals

Itraconazole was kindly provided by Janssen Pharmaceutica (Geel, Belgium) and dissolved in dimethylacetamide (DMA) to give a stock concentration of 10 mM. Subsequent dilutions were made in the same solvent and the final concentrations of itraconazole used in the assays described below ranged from 0·1 to 5 μM, while that of DMA was 0·1%. Unless indicated, all other chemicals and reagents were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Neutrophils

The study was approved by the Faculty of Health Sciences Research Ethics Committee of the University of Pretoria (Protocol 43/2006), and prior informed consent was obtained from all participants.

Purified neutrophils were prepared from heparinized (5 units of preservative-free heparin/ml) venous blood of healthy, non-smoking adult human volunteers and separated from mononuclear leucocytes by centrifugation on Histopaque®-1077 (Sigma Diagnostics) cushions at 400 ***g*** for 25 min at room temperature. The resultant pellet was suspended in phosphate-buffered saline (PBS; 0·15 M, pH 7·4) and sedimented with 3% gelatine to remove most of the erythrocytes. After centrifugation, erythrocytes were removed by selective lysis with 0·84% ammonium chloride at 4°C for 10 min. The neutrophils, which were routinely of high purity (> 90%) and viability (> 95%), were resuspended at 1×10^7/ml in PBS and held on ice until used.

Measurement of LTB_4

A competitive binding enzyme immunoassay procedure (Correlate-EIA™; Assay Designs Inc., Ann Arbor, MI, USA) was used to measure LTB_4 in the supernatants of neutrophils activated with PAF (200 nM) in the absence or presence of itraconazole (0·1–5 μM). Neutrophils (2×10^6/ml, final) in Hank's balanced salt solution (HBSS) were preincubated for 10 min at 37°C with itraconazole after which the chemoattractant, platelet-activating factor (PAF; 200 nM, final) was added to the cells and the reactions stopped after 3 min incubation at 37°C (predetermined in preliminary time–course experiments) by the addition of an equal volume of ice-cold HBSS to the tubes which were then held in an ice-bath prior to pelletting the cells by centrifugation. The cell-free supernatants were then assayed for LTB_4 using the enzyme immunoassay (EIA) procedure. Supernatants from cells activated with PAF were diluted 1 : 4 prior to assay. These results are expressed as picograms (pg)/10^7 cells.

The effects of LY255283 (1 μM) and MK886 (0·5 μM), an LTB_4 receptor antagonist [12] and 5'-lipoxygenase-activating protein (FLAP) inhibitor [13], respectively (both from the Cayman Chemical Co., Ann Arbor, MI, USA), on LTB_4 production by PAF-activated neutrophils were also investigated. The concentrations of MK886 and LY255283 used for these experiments (0·5 μM and 1 μM, respectively) were based on data derived from preliminary experiments. At a concentration of 0·5 μM, MK886 effectively attenuated the generation of LTB_4 by neutrophils activated with the calcium ionophore, A23187 (1 μM), while LTB_4 (50 nM)-mediated mobilization of Ca^{2+} from neutrophil intracellular stores was completely antagonized by 1 μM LY255283 (data not shown).

The effects of itraconazole (5 μM) on the production of LTB_4 by PAF-activated neutrophils suspended in Ca^{2+} replete and Ca^{2+} free HBSS were also investigated. To minimize loss of Ca^{2+} from intracellular stores, neutrophils were preincubated in Ca^{2+} replete HBSS for 10 min at 37°C, pelleted by centrifugation and resuspended in either Ca^{2+} replete HBSS (control cells) or HBSS containing 50 μM $CaCl_2$. The cells were then preincubated at 37°C for 9 min, after which the Ca^{2+} chelating agent, ethylene glycol tetraacetic acid (EGTA) (500 μM final) was added to cells suspended in HBSS containing 50 μM $CaCl_2$, followed by transfer to cuvettes and activation 1–2 min later with PAF. With this procedure we were unable to detect influx of Ca^{2+} into neutrophils treated with the pneumococcal

pore-forming toxin, pneumolysin (8·37 ng/ml, final), which causes influx of extracellular Ca^{2+} without mobilizing the cation from neutrophil intracellular stores [14].

To exclude possible interference of itraconazole, LY255283, and MK886 at concentrations of 5, 1 and 0·5 μM, respectively, with LTB_4 in the EIA, we investigated the reactivity of a fixed concentration (3000 pg/ml) of the LTB_4 standard in the presence and absence of the LTB_4 receptor antagonist.

Activity of purified 5'-lipoxygenase (5-LO)

The effects of itraconazole (5–50 μM, final) or the DMA solvent (1% final in this system) on the reactivity of purified 5-LO were investigated using the Lipoxygenase Inhibitor Screening Assay Kit from the Cayman Chemical Co., which is a colorimetric assay using purified potato 5-LO (5 units/assay) and linoleic acid (100 μM, final) as enzyme and substrate, respectively (as recommended by the manufacturer). The enzyme and DMA or itraconazole in assay buffer were preincubated for 15 min at room temperature, followed by addition of substrate and incubation for an additional period of 5 min. Enzyme activity was monitored spectrophotometrically at 490 nm according to the magnitude of chromogen-reactive hydroxperoxide formation. Substrate and enzyme were omitted from negative control systems. The results of these experiments are expressed as the mean percentage activity of the itraconazole-free control systems.

Spectrofluorimetric measurement of Ca^{2+} fluxes

Fura-2/AM was used as the fluorescent Ca^{2+} sensitive indicator for these experiments [15]. Neutrophils (1×10^7/ml) suspended in PBS were prewarmed for 5 min at 37°C followed by addition of fura-2/AM (2 μM, final) and reincubated for 25 min at 37°C. The fura-2-loaded neutrophils were then pelleted by centrifugation and resuspended in indicator-free, Ca^{2+} replete (1·25 mM $CaCl_2$) HBSS, pH 7·4. The neutrophils (2×10^6/ml) were then preincubated for 10 min at 37°C with one of the following: itraconazole (0·1–5 μM); MK886 (0·5 μM) or LY255283 (1 μM); an equivalent amount of the relevant DMA (itraconazole) or dimethylsulphoxide (DMSO; MK886, LY255283) solvent control systems. The cells were then transferred to disposable reaction cuvettes which were maintained at 37°C in a Hitachi 650 10S fluorescence spectrophotometer with excitation and emission wavelengths set at 340 and 500 nm, respectively. After a stable baseline was obtained (1 min), the neutrophils were activated by addition of PAF (200 nM). Alterations in fluorescence intensity were then monitored over a 5–10-min period. The final volume in each cuvette was 3 ml containing a total of 6×10^6 neutrophils.

In a related series of experiments, the involvement of LTB_4 in sustaining cytosolic Ca^{2+} transients in neutrophils activated with PAF was also probed using an anti-LTB_4 antibody produced in rabbit whole anti-serum (Sigma). The antibody (60 μl of a 5-mg/ml solution) was added to the cells 1 min prior to the activators. An equivalent concentration of a control antibody to prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (rabbit whole anti-serum; Sigma) was added to control systems. The efficacy of the LTB_4 antibody was demonstrated in preliminary experiments in which it was found to antagonize the mobilization of Ca^{2+} from neutrophil intracellular stores following addition of purified LTB_4 (50 nM) to the cells.

In an attempt to clarify the possible association between itraconazole-mediated inhibition of LTB_4 production and Ca^{2+} influx, an additional series of experiments was designed to investigate the potential of added, purified LTB_4 to reverse the inhibitory effects of itraconazole (5 μM) on the uptake of Ca^{2+} by PAF-activated neutrophils. Three strategies were used: (i) LTB_4 (75 nM) and PAF (200 nM) were added simultaneously to control or itraconazole-treated cells; (ii) LTB_4 (75 nM) was added 10 s after PAF; and (iii) LTB_4 (15 nM) and PAF were added simultaneously, followed 10 s later by addition of a second, higher dose of LTB_4 (75 nM) only.

Cellular adenosine triphosphate (ATP) levels

The cytotoxic potential of itraconazole (5 μM), LY255283 (1 μM) and MK886 (0·5 μM) was determined by measurement of cellular ATP in the lysates of neutrophils (10^6/ml) which had been exposed to the

test agents for 10 min at 37°C using a luciferin/luciferase chemiluminescence procedure [16]. The results are expressed as picomoles ATP/10^7 cells.

Expression and statistical analysis of results

The results of each series of experiments are expressed as the mean values ± s.e.m., with the exception of several of the fura-2 experiments for which the traces are shown. Statistical analysis was performed using Student's *t*-test when comparing two groups or by analysis of variance with subsequent Tukey–Kramer multiple comparisons test for multiple comparisons.

Results

Effects of itraconazole on LTB_4 production by activated neutrophils

The effects of itraconazole at concentrations of 0·1–5 μM on the production of LTB_4 by neutrophils activated with PAF are shown in Fig. 1. Itraconazole caused dose-related inhibition of the production of LTB_4 by the chemoattractant, which attained statistical significance at concentrations of 0·1 μM.

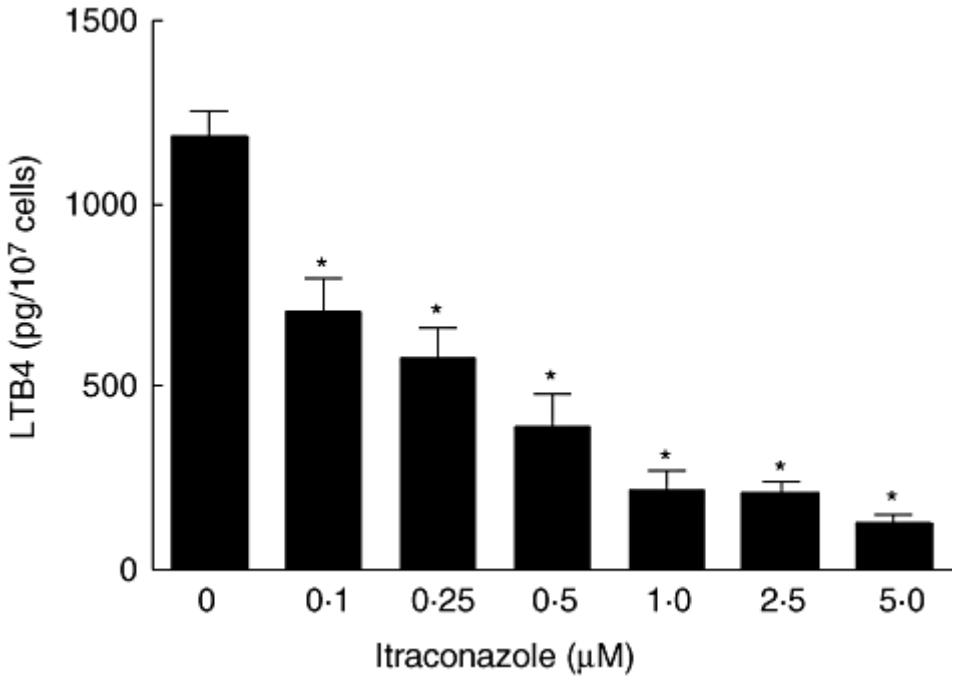

Fig. 1. Effects of itraconazole (0·1–5 μM) on the production of leukotriene B_4 (LTB_4) by platelet-activating factor (PAF) (200 nM)-activated neutrophils. The results of three experiments with five replicates for each system are expressed as the mean values ± s.e.m. The values for the unstimulated control, and for the PAF-activated control systems were 43 ± 5 and 1190±67 pg $LTB_4/10^7$ cells, respectively. **P* < 0·05 for comparison with the corresponding itraconazole-free control system.

A comparison of the effects of LY255283 and MK886 on PAF-activated production of LTB_4 by neutrophils is shown in Table 1. Both agents caused statistically significant inhibition of LTB_4 production. Importantly, itraconazole (5 μM), LY255283 (1 μM) and MK886 (0·5 μM) did not affect the reactivity of LTB_4 in the EIA (data not shown).

PAF-activated neutrophils incubated with	Leukotriene B_4 (pg/10^7 cells)
Solvent only (control)	1054 ± 49[a]
1·0 μM LY255283	396 ± 42*
0·5 μM MK886	78 ± 16*
[a]The results of three different experiments with five replicates for each system are expressed as the mean values ± s.e.m. The basal values for unstimulated cells was 59 ± 10 pg $LTB_4/10^7$. *P < 0·05.	

Table 1. Effects of LY255283 (1 μM) and MK886 (0·5 μM) on the production of leukotreine B_4 (LTB_4) by platelet-activating factor (PAF)-activated neutrophils.

Comparison of the effects of itraconazole on LTB_4 production by PAF-activated neutrophils suspended in Ca^{2+} replete and Ca^{2+} free HBSS

These results are shown in Fig. 2. Although markedly reduced relative to the corresponding responses in Ca^{2+} replete HBSS, the production of LTB_4 by PAF-activated neutrophils was still detectable and increased significantly above basal, unstimulated values. The production of LTB_4 by PAF-activated neutrophils was significantly (P < 0·05) inhibited by itraconazole irrespective of the Ca^{2+} content of the cell-suspending medium (Fig. 2).

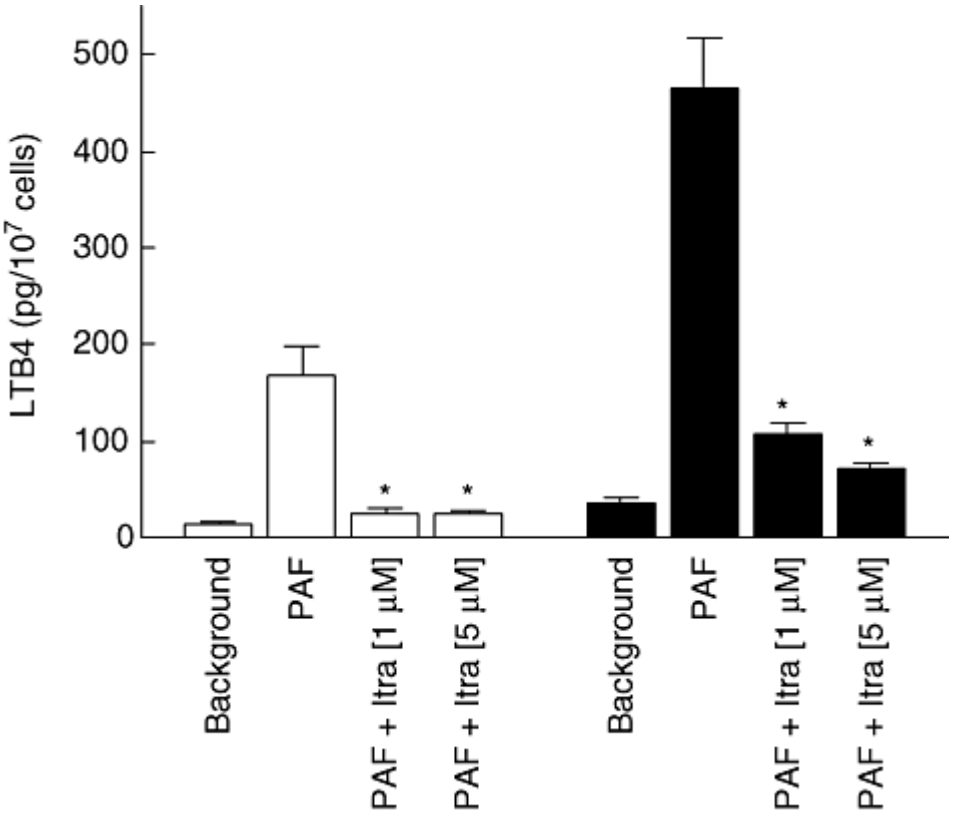

Fig. 2. Effects of itraconazole (Itra) at concentrations of 1 and 5 μM on the production of leukotriene B_4 (LTB_4) by platelet-activating factor (PAF) (200 nM)-activated, Ca^{2+} loaded neutrophils (to ensure that intracellular Ca^{2+} stores are full) suspended in Ca^{2+} free (□) or Ca^{2+} replete (■) medium. The results of three experiments with five replicates for each system in each experiment are expressed as the mean values ± s.e.m. *P < 0·05 for comparison with the corresponding itraconazole-free, PAF-activated system.

Cytosolic Ca^{2+} fluxes of neutrophils suspended in either Ca^{2+} replete or Ca^{2+} free HBSS and treated with pneumolysin or PAF are shown in Fig. 3. The influx of Ca^{2+} which accompanies treatment of neutrophils with pneumolysin was abolished completely in the Ca^{2+} free system, while the abrupt increase in cytosolic Ca^{2+} remained intact in PAF-activated cells, but was associated with attenuation of the plateau phase and a rapid decline in fura-2 fluorescence intensity due, presumably, to absence of influx of Ca^{2+} as described in detail in the section on cytosolic Ca^{2+} fluxes below. These observations validate the experimental design used to eliminate Ca^{2+} influx during activation of the cells with PAF, in the setting of retention of mobilization of the cation from intracellular stores.

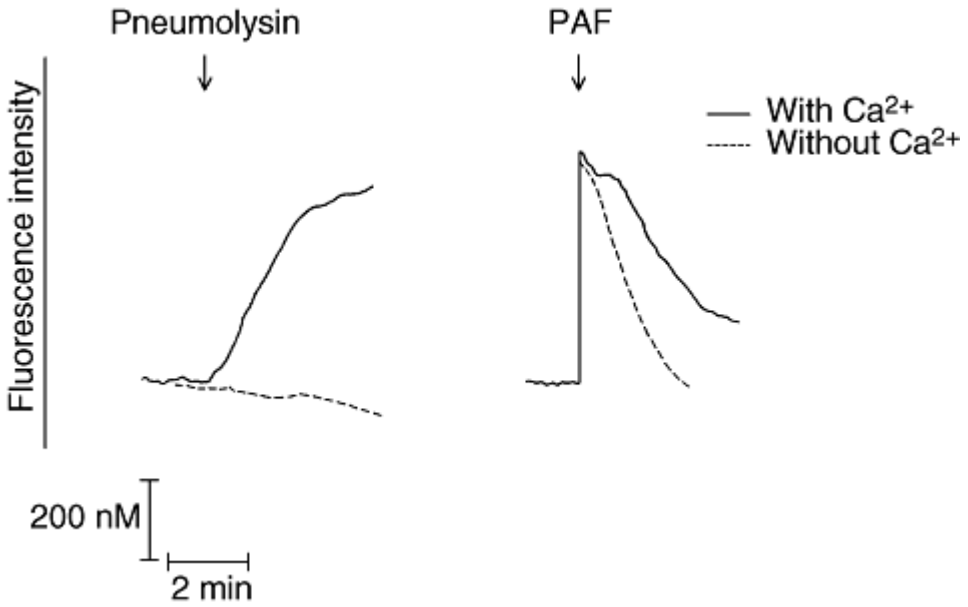

Fig. 3. Traces showing the fura-2 fluorescence responses of Ca^{2+} loaded neutrophils (to ensure that intracellular Ca^{2+} stores are full) suspended in either Ca^{2+} free or Ca^{2+} replete Hank's balanced salt solution, followed by addition of either pneumolysin (8·37 ng/ml) or platelet-activating factor (PAF) (200 nM) as denoted by the arrow (↓).

Effects of itraconazole on purified 5-LO

Itraconazole caused dose-related inhibition of the activity of purified 5-LO in a cell-free assay system with linoleic acid as substrate. The mean percentages of the DMA-treated control systems were 83 ± 4, 77 ± 4, 71 ± 6, and 55 ± 5% for systems containing 5, 10, 20 and 50 μM itraconazole, respectively (data from six experiments; $P < 0{\cdot}05$ for comparison of each of the itraconazole-treated systems with the control system). These experiments were complicated by the insolubility of itraconazole in aqueous, cell-free systems, and may have under-estimated the inhibitory potency of itraconazole for 5-LO.

Effects of itraconazole, LY255283, MK886 and LTB_4-antibody on cytosolic Ca^{2+} transients in activated neutrophils

The effects of itraconazole at concentrations of 0·1–5 μM on cytosolic Ca^{2+} fluxes in PAF-activated neutrophils are shown in Fig. 4 and Table 2, while the comparative effects of itraconazole, LY255283, MK886 and the LTB4-antibody are shown in Fig. 5 and Table 2. Addition of PAF to neutrophils was accompanied by the typical, abrupt increase in fluorescence intensity which accompanies G-protein-coupled receptor activation of phospholipase C and inositol triphosphate-mediated release of Ca^{2+} from intracellular stores. Peak fluorescence intensity was sustained for about 1–1·5 min, subsiding gradually

thereafter, compatible with the previously described early influx of Ca^{2+} into PAF-activated neutrophils and slow clearance of cytosolic Ca^{2+} [17]. Pretreatment of neutrophils with itraconazole did not affect the magnitude of the immediate peak cytosolic Ca^{2+} response of PAF-activated neutrophils (535 ± 41 nM for the control system, rising from a basal value of 116 ± 12 nM). However, the duration of the plateau phase of peak fluorescence intensity was shortened significantly by treatment of the cells with the anti-mycotic, compatible with inhibition of Ca^{2+} influx. These effects of itraconazole were statistically significant at concentrations of ≥ 0·5 μM (Fig. 4 and Table 2).

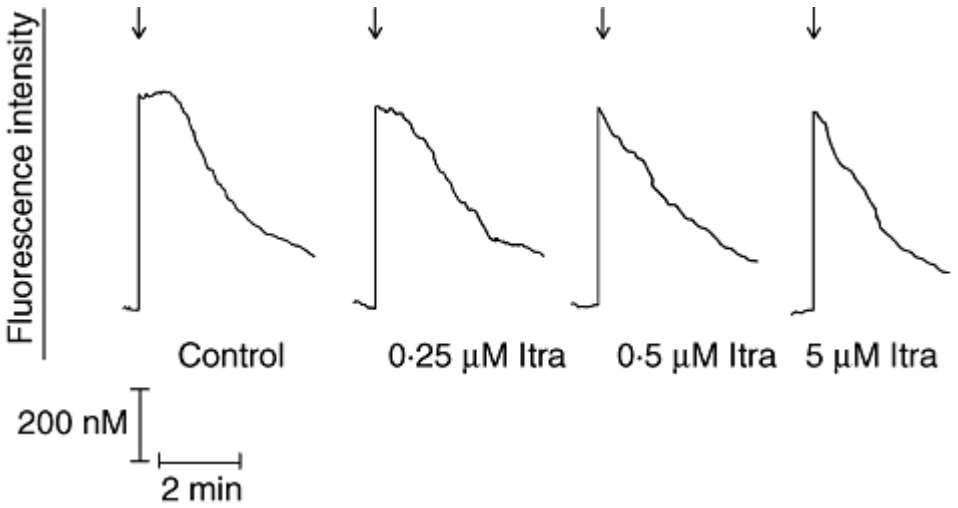

Fig. 4. Traces from a single representative experiment (six in the series) showing the effects of itraconazole (Itra) at concentrations of 0·25, 0·5 and 5 μM on the fura-2 fluorescence responses of platelet-activating factor (PAF) (200 nM)-activated neutrophils added as denoted by the arrow (↓).

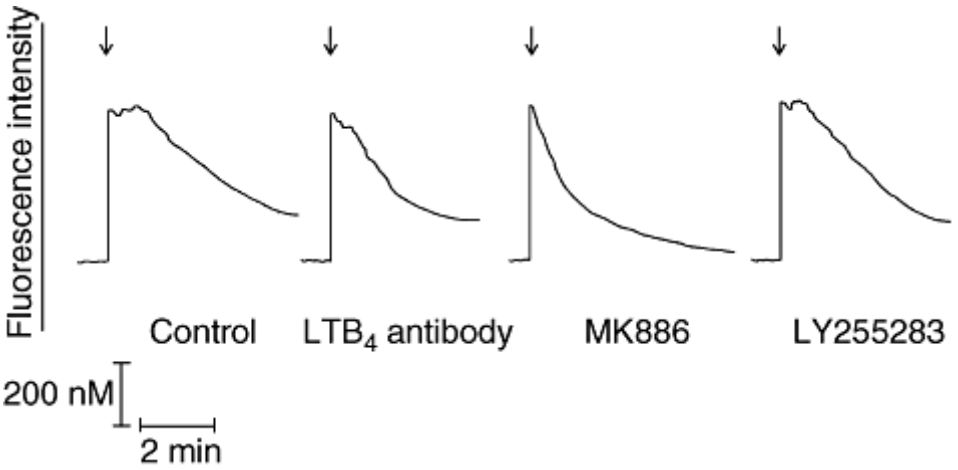

Fig. 5. Traces from a single representative experiment (six to eight in the series) showing the effects of an anti-leukotriene B_4 (LTB_4) antibody (60 μg/ml), MK886 (0·5 μM) and LY255283 (1 μM) on the fura-2 fluorescence responses of platelet-activating factor (PAF) (200 nM)-activated neutrophils added as denoted by the arrow (↓).

PAF-activated neutrophils incubated with	Duration of the peak cytosolic Ca^{2+} response (min)
Solvent only (control)	1·20 ± 0·05[a]
0·1 μM itraconazole	1·00 ± 0·10
0·25 μM itraconazole	0·80 ± 0·13
0·5 μM itraconazole	0·23 ± 0·08*
1·0 μM itraconazole	0·25 ± 0·03*
2·5 μM itraconazole	0·30 ± 0·03*
5·0 μM itraconazole	0·33 ± 0·02*
1 μM LY255283	0·60 ± 0·03*
0·5 μM MK886	0·13 ± 0·02*
LTB_4-antibody	0·70 ± 0·03*
[a]The results of six different experiments are presented as the mean duration (min) ± s.e.m. of the peak cytosolic Ca^{2+} response. The mean peak cytosolic Ca^{2+} response of the control system was 535 ± 41 nM, rising from a basal value of 116 ± 41 nM. *P < 0·05 for comparison with the itraconazole-free control system.	

Table 2. Effect of itraconazole, LY255283, MK886 and the leukotreine B_4 (LTB_4) antibody on duration of the peak cytosolic response of platelet-activating factor (PAF)-activated neutrophils.

As shown in Fig. 5 and Table 2, treatment of neutrophils with LY255283, MK886 or the LTB_4-antibody also (P < 0·05) shortened significantly the duration of the peak plateau phase of fura-2 fluorescence intensity in PAF-activated cells, without affecting the magnitude of the immediate peak increase in cytosolic Ca^{2+}. The duration of the plateau phase was not affected by treatment of the cells with the control, $PGF_{2\alpha}$-antibody (not shown).

As shown in Table 3, pulsed addition of purified LTB_4 (i.e. PAF + 15 nM LTB_4, followed 10 s later by 75 nM LTB_4) substantially restored Ca^{2+} influx to itraconazole (5 μM)-treated neutrophils, without affecting the fura-2 responses of itraconazole-free control cells. On the other hand, simultaneous addition of LTB_4 (75 nM) with PAF only, or addition of LTB_4 (75 nM) 10 s after PAF, was less effective in counteracting the inhibitory effects of itraconazole on Ca^{2+} influx (data not shown).

Although not shown, we also observed that treatment of neutrophils with itraconazole (5 μM) did not affect the fura-2 fluorescence responses of cells activated with purified LTB_4 (50 nM), excluding possible LTB_4 receptor blockading effects of the anti-mycotic.

System	Duration of the peak cytosolic Ca^{2+} response (min)
Neutrophils + PAF	1·05 ± 0·03[a]
Neutrophils + itraconazole + PAF	0·25 ± 0·02
Neutrophils + PAF + LTB_4	1·09 ± 0·03
Neutrophils + itraconazole + PAF + LTB_4	0·82 ± 0·03*
[a]Results are expressed as the mean values ± s.e.m. for the duration of the abruptly occurring peak Ca^{2+} transients in neutrophils activated with PAF only, or with PAF + LTB_4 (15 nM) added simultaneously followed 10 s later by 75 nM LTB_4 in the absence or presence of 5 μM itraconazole (data from 10 determinations). *P < 0·05 for comparison between the itraconazole-treated systems activated with PAF only or with PAF + LTB_4.	

Table 3. Effects of pulsed addition of leukotriene B_4 (LTB_4) on itraconazole (5 μM)-mediated attenuation of the sustained, peak cytosolic Ca^{2+} transient in platelet-activating factor (PAF) (200 nM)-activated neutrophils.

ATP levels

At the maximum concentrations used, ATP was unaffected by treatment with itraconazole (5 μM), LY255283 (1 μM) or MK886 (0·5 μM). In the case of itraconazole the values for the solvent control (0·1% DMA) and drug-treated systems were 29·4 ± 2·7 and 30·0 ± 2·1 pmoles ATP/10^7 cells, respectively, while the corresponding values for the DMSO (0·1%) and LY255283- and MK886-treated systems were 25·5 ± 2·4, 25·2 ± 1·8 and 27·0 ± 2·1 pmoles ATP/10^7 cells. At the concentrations used (0·1%), neither of the solvents affected neutrophil ATP levels (not shown).

Discussion

In the current study, we have observed that itraconazole attenuates both LTB_4 production and Ca^{2+} influx following activation of human neutrophils with the chemoattractant, PAF. Assuming a mechanistic relationship between these events, two possibilities were evident. Firstly, the anti-mycotic acts primarily as an antagonist of Ca^{2+} influx leading to secondary inhibition of LTB_4 production; alternatively, the inhibitory effects of the anti-mycotic on Ca^{2+} influx are indirect, resulting from interference with production of LTB_4.

Taken together, the following observations clearly supported a primary inhibitory effect of itraconazole on LTB_4 production, with consequent secondary inhibition of Ca^{2+} influx in PAF-activated neutrophils; (i) although the magnitude of the LTB_4 response was dependent upon the Ca^{2+} content of the cell-suspending medium, the inhibitory effects of the anti-mycotic were evident irrespective of the presence or absence of the cation in the medium; (ii) the inhibitory effects of itraconazole on Ca^{2+} uptake were mimicked by treatment of the cells with an antibody to LTB_4, as well as by non-cytotoxic concentrations of LY255283 and MK886, antagonists of LTB_4 receptors and FLAP, respectively; (iii) the inhibitory effects of itraconazole on Ca^{2+} influx were negated largely by addition of LTB_4, while the responses of itraconazole-free control cells were unaffected by the eicosanoid; and (iv) itraconazole antagonized the activity of purified 5'-lipoxygenase (5-LO) in a cell-free system.

In addition to 5-LO, other possible targets of itraconazole which would result in decreased production of LTB_4 by activated neutrophils include cytosolic phospholipase A_2 and FLAP. Cytosolic phospholipase A_2, however, appears to be an improbable target of the anti-mycotic. This contention is based on a previous report that the production of prostaglandin E_2 by human neutrophils activated with the calcium

ionophore, A23187 is unaffected by itraconazole [10], and our own unpublished observations that the generation of arachidonic acid by A23187 (1 μM)-activated human neutrophils is insensitive to the anti-mycotic. Although involvement of FLAP cannot be excluded, the direct inhibitory effects of itraconazole on purified 5-LO suggest that this is the primary target of the anti-mycotic, which is in keeping with the well-recognized inhibitory interactions of pharmacological agents which possess an imidazole moiety with this enzyme [18,19]. It is noteworthy, however, that the inhibitory effects of itraconazole on purified 5-LO were of lesser magnitude than those observed with LTB_4 production by PAF-activated neutrophils. This may simply reflect the strongly lipophilic properties of itraconazole, resulting in intracellular accumulation of the anti-mycotic. Alternatively, other intermediates of arachidonic acid metabolism, such as 5-hydroperoxyeicosatetraenoic acid, may also contribute to Ca^{2+} uptake by PAF-activated neutrophils.

The inhibitory effects of LY255283 on the production of LTB_4 by PAF-activated neutrophils are compatible with a sequence of events which result in a protracted, autocrine activation of the cells. Exposure of neutrophils to the chemoattractant results in mobilization of Ca^{2+} from both intracellular and extracellular reservoirs and consequent production of LTB_4; the eicosanoid in turn interacts with LTB_4 receptors and initiates a secondary, autocrine amplification cascade resulting in sustained Ca^{2+} influx and prolonged activation of the cells.

Unlike the well-recognized involvement of cysteinyl leukotrienes in the pathogenesis of bronchial asthma, the clinical importance of LTB_4-mediated autocrine amplification cascades remains to be established. Very recently, however, LTB_4 has been reported to be involved critically in both the initiation and perpetuation of neutrophil influx and synovial damage in experimental inflammatory arthritis [20,21], while membrane and nuclear receptors for LTB_4 are up-regulated in the lungs of smokers who are susceptible to the development of chronic obstructive pulmonary disease [22]. These reports, together with recent studies which have identified an important role for LTB_4 in the pathogenesis of asthma [23,24], clearly underscore the validity of pharmacological anti-inflammatory strategies which target LTB_4 and its receptors, although no such agents are currently available for widespread clinical use. In this regard it is noteworthy that the fenamate subgroup of non-steroidal anti-inflammatory agents has been reported to inhibit both leukotriene B_4 production and Ca^{2+} uptake by activated human neutrophils; however, the relationship between these two events was not investigated in these studies [25,26].

Notwithstanding conventional anti-fungal activity, the inhibitory effects of itraconazole on LTB_4 production by activated neutrophils described in the current study, together with a possible small contribution from contaminating cells, may also contribute to the usefulness of this and other imidazole anti-mycotics in the prophylaxis and/or therapy of both acute and chronic inflammatory disorders, including septic shock [27], chronic granulomatous disease [5,28] and allergic bronchopulmonary aspergillosis [29]. Moreover, PAF, and by implication LTB_4, is considered to be a key proinflammatory mediator of both hyperacute and chronic inflammatory disorders such as septic shock and bronchial asthma [30,31].

In conclusion, itraconazole at therapeutically attainable concentrations of 0·5 μM [32], antagonizes the activity of 5-LO in PAF-activated neutrophils, preventing the initiation of a secondary amplification mechanism with the potential to intensify and prolong harmful inflammatory responses.

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