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# Activation of the human neutrophil 5-lipoxygenase by exogenous arachidonic acid: involvement of pertussis toxin-sensitive guanine nucleotide-binding proteins

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**1** The mechanism by which incubation of human peripheral blood neutrophils with exogenous arachidonic acid leads to 5-lipoxygenase product synthesis was investigated.

**2** Incubation of neutrophils with arachidonic acid caused a concentration- and time-dependent synthesis of leukotriene $B_4$, its Ω-oxidation products, and 5-hydroxyeicosatetraenoic acid.

**3** The threshold concentration of arachidonic acid required for this effect was equal to, or greater than 3.3 μM and the synthesis increased with up to 33 μM arachidonic acid, the highest concentration used. Synthesis induced by arachidonic acid increased with time for up to 15 min and the major products detected were the Ω-oxidation products of leukotriene $B_4$.

**4** Pre-incubation of neutrophils with pertussis toxin inhibited the synthesis of 5-lipoxygenase products induced by arachidonic acid by 75% or more, but had no effect on either arachidonic acid-induced synthesis of the 15-lipoxygenase product, 15-hydroxyeicosatetraenoic acid, or activation of the 5-lipoxygenase induced by the calcium ionophore A23187.

**5** Pre-incubation of neutrophils with granulocyte-macrophage colony-stimulating factor lead to enhanced leukotriene synthesis in response to arachidonic acid.

**6** These results imply that exogenous arachidonic acid is not only used as a substrate, but also activates the 5-lipoxygenase. Possible mechanisms of action are discussed.

## Introduction

By virtue of their potent effects on cells of the immune system, the 5-lipoxygenase metabolites of arachidonic acid are believed to play important roles in inflammation and allergy (Sirois & Borgeat, 1984; Lewis & Austen, 1984). In particular, leukotriene $B_4$ is a stereospecific activator of neutrophil chemotaxis (Ford-Hutchinson *et al.*, 1980; Malmsten *et al.*, 1980), calcium mobilization (Naccache *et al.*, 1981; White *et al.*, 1983), aggregation (Ford-Hutchinson *et al.*, 1980) and degranulation (Hafstrom *et al.*, 1981). The actions of leukotriene $B_4$ are receptor-dependent (Goldman & Goetzl, 1984) and are blocked by the guanine nucleotide-binding protein (G-protein) inhibitor, pertussis toxin (Becker *et al.*, 1985).

For 5-lipoxygenase product generation to occur, both release of endogenous arachidonic acid and activation of the 5-lipoxygenase are required. These two events are calcium-dependent (Feinstein & Sha'afi, 1983; Borgeat *et al.*, 1983; Samuelsson *et al.*, 1987). Furthermore, it has recently been demonstrated that activation of the 5-lipoxygenase may require its calcium-dependent translocation to the cell membrane (Rouzer & Samuelsson, 1987; Rouzer & Kargman, 1988). Therefore, although the requirement of calcium for release of endogenous substrate may be overcome by supplying exogenous substrate, it is likely that intracellular calcium mobilization is still necessary for significant activation of the 5-lipoxygenase.

Recently, there has been considerable focus on the action of arachidonic acid on neutrophils. At high concentrations (50–100 μM), arachidonic acid stimulates translocation of protein kinase C (McPhail *et al.*, 1984; Cox *et al.*, 1987). At lower concentrations (0.1–50 μM), incubation of neutrophils with arachidonic acid results in mobilization of intracellular

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calcium (Smith et al., 1987; Sha'afi et al., 1980), aggregation (O'Flaherty et al., 1979), production of superoxide (Maridonneau-Parini & Tauber, 1986; Badwey et al., 1987; Clark et al., 1987), and degranulation (Smith et al., 1987; O'Flaherty et al., 1979). Although the exogenous concentrations of arachidonic acid required to activate these cells are considerably higher than those for other compounds such as Paf, fMet-Leu-Phe and leukotriene $B_4$, they may be achieved in inflamed tissue (Hammarstrom et al., 1975; Greaves, 1986).

In light of these previous reports which have characterized arachidonic acid as a neutrophil stimulus, we investigated and characterized the effect of exogenous arachidonic acid on the 5-lipoxygenase. The results indicate that the interaction of exogenous arachidonic acid with the human neutrophil 5-lipoxygenase system involves not only the presentation of substrate, but also activation of the 5-lipoxygenase enzyme that is mediated, at least in part, via pertussis toxin-sensitive G-proteins.

## Methods

### *Cell separation*

Whole blood was obtained by venepuncture, and following dextran sedimentation of erythrocytes, neutrophils were purified by centrifugation on Ficoll-Paque cushions (Boyum, 1968). The erythrocytes remaining in the final pellet were removed by hypotonic lysis with water (20 s), and the cells were resuspended at a final concentration of $10^7$ cells $ml^{-1}$. The entire cell separation was carried out at room temperature. The percentage of neutrophils in the cell suspensions used in this study exceeded 97%, and cell viability as determined by trypan blue exclusion was greater than 98%.

### *Cell incubations*

The cell suspensions were warmed to 37°C for 5 min before incubation with arachidonic acid. Arachidonic acid, A23187 and 15-HPETE were dissolved in dimethylsulphoxide (DMSO) at a 1000 fold higher concentration than was finally required. The final concentration of DMSO present (maximum of 0.2%) did not stimulate detectable leukotriene synthesis. After the desired incubation time with the stimuli, the cells were inactivated by the addition of 500 $\mu$l of an ice-cold mixture of methanol/acetonitrile (50/50, v/v), containing internal standards (25 ng $ml^{-1}$ of prostaglandin $B_2$ and 19-OH prostaglandin $B_2$). The samples were then diluted by the addition of 500 $\mu$l of distilled water, and stored at −20°C until analysed by r.p.-h.p.l.c.

In the experiments involving pertussis toxin, the cell suspensions were pre-incubated with the toxin at a final concentration of 0.25 $\mu$g $ml^{-1}$ for 2 h at 37°C. During this period of time, the cells were gently swirled in a rotary water bath (New Brunswick Scientific, Edison, New Jersey, U.S.A.). After 2 h, the cells were incubated under the desired conditions, inactivated and stored as described above prior to r.p.-h.p.l.c. analysis.

To examine the effect of granulocyte-macrophage colony-stimulating factor (GM-CSF) on 5-lipoxygenase product synthesis in response to arachidonic acid, neutrophils were pre-incubated with 200 pM GM-CSF at room temperature for 1 h. We have previously shown that these incubation conditions are optimal for maximal enhancement of leukotriene synthesis in response to Paf, fMet-Leu-Phe and A23187 (DiPersio et al., 1988). The cells were then warmed to 37°C for 5 min before stimulation with arachidonic acid.

### *Reverse phase-h.p.l.c. analysis of lipoxygenase products*

Analysis of lipoxygenase products was carried out by r.p.-h.p.l.c. as described previously, using an on-line extraction procedure (Borgeat & Picard, 1988). Briefly, the denatured cell suspensions were centrifuged at 600 $g$ for 10 min to remove precipitated material, and the supernatants were injected directly onto a Nova-Pak $C_{18}$ (5 × 100 mm, 4 $\mu$m particles, Waters Millipore) protected with a Guard-Pak ($\mu$BondaPak $C_{18}$) cartridge (Waters Millipore). The various lipoxygenase products were eluted at 1.5 ml $min^{-1}$ with gradients of organic solvents. Elution was monitored with fixed wavelength u.v. photometers at 229 and 280 nm. The compounds were identified by comparison of their retention times with those of authentic standards and by the ratio of their u.v. absorbances at 229 and 280 nm. 5,15 diHETE was measured at 229 nm under the same assay conditions as described above. Quantitation was performed by comparing peak heights of calibrated standards and corresponding compounds in samples after correction for recovery using the internal standard $PGB_2$. The lower level of detection was 0.5–1 ng.

### *Chemicals*

Calcium ionophore A23187 and 5, 8, 11, 14 (all-*cis*) eicosatetraenoic acid (arachidonic acid) were obtained from the Sigma Chemical Company (St. Louis, Missouri, U.S.A.). Hank's Balanced Salt Solu-

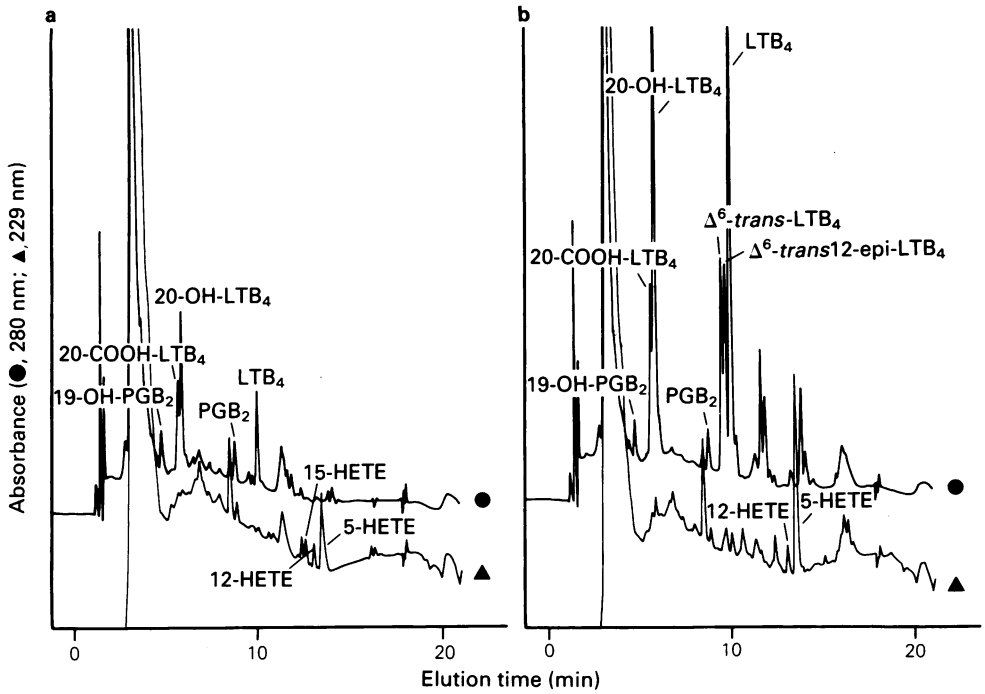


**Figure 1** Reverse-phase h.p.l.c. chromatograms of lipoxygenase products from human neutrophils. Neutrophils from healthy human donors were prepared as described (see Methods), resuspended at a final concentration of $10^7$ cells $ml^{-1}$, and incubated at 37°C for 15 min under the following conditions: (a) addition of 33 $\mu$M arachidonic acid, or (b) addition of 1.0 $\mu$M calcium ionophore A23187. After 15 min, the reactions were stopped by the addition of 500 $\mu$l of an ice-cold mixture of methanol/acetonitrile containing 12.5 ng of the internal standards ($PGB_2$ and 19-OH-$PGB_2$), and diluted to approximately 2 ml with 500 $\mu$l of distilled water. After centrifugation, the entire supernatant was injected. The amounts of $PGB_2$ and 19-OH-$PGB_2$ present in each chromatogram are therefore 12.5 ng. Elution, detection and quantitation were achieved as described (see Methods). Attenuation settings were 0.02 and 0.05 a.u.f.s. for 280 nm and 229 nm respectively. These results are from one experiment that is representative of at least ten others.

tion (HBSS) was from GIBCO (Burlington, Ontario, Canada), Ficoll-Paque was purchased from Pharmacia (Dorval, Québec, Canada) and all solvents were h.p.l.c. grade from Anachemia (Montréal, Canada). Granulocyte-macrophage colony-stimulating factor (GM-CSF) was a generous gift from the Genetics Institute (Cambridge, Massachussetts, U.S.A.), fura-2 was purchased from Molecular Probes Inc. (Eugene, Oregon, U.S.A.). 15S-hydroperoxy-eicosatetraenoic acid (15-HPETE) was synthesized from soy-bean lipoxygenase as previously described (Gardner, 1975), using arachidonic acid as substrate. The fatty acids (arachidonic acid and 15-HPETE) were purified either by silicic acid chromatography or reverse-phase high performance liquid chromatography (r.p.-h.p.l.c.) prior to use. Pertussis toxin was purchased from List Biological Labs Incorporated (Campbell, California, U.S.A.).

## Results

### *Reverse phase-h.p.l.c. lipoxygenase products from arachidonic acid-stimulated human neutrophils*

When human peripheral blood neutrophils were exposed to arachidonic acid they produced leukotriene $B_4$, its Ω-oxidation products (20-OH and 20-COOH-leukotriene $B_4$) and 5-HETE. Occasionally, we also observed synthesis of the non-enzymatically derived all-*trans* isomers of leukotriene $B_4$. However, this was not a consistent response during the course of this study. Figure 1a shows the characteristic arachidonic acid metabolite profile when the cell suspensions were exposed to 33 $\mu$M arachidonic acid for 15 min. Under these conditions, the major product was leukotriene $B_4$ (taking into account the combined synthesis of leukotriene $B_4$ and its Ω-

oxidation products). Production of the 15-lipoxygenase metabolite, 15-HETE was also observed under these conditions as was 12-HETE, which was derived from contaminating platelets. The 5-lipoxygenase product synthesis induced by arachidonic acid was not as powerful as that observed with the calcium ionophore A23187 (Figure 1b), where larger quantities of leukotriene $B_4$ and its metabolites as well as 5-HETE were detected (no 15-HETE was observed when A23187 was used as a stimulus). In addition to these compounds, A23187 also induced the production of the non-enzymatically-derived diHETEs.

*Concentration-dependence of the effect of arachidonic acid on 5-lipoxygenase product synthesis*

To characterize further the effect of exogenous arachidonic acid on 5-lipoxygenase product synthesis, neutrophils were incubated with increasing concentrations of the fatty acid for 15 min (Figure 2). A concentration-dependence was observed, with maximal formation of 5-lipoxygenase products at the highest dose of arachidonic acid used (33 $\mu$M). The threshold in all experiments was between 3.3 $\mu$M and 10 $\mu$M, depending on the donor. The major products were 20-OH- and 20-COOH-leukotriene $B_4$. The synthesis of the 15-lipoxygenase product, 15-HETE, also increased with increasing concentrations of arachidonic acid. Maximal stimulation of leukotriene synthesis by arachidonic acid was observed after an incubation time of 15 min. At this time point, the product profile was characterized by a marked increase in the levels of 20-OH and 20-COOH-leukotriene $B_4$ while levels of 5-HETE, 15-HETE and leukotriene $B_4$ had plateaued (data not shown).

*Effect of pre-incubation of neutrophils with pertussis toxin on the 5-lipoxygenase product synthesis induced by arachidonic acid*

To examine the hypothesis that arachidonic acid not only acts as a substrate but also stimulates 5-lipoxygenase product synthesis via a pertussis toxin-sensitive G-protein, neutrophils were preincubated with 0.25 $\mu g\,ml^{-1}$ of pertussis toxin for 2 h at 37°C before stimulation. These incubation conditions with pertussis toxin have been previously shown to cause significant inhibition of G-protein function in other systems (Becker *et al.*, 1985; Bradford & Rubin, 1985). These experimental conditions were chosen in order to maximize cell viability and experimental reproducibility. Pre-incubation of neutrophils with pertussis toxin prior to stimulation with arachidonic acid significantly inhibited 5-lipoxygenase product synthesis (Figure 3). The inhibitory effect of the toxin was observed even at the highest dose of arachidonic

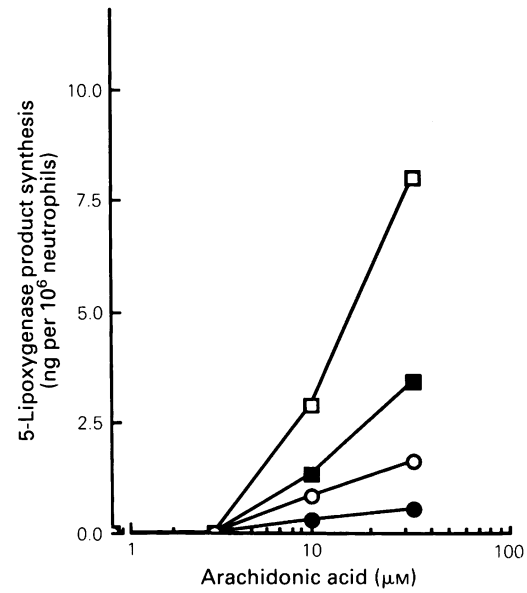


**Figure 2** Concentration-dependence of the transformation of arachidonic acid by human neutrophils into 5-lipoxygenase products. Neutrophils ($10^7$ cells per ml) were stimulated at 37°C for 15 min with increasing concentrations of arachidonic acid. The reactions were then stopped and the resultant 5-lipoxygenase product synthesis was determined by r.p.-h.p.l.c. as described (see Methods): (●) leukotriene $B_4$; (■) 5-HETE; (○) 15-HETE; (□) Σ(20-OH- and 20-COOH-leukotriene $B_4$). The values are expressed as the mean of triplicate determinations from a single experiment which is representative of five others. Standard deviations of the mean were less than 5% of the mean.

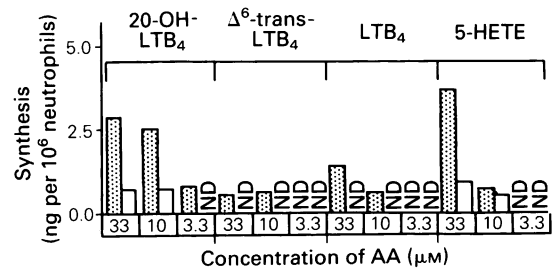


**Figure 3** Effect of pre-treatment of human neutrophils with pertussis toxin (PT) on the 5-lipoxygenase product synthesis induced by arachidonic acid (AA). Neutrophils were pre-incubated for 2 h at 37°C with either 0.25 $\mu g\,ml^{-1}$ PT or an equivalent volume of diluent control, prior to stimulation for 15 min with one of 3 different concentrations of arachidonic acid. The results are expressed as described in the legend for Figure 2. ND = not detectable. Open column with PT; stippled column without PT.

acid used (33 $\mu$M), where production of leukotriene $B_4$ and its all-*trans* isomers was not detected in the cells pre-treated with pertussis toxin. Furthermore, levels of Ω-oxidation products of leukotriene $B_4$ and 5-HETE were reduced to less than 30% of control. The same trend was observed when 10 $\mu$M arachidonic acid was used as a stimulus, and pre-treatment of the cells with pertussis toxin completely inhibited the response to 3.3 $\mu$M arachidonic acid.

In support of a specific effect of pertussis toxin on 5-lipoxygenase product synthesis, pre-incubation of neutrophils with pertussis toxin had no effect on the arachidonic acid-induced synthesis of the 15-lipoxygenase product 15-HETE while the synthesis of 5-lipoxygenase products was inhibited by more than 60% under the same conditions (data not shown).

*Effect of pre-incubation of human neutrophils with pertussis toxin on the transformation of 15-HPETE into 5,15 diHETE induced by A23187 or arachidonic acid*

In order to determine whether the toxin was blocking the activation pathway of the 5-lipoxygenase or directly inhibiting the enzyme, we studied its effect on the activation of the 5-lipoxygenase induced by the calcium ionophore A23187, a non receptor-dependent agonist. In these experiments, 5-lipoxygenase activity was assessed by the measurement of the formation of 5,15 diHETE from exogenous 15-HPETE, a compound previously shown to be an excellent substrate for the 5-lipoxygenase in A23187-stimulated neutrophils (Borgeat *et al.*, 1983). By using an exogenous substrate, the possibility that pertussis toxin reduced 5-lipoxygenase product synthesis via an effect at the level of substrate release was eliminated. Neutrophils were pre-incubated with or without pertussis toxin under the conditions described above, and then incubated with both 10 nM A23187 and 3.3 $\mu$M 15-HPETE for 15 min at 37°C (Figure 4). Similar experiments were carried out on neutrophils incubated with both 3.3 $\mu$M arachidonic acid and 3.3 $\mu$M 15-HPETE. At the concentrations used, neither A23187 nor arachidonic acid stimulated detectable synthesis of 5,15 diHETE when added alone (data not shown). Incubation of neutrophils with 15-HPETE alone resulted in the formation of small amounts of 5,15 diHETE (Figure 4), a response that was unaffected by pre-treatment with pertussis toxin. In combination with 15-HPETE, both arachidonic acid and A23187 induced a significant increase in the conversion of 15-HPETE to 5,15 diHETE, indicating that both compounds stimulate 5-lipoxygenase mediated metabolism of 15-HPETE. Pre-incubation of neutrophils with pertussis toxin had no significant

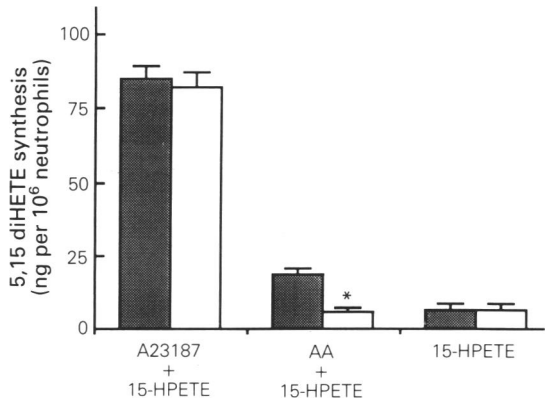


**Figure 4** Effect of pre-treatment of human neutrophils with pertussis toxin (PT) on the activation of the 5-lipoxygenase by the calcium ionophore A23187 or arachidonic acid. Neutrophils were pre-incubated for 2 h at 37°C with 0.25 $\mu$g ml$^{-1}$ PT (open columns) or the equivalent volume of diluent control (solid columns). The cells were then incubated under the following conditions: 3.3 $\mu$M 15-HPETE and 10 nM A23187, 3.3 $\mu$M 15-HPETE and 3.3 $\mu$M arachidonic acid (AA), or 3.3 $\mu$M 15-HPETE alone. After 15 min, the reactions were stopped and the resulting synthesis of 5,15 diHETE measured by r.p.-h.p.l.c. as described (see Methods). Synthesis of 5,15 diHETE under stimulation by either 10 nM A23187 or 3.3 $\mu$M arachidonic acid alone was not detectable. The results are expressed as mean of triplicate determinations of a single experiment that is representative of three; vertical bars show s.d. Significantly different from control values at $^*P < 0.01$ (Student's $t$ test, $n = 3$).

effect on the synthesis of 5,15 diHETE induced by A23187 while under the same conditions, the synthesis of 5,15 diHETE induced by arachidonic acid was completely inhibited.

*Effect of pre-incubation of neutrophils with GM-CSF on leukotriene synthesis in response to arachidonic acid*

Neutrophils ($5 \times 10^6$ ml$^{-1}$) were pre-incubated with GM-CSF (200 pM) for 1 h at room temperature before stimulation with increasing concentrations of arachidonic acid for either 5 or 15 min. The resulting synthesis of leukotriene $B_4$ and its Ω-oxidation products are shown in Figures 5a and b respectively. As shown in the previous results, increased leukotriene synthesis was observed with increasing concentrations of arachidonic acid. In these experiments, the lowest concentration of arachidonic acid with which we could detect leukotriene synthesis was 10 $\mu$M. However, when the cells were pre-incubated with GM-CSF, leukotriene synthesis was consistently detected when the cells were incubated with 3.3 $\mu$M

and sometimes as little as 1.0 μM arachidonic acid (data not shown). Furthermore, pre-treatment of the cells with GM-CSF lead to enhancement (between two and ten fold depending on the concentration of exogenous arachidonic acid) of leukotriene synthesis in response to concentrations of arachidonic acid which induced leukotriene synthesis in the cells untreated with GM-CSF.

## Discussion

It has previously been shown that incubation of several different cell types with exogenous arachidonic acid results in the formation of 12- and 15-lipoxygenase products (Jorg *et al.*, 1982; Goldyne *et al.*, 1984; Lagarde *et al.*, 1984). In the case of 5-lipoxygenase products, however, the situation is different, and it is generally accepted that leukotriene synthesis in neutrophils (the system most extensively studied) requires activation of the enzyme (Borgeat *et al.*, 1983). The calcium ionophore A23187 has been shown to be a strong stimulus of the neutrophil 5-lipoxygenase (Borgeat & Samuelsson, 1979a), whereas inflammatory stimuli such as Paf and fMet-Leu-Phe activate the enzyme to a lesser degree (Salari *et al.*, 1985; Borgeat *et al.*, 1988). It is also recognized that exogenous arachidonic acid induces a small but consistent synthesis of 5-lipoxygenase products in neutrophils (Borgeat & Samuelsson, 1979b). In the present work, through the use of pertussis toxin, we have clearly demonstrated that in addition to being used as substrate, exogenous arachidonic acid also activates the human neutrophil 5-lipoxygenase, thereby reinforcing the concept that 5-lipoxygenase product synthesis requires activation of the 5-lipoxygenase.

Our studies with the G-protein inhibitor, pertussis toxin, provide strong evidence for receptor-dependent activation of the 5-lipoxygenase by exogenous arachidonic acid. G-proteins have been identified as early elements in the excitation response sequence of many cell types, and have been invariably associated with the transduction of receptor occupancy into cellular responses (Gilman, 1984). To date, the G-proteins represent the earliest known molecular components interacting with cell surface receptors (Gilman, 1984). The signalling transduction pathway in human neutrophils contains a G-protein which is linked to the cell surface receptors for many neutrophil agonists including leukotriene $B_4$ (Becker *et al.*, 1985), Paf (Naccache *et al.*, 1986) and fMet-Leu-Phe (Lad *et al.*, 1985), and the association between this G-protein and these receptors is specifically inhibited by pertussis toxin (Becker *et al.*, 1985; Lad *et al.*, 1985; Naccache *et al.*, 1986).

In the present work, the inhibitory effects of the toxin were specifically directed towards the stimulation of 5-lipoxygenase product synthesis by arachidonic acid, since we did not observe inhibition of the synthesis of the 15-lipoxygenase product 15-HETE. In addition, using the calcium ionophore A23187 as a 5-lipoxygenase stimulus, we were able to determine that pertussis toxin did not directly inhibit the 5-lipoxygenase enzyme. In this assay, we used the hydroperoxy fatty acid 15-HPETE as a substrate and measured its conversion into 5,15 diHETE as an indication of 5-lipoxygenase activation (Borgeat *et al.*, 1983). 15-HPETE was metabolized by neutrophils to 5,15 diHETE only to a minor extent unless A23187 or arachidonic acid were also added (Figure 5). Pertussis toxin had no effect on the ability of A23187 to activate the 5-lipoxygenase and stimulate

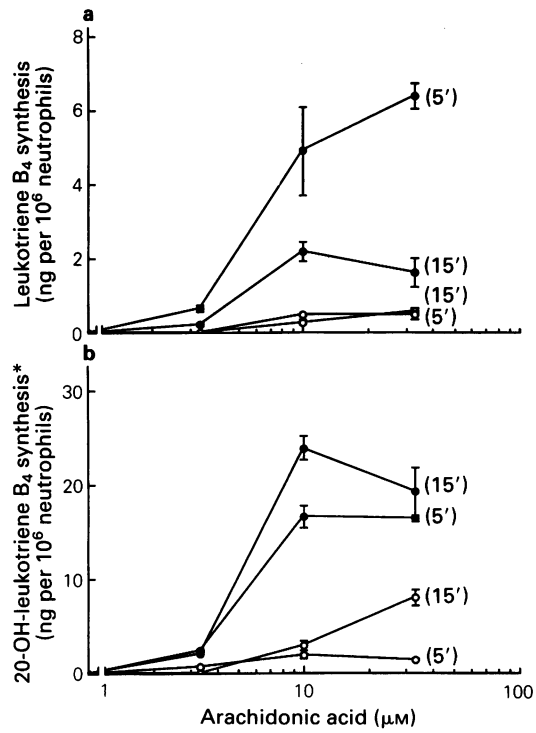


**Figure 5** The effect of pre-incubation of neutrophils with GM-CSF on the synthesis of 5-lipoxygenase products induced by exogenous arachidonic acid. Neutrophils were pre-treated with 200 pM GM-CSF for 1 h at room temperature prior to stimulation with increasing concentrations of arachidonic acid for either 5 or 15 min: (●) plus GM-CSF; (○) without GM-CSF. The resultant synthesis of leukotriene $B_4$ and 20-OH-leukotriene $B_4^*$ (i.e. Σ20-OH- and 20-COOH-leukotriene $B_4$) are shown in (a) and (b) respectively. The results are expressed as mean of triplicate determinations from one experiment that is representative of four; vertical bars show s.d.

the conversion of 15-HPETE into 5,15 diHETE, indicating that it does not directly inhibit the 5-lipoxygenase or interfere with activation of the 5-lipoxygenase by A23187. It is also important to note that arachidonic acid stimulated the transformation of 15-HPETE into 5,15 diHETE, and furthermore, that this transformation was inhibited by pertussis toxin. These results confirm our previous observation that pertussis toxin inhibits arachidonic acid-induced leukotriene synthesis and further supports our hypothesis that arachidonic acid activates the 5-lipoxygenase via a G-protein-dependent mechanism. Our results demonstrating that pre-incubation of neutrophils with GM-CSF enhanced the synthesis of leukotrienes in response to arachidonic acid also provide support to our hypothesis that arachidonic acid stimulates the 5-lipoxygenase, since we and others have recently shown that pre-incubation of neutrophils with GM-CSF enhances the ability of several other neutrophil agonists such as fMet-Leu-Phe (DiPersio *et al.*, 1988), C5a (Dahinden *et al.*, 1988) and Paf to stimulate leukotriene synthesis.

In the present study, it has proved difficult to establish the mechanism(s) involved in the activation of the 5-lipoxygenase and the stimulation of leukotriene synthesis by arachidonic acid. However, considering the established role of calcium in activation of the 5-lipoxygenase, our recent investigations on the effects of arachidonic acid on the mobilization of intracellular calcium, and the observation that leukotriene $B_4$ can also activate the 5-lipoxygenase, it is possible that activation of the 5-lipoxygenase by arachidonic acid is related to its ability to promote calcium mobilization, and involves activation of the leukotriene $B_4$ receptor, probably by a positive feedback loop due to *de novo* synthesis of leukotriene $B_4$ (Figure 6). The latter mechanism could be due to transformation of exogenous arachidonic acid into leukotriene $B_4$ by 5-lipoxygenase which is already active, or by a direct effect of arachidonic acid on intracellular calcium stores (Beaumier *et al.*, 1987), resulting in the activation of the 5-lipoxygenase, leukotriene $B_4$ synthesis from exogenous arachidonic acid, and subsequent activation of the leukotriene $B_4$ receptor. In both cases, stimulation of the leukotriene $B_4$ receptor would enhance the activation of the 5-lipoxygenase and consequently, leukotriene synthesis from exogenous arachidonic acid. In a separate study, we have obtained evidence that both of these mechanisms exist and account for the effect of arachidonic acid on calcium mobilization. Additional support for this hypothesis arises from the fact that the characteristics of arachidonic acid and leukotriene $B_4$ as neutrophil agonists are strikingly similar. Indeed, stimulation of human neutrophils by leukotriene $B_4$ elicits a similar range of functions as those previously described for arachidonic acid

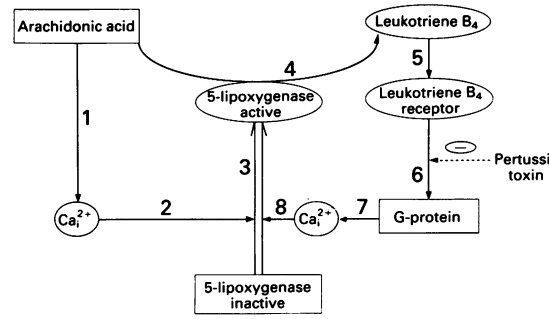


**Figure 6** Possible mechanism for the stimulation of the 5-lipoxygenase by exogenous arachidonic acid. Numbers represent the postulated sequence of events. This scheme implies a critical role for calcium in the activation of the 5-lipoxygenase, and also that leukotriene $B_4$ activates this enzyme. In this scheme, the triggering event for the induction of 5-lipoxygenase product synthesis by arachidonic acid would be the direct effect of arachidonic acid on intracellular levels of calcium, which in turn would lead to the activation of the 5-lipoxygenase (events 1 to 3). Formation of leukotriene $B_4$ would occur, leading to a positive feedback effect of leukotriene $B_4$ on the activation of the 5-lipoxygenase (events 4 to 9). The positive feedback could eventually be switched off due to desensitization of the leukotriene $B_4$ receptor by leukotriene $B_4$.

(O'Flaherty *et al.*, 1979; Hafstrom *et al.*, 1981; White *et al.*, 1983; Smith *et al.*, 1987). Furthermore, and most importantly, cellular responses to both leukotriene $B_4$ (Becker *et al.*, 1985) and arachidonic acid are sensitive to pertussis toxin. The positive feedback loop could finally be switched off as a consequence of desensitization of the leukotriene $B_4$ receptor by leukotriene $B_4$, as has previously been reported (Sha'afi *et al.*, 1981).

The ability of arachidonic acid to activate the human neutrophil 5-lipoxygenase may be of considerable patho-physiological importance *in vivo*. The fact that micromolar concentrations of arachidonic acid are required to observe these effects *in vitro* does not diminish the importance of this finding since large quantities of arachidonic acid have been described in inflammatory lesions (Hammarstrom *et al.*, 1975; Greaves, 1986). Furthermore, in this work, we have demonstrated that exposure of neutrophils to GM-CSF *in vitro* increases the sensitivity of these cells to arachidonic acid (Figure 5). If GM-CSF has the same effect *in vivo*, it may have a dramatic influence on inflammation by increasing the activation of the 5-lipoxygenase not only in response to agents such as Paf and C5a (Dahinden *et al.*, 1988), but also arachidonic acid.

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## References

BADWEY, J.A., CURNUTTE, J.T., ROBINSON, J.M., BERDE, C.B., KARNOVSKY, M.J. & KARNOVSKY, M.L. (1987). Effects of free fatty acids on release of superoxide and on change of shape by human neutrophils. Reversibility by albumin. *J. Biol. Chem.*, **259**, 7870–7877.

BEAUMIER, L., FAUCHER, N. & NACCACHE, P.H. (1987). Arachidonic acid-induced release of calcium in permeabilized human neutrophils. *FEBS Letts.*, **221**, 289–292.

BECKER, E.L., KERMODE, J.C., NACCACHE, P.H., YASSIN, R., MARSH, M.L., MUNOZ, J.J. & SHA'AFI, R.I. (1985). The inhibition of neutrophil granule enzyme secretion and chemotaxis by pertussis toxin. *J. Cell Biol.*, **100**, 1641–1646.

BORGEAT, P. & PICARD, S. (1988). 19-hydroxy-prostaglandin $B_2$ as an internal standard for on-line extraction-high-performance liquid chromatography analysis of lipoxygenase products. *Anal. Biochem.*, **171**, 283–289.

BORGEAT, P. & SAMUELSSON, B. (1979a). Arachidonic acid metabolism in polymorphonuclear leukocytes: Effects of ionophore A23187. *Proc. Natl. Acad. Sci. U.S.A.*, **76**, 2148–2152.

BORGEAT, P. & SAMUELSSON, B. (1979b). Metabolism of arachidonic acid in polymorphonuclear leukocytes. Structural analysis of novel hydroxylated compounds. *J. Biol. Chem.*, **254**, 7865–7869.

BORGEAT, P., FRUTEAU DE LACLOS, B. & MACLOUF, J. (1983). New concepts in the modulation of leukotriene synthesis. *Biochem. Pharmacol.*, **32**, 381–387.

BORGEAT, P., NADEAU, M., ROULEAU, G., SIROIS, P., BRAQUET, P. & POUBELLE, P.E. (1988). PAF-induced leukotriene synthesis in human polymorphonuclear leukocytes: Inhibition by Ginkgolide B (BN52021) In *The Ginkgolides: Chemistry, Biology, Pharmacology and Clinical Aspects.* ed. Braquet, P. pp. 171–180. Barcelona, Spain: J.R. Prous Sciences Publishers.

BOYUM, A. (1968). Isolation of mononuclear cells and granulocytes from human blood. *Scand. J. Clin. Lab. Invest.*, **97**(S29), 77–89.

BRADFORD, P.G. & RUBIN, R.P. (1985). Pertussis toxin inhibits chemotactic factor induced phospholipase C stimulation and lysosomal enzyme release in rabbit neutrophils. *FEBS Lett.*, **183**, 317–320.

CLARK, R.A., LEIDAL, K.G., PEARSON, D.W. & NAUSEEF, W.M. (1987). NADPH oxidase of human neutrophils. Subcellular localization and characterization of an arachidonate-activatable superoxide-generating system. *J. Biol. Chem.*, **262**, 4065–4074.

COX, J.A., JENG, A.U., BLUMBERG, P.M. & TAUBER, A.I. (1987). Comparison of subcellular activation of the human neutrophil NADPH-oxidase by arachidonic acid, sodium dodecyl sulfate (SDS), and phorbol myristate acetate. *J. Immunol.*, **138**, 1884–1888.

DAHINDEN, C.A., ZINGG, J., MALY, F.E. & WECK, A.L. (1988). Leukotriene production in human neutrophils primed by recombinant human granulocyte/macrophage colony-stimulating factor and stimulated with the complement component C5a and FMLP as second signals. *J. Exp. Med.*, **167**, 1281–1295.

DiPERSIO, J.F., NACCACHE, P.H., BORGEAT, P., GASSON, J.C., NGUYEN, M-H. & McCOLL, S.R. (1988). Granulocyte-macrophage colony-stimulating factor enhances the production of leukotrienes in human neutrophils in response to A23187 and N-formyl-methionyl-leucyl-phenylalanine. *Prostaglandins*, **36**, 673–691.

FEINSTEIN, M.B. & SHA'AFI, R.I. (1983). Role of calcium in arachidonic acid metabolism and in the actions of arachidonic acid-derived metabolites. In *Calcium and Cell function*, ed. Cheung, W.Y. pp. 337–376. New York: Academic Press.

FORD-HUTCHINSON, A.W., BRAY, M.A., DOIG, M.V., SHIPLEY, M.E. & SMITH, M.J.H. (1980). Leukotriene $B_4$, a potent chemokinetic and aggregating substance released from polymorphonuclear leukocytes. *Nature*, **286**, 264–265.

GARDNER, H.W. (1975). Isolation of a pure isomer of linoleic acid hydroperoxide. *Lipids*, **10**, 248–252.

GILMAN, A.G. (1984). Guanine nucleotide binding regulatory proteins and dual control of adenylate cyclase. *Cell*, **36**, 577–579.

GOLDMAN, D.W. & GOETZL, E.J. (1984). Heterogeneity of human polymorphonuclear leukocyte receptors for leukotriene $B_4$. Identification of a subset of high affinity receptors that transduce the chemotactic response. *J. Exp. Med.*, **159**, 1027–1041.

GOLDYNE, M., BURRISH, G.F., POUBELLE, P.E. & BORGEAT, P. (1984). Arachidonic acid metabolism among human mononuclear leukocytes. *J. Biol. Chem.*, **259**, 8815–8819.

GREAVES, M.W. (1986). Leukotrienes in psoriasis and other dermatoses. In *The Leukotrienes: their Biological Significance.* ed. Piper, P. pp. 175–184. New York: Raven Press.

HAFSTROM, I., PALMBLAD, J., MALMSTEN, C.L., RADMARK, O. & SAMUELSSON, B. (1981). Leukotriene $B_4$ – a stereospecific stimulator for release of lysosomal enzymes from neutrophils. *FEBS Lett.*, **130**, 146–148.

HAMMARSTROM, S., HAMBERG, M., SAMUELSSON, B., DUELL, E.A., STAWISKI, M. & VOORHEES, J.J. (1975). Increased concentration of non-esterified arachidonic acid, 12L-hydroxy-5,8,10,14-eicosatetraenoic acid, prostaglandin E, and prostaglandin $F_2$alpha in epidermis of psoriasis. *Proc. Natl. Acad. Sci. U.S.A.*, **72**, 5130–5134.

JORG, A., HENDERSON W.R., MURPHY, R.C. & KLEBANOFF, S.J. (1982). Leukotriene generation by eosinophils. *J. Exp. Med.*, **155**, 390–402.

LAD, P.M., OLSON, C.V., GREWAL, I.S. & SCOTT, S.J. (1985). A pertussis toxin-sensitive GTP-binding protein in the human neutrophil regulates multiple receptors, calcium mobilization and lectin-induced capping. *Proc. Natl. Acad. Sci. U.S.A.*, **82**, 8643–8647.

LAGARDE, M., CROSET, M., BOUKHACHE, D., GREFFE, A., DECHAUNNE, M. & RENAUD, S. (1984). Lipoxygenase activity of intact platelets. *Prost. Leuk. Med.*, **13**, 61–66.

LEWIS, R.A. & AUSTEN, K.F. (1984). The biologically active leukotrienes. Biosynthesis, metabolism, receptors, function and pharmacology. *J. Clin. Invest.*, **73**, 889–897.

MALMSTEN, C.L., PALMBLAD, J., UDEN, A.M., RADMARK, O., ENGSTEDT, L. & SAMUELSSON, B. (1980). Leukotriene $B_4$: a highly potent and stereospecific factor stimulating migration in PMNs. *Acta Physiol. Scand.*, **110**, 449–451.

MARIDONNEAU-PARINI, I. & TAUBER, A.I. (1986). Activation of NADPH-oxidase by arachidonic acid involves phospholipase $A_2$ in intact human neutrophils but not in the cell free system. *Biochem. Biophys. Res. Commun.*, **138**, 1099–1105.

McPHAIL, L.C., CLAYTON, C.C. & SNYDERMAN, R. (1984). A potential second messenger role for unsaturated fatty acids: activation of Ca2+-dependent protein kinase. *Science*, **224**, 622–625.

NACCACHE, P.H., MOLSKI, M.M., VOLPI, M., SHEWFCYK, J., MOLSKI, T.F.P., LOEW, L., BECKER, E.L. & SHA'AFI, R.I. (1986). Biochemical events associated with the stimulation of rabbit neutrophils by platelet-activating factor. *J. Leuk. Biol.*, **40**, 533–548.

NACCACHE, P.H., SHA'AFI, R.I., BORGEAT, P. & GOETZL, E.J. (1981). Mono- and dihydroxy-eicosatetraenoic acids alter calcium homeostasis in rabbit neutrophils. *J. Clin. Invest.*, **64**, 1584–1587.

O'FLAHERTY, J.T., SHOWELL, H.J., BECKER, E.L. & WARD, P.A. (1979). Neutrophil aggregation and degranulation. Effect of arachidonic acid. *Am. J. Pathol.*, **95**, 435–444.

ROUZER, C.A. & SAMUELSSON, B. (1987). Reversible, calcium-dependent membrane association of human leukocyte 5-lipoxygenase. *Proc. Natl. Acad. Sci. U.S.A.*, **84**, 7393–7397.

ROUZER, C.A. & KARGMAN, S. (1988). Translocation of 5-lipoxygenase to the membrane in human leukocytes challenged with ionophore A23187. *J. Biol. Chem.*, **263**, 10980–10988.

SALARI, H., BRAQUET, P., NACCACHE, P.H. & BORGEAT, P. (1985). Characterization of effect of N-formyl-methionyl-leucyl-phenylalanine on leukotriene synthesis in human polymorphonuclear leukocytes. *Inflammation*, **9**, 127–138.

SAMUELSSON, B., DAHLEN, S.E., LINDGREN, J.A., ROUZER, C.A. & SERHAN, C.N. (1987). Leukotrienes and lipoxins: structures, biosynthesis and biological effects. *Science*, **237**, 1171–1176.

SHA'AFI, R.I., MOLSKI, T.F.P., BORGEAT, P. & NACCACHE, P.H. (1981). Deactivation of the effects of F-Met-Leu-Phe and leukotriene $B_4$ on calcium mobilization in rabbit neutrophils. *Biochem. Biophys. Res. Commun.*, **103**, 766–773.

SHA'AFI, R.I., NACCACHE, P.H., ALOBAIDI, T., MOLSKI, T.F.P. & VOLPI, M. (1980). Effect of arachidonic acid and the chemotactic factor fMet-Leu-Phe on cation transport in rabbit neutrophils. *J. Cell. Physiol.*, **106**, 215–223.

SIROIS, P. & BORGEAT, P. (1984). Pharmacology of the leukotrienes. *J. Pharmacol.*, **15** (Suppl.), 53–68.

SMITH, R.J., SAM, L.M., JUSTEN, J.M., LEACH, K.L. & EPPS, D.E. (1987). Human polymorphonuclear neutrophil activation with arachidonic acid. *Br. J. Pharmacol.*, **91**, 641–649.

WHITE, J.R., NACCACHE, P.H., MOLSKI, T.F.P., BORGEAT, P. & SHA'AFI, R.I. (1983). Direct demonstration of increased intracellular concentration of free calcium in rabbit and human neutrophils following stimulation by chemotactic factors. *Biochem. Biophys. Res. Commun.*, **113**, 44–50.

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