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AURANOFIN STIMULATES LTA HYDROLASE AND INHIBITS 5-LIPOXYGENASE/LTA SYNTHASE ACTIVITY OF ISOLATED HUMAN NEUTROPHILS

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Abstract—The effect of auranofin on the 5-lipoxygenase pathway was studied in human neutrophils stimulated with either fMLP or A23187 (with or without arachidonic acid). The synthesis of leukotriene B_4 (LTB_4), 5-HETE and the all-trans isomers of LTB_4 was measured by HPLC. At low concentrations (0.5–2.0 μM), auranofin stimulated LTB_4 synthesis, but inhibited it at higher concentrations (100% inhibition at $<10\ \mu$M). In contrast auranofin caused dose-dependent inhibition of the synthesis of 5-HETE and the all-trans isomers of LTB_4. Similar observations were made with each agonist. The stimulation of LTB_4 synthesis and inhibition of the trans isomer production suggests that auranofin at low concentrations stimulates LTA hydrolase—the enzyme that converts LTA_4 to LTB_4, whereas the inhibition of synthesis of all lipoxygenase products at higher auranofin concentrations, suggests inhibition of 5-lipoxygenase/LTA synthase.

Leukotrienes are potent inflammatory mediators generated by a variety of cells including neutrophil polymorphonuclear leukocytes (PMN) and have been implicated in the pathogenesis of a number of inflammatory conditions including rheumatoid arthritis (RA). In human PMN, the 5-lipoxygenase pathway of leukotriene synthesis leads mainly to the production of the chemoattractant leukotriene B_4 (LTB_4†) and the hydroxy acid 5-hydroxyeicosatetraenoic acid (5-HETE), (Fig. 1).

Auranofin (2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranosato-*S*-[triethylphosphine]gold) (AF) has been used widely in the treatment of RA and has been shown *in vitro* to modulate a number of phagocyte functions [1]. While there have been a number of studies demonstrating that AF inhibits human PMN leukotriene synthesis, there are considerable discrepancies between the results, and the studies have not determined the specific site(s) of action of AF in the 5 lipoxygenase (5-LO) pathway (see Fig. 1). For example, in A23187-stimulated PMN, Elmgreen *et al.* [2] found 50% inhibition of LTB_4 and 5-HETE synthesis at 17.4 μM AF and Honda *et al.* [3] found that 8 μM AF was required to inhibit LTB_4 synthesis by 41%, respectively, although there was no inhibition at 6 μM AF. Similarly, Hafstrom *et al.* [4] also found no effect of 6 μM AF on LTB_4, 5-HETE and 15-HETE production, and Herlin *et al.* [5] found no effect of AF up to 11 μM on either LTB_4 or 15-HETE synthesis. When the chemotactic peptide fMLP was used to stimulate PMN, Parente *et al.* [6] found that 20 μM AF (4 μg/mL elemental gold) caused only a 33% inhibition of LTB_4 synthesis and a 75% inhibition of LTC_4 synthesis, in contrast to the 70% inhibition of LTB_4 synthesis at 11 μM AF found by Herlin *et al.* [5]. The reasons for these differences are unknown. Possibilities include the use of different PMN isolation procedures, different experimental conditions (e.g. different PMN counts, buffers and pH), or different techniques to quantify the leukotrienes (e.g. HPLC [3–5], RIA [6] and TLC-radiolabels [2]).

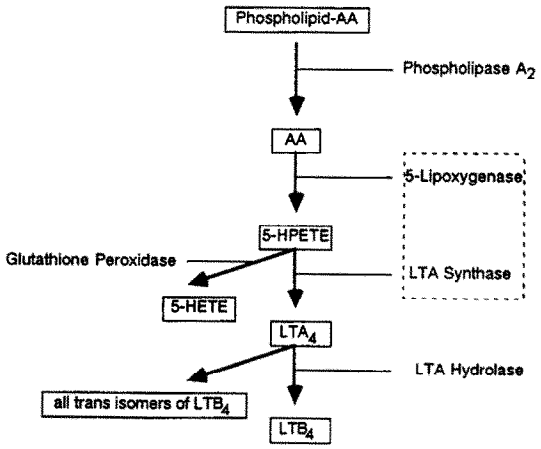

Fig. 1. The 5-lipoxygenase pathway of PMN. Phospholipase A_2 releases arachidonic acid (AA) from the membrane phospholipids. This AA is then metabolized by 5-lipoxygenase to 5-HPETE, which is further metabolized by LTA synthase to LTA_4 and by LTA hydrolase to LTB_4. 5-HPETE is also converted to 5-HETE in the presence of glutathione peroxidase, and LTA_4 is converted nonenzymatically to the all-trans isomers of LTB_4. 5-Lipoxygenase and LTA hydrolase probably reside on the same protein. The relative proportions of these metabolites reflects the relative activities of the enzymes in the pathway.

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† Abbreviations: AA, arachidonic acid; AF, auranofin; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; PMA, phorbol 12-myristate 13-acetate; 5-HETE, (5*S*)-hydroxy-6,8,11,14-eicosatetraenoic acid; LTB_4, (5*S*,12*R*)-dihydroxy,6,8,10,14-eicosatetraenoic acid; DMSO, dimethylsulfoxide.

Furthermore, in one of the above studies [6], LTB_4 and LTC_4 synthesis was significantly ($P < 0.05$) enhanced by 2.5 μM AF, although the authors made no comment. This is important because in a number of studies of the effects of AF on the functions of a variety of cells, AF has biphasic effects (i.e. stimulation at low concentrations and inhibition at higher concentrations). For example, AF has biphasic effects on PMN superoxide production [7, 8], PMN aggregation, β-glucuronidase and lysozyme secretion [5] and protein phosphorylation in PMN [8] and platelets [9].

We have therefore re-examined the effects of AF on the 5-LO pathway over a wider and more effective concentration range than previously reported, using several different stimuli. Furthermore, we used an assay that measures 5-HETE, LTB_4 and the all-trans isomers of LTB_4, so that information on the differential effects of AF on the activities of the each of the enzymes of the 5-LO pathway (Fig. 1) could be obtained.

MATERIALS AND METHODS

Chemicals. Calcium ionophore A23187 was purchased from Boehringer Mannheim (Mannheim, F.R.G.); *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), arachidonic acid (free acid) and phorbol 12-myristate 13-acetate (PMA) from the Sigma Chemical Co. (St Louis, MO). Auranofin was supplied by courtesy of Smith Kline and French Laboratories (Sydney, Australia) Ltd. LTB_4 was purchased from Upjohn (Kalamazoo, MI): 15-HETE was synthesized from arachidonic acid using 15-lipoxygenase [10], and 5-HETE was a gift from the Organic Chemistry Department at the University of Adelaide. Stock solutions of AF, arachidonic acid (AA), A23187, LTB_4, 5-HETE and 15-HETE were prepared in methanol. AF, AA and A23187 were all added to experimental solutions from methanol stocks or dilutions in methanol, and in the case of controls, the equivalent volume of methanol was added. fMLP was stored as a stock solution of 20 mM in DMSO at 4° and was diluted in modified Dulbecco's to 80 μM immediately prior to each experiment.

Isolation of human PMN. PMN were isolated from peripheral blood of healthy volunteers (N = 6), and patients (N = 5) with rheumatoid arthritis who were about to commence on AF therapy. The blood (30 mL) was collected by venepuncture and anticoagulated with 6 mL 4.5% EDTA (in water), pH 7.4 and sedimented with 6 mL 6% Dextran (Pharmacia, Uppsala, Sweden) for 30 min at 37°. The white cell-rich supernatant was then layered onto double Percoll (Pharmacia) layers with specific gravities of 1.070 and 1.092, and spun at 450 *g* for 20 min. The PMN were collected from the interface between the two Percoll layers, washed twice in Ca^{2+}, Mg^{2+}-free Dulbecco's, before final suspension at a cell count of $1–2 \times 10^6$ PMN/mL, in modified Dulbecco's buffer. This buffer contained 138 mM NaCl, 2.7 mM KCl, 16.2 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 0.6 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 7.5 mM glucose at pH 7.4. Using this procedure, cell suspensions that contained greater than 95% PMN, were obtained.

Measurement of activity of 5-lipoxygenase pathway. One millilitre of PMN ($1–2 \times 10^6$/mL) in modified Dulbecco's was incubated without AF (controls) or with AF for 10 min at 37°, prior to activation with either 4 μM fMLP plus 5 μM AA, 5 μM A23187 or 0.5 μM A23187 plus 5 μM AA. The concentrations of the different stimuli were chosen because they induce maximal activation of LTB_4 synthesis. Leukotriene synthesis was stopped after 5 min by the addition of 250 μL of 100 mM citric acid (which lowered the pH to less than 3). Seventy nanograms of 15-HETE was added as internal standard. (Note: no 15-HETE was produced by PMN, prepared using our separation technique and experimental conditions.) The leukotrienes and hydroxy acids were extracted into chloroform by the addition of 2.5 mL of 70:30 chloroform:methanol to the acidified cell suspension. The mixture was then vortexed for 30 sec and centrifuged at 800 *g* for 10 min. The bottom chloroform layer was removed and evaporated to dryness in a Savant (2000) centrifugal evaporator. The extract was then reconstituted in 100 μL of methanol for injection into the HPLC.

The HPLC was a Waters Millipore system with a Wisp 710B autoinjector and a 490 multichannel variable wavelength detector. The column was a Waters C_{18} Novapak. The mobile phase consisted of a 72:28:0.02 mixture of methanol:water:acetic acid, with the pH adjusted to 5.6 with ammonium hydroxide. Twenty-five microlitres of sample was injected, the flow rate was 1.5 mL/min. Under these conditions we were able to quantify the all trans isomers of LTB_4, LTB_4, and 5-HETE, which eluted from the column at approximately 4.0, 5.2 and 20 min, respectively. 15-HETE eluted at 16 min. LTB_4 and its isomers were detected at 270 nm: at 10 min the 490 detector switched to 234 nm for the detection of 15-HETE and 5-HETE. Each component was quantified by the ratio of component peak height to peak height of 15-HETE. These ratios were then compared with ratios obtained from 5 point standard curves (0–50 ng for LTB_4 and 0–250 ng for 5-HETE). The isomers of LTB_4 were quantified using the LTB_4 standard curve.

Data presentation. Results expressed as mean ± SD of four replicates. Figures 2–4 are data from one experiment from one individual and are representative of the results obtained from all other individuals, both control and patients. Significance of results was tested using the unpaired Student's *t*-test.

RESULTS

Preincubation of PMN with AF (0.5–10 μM) at 37° for 10 min prior to stimulation with either 0.5 μM A23187 plus 5 μM AA or 4 μM fMLP plus 5 μM AA, resulted in a biphasic effect on LTB_4 synthesis. Significant enhancement ($P < 0.05$) was seen at concentrations in the range 0.5 to 2 μM AF and inhibition at higher concentrations (Figs 2 and 3). The enhancing effect of AF on LTB_4 was maximal at

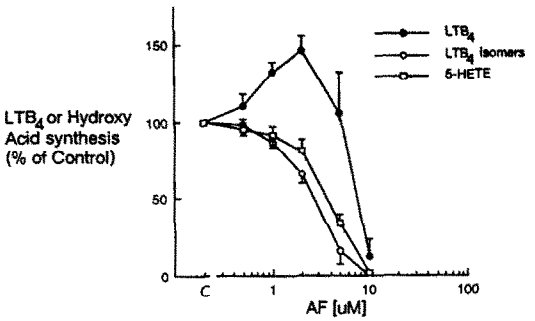

Fig. 2. Effect of increasing *in vitro* AF concentrations on 5-lipoxygenase pathway metabolite synthesis, when PMN were activated with 0.5 μM A23187 plus 5.0 μM AA. PMN were pretreated with AF for 10 min prior to activation. Control levels for LTB_4, LTB_4 isomers and 5-HETE were 13.7 ± 1.1, 22.9 ± 2.6 and 126 ± 7 ng/10^6 PMN, respectively. Values are mean ± SD of four replicate samples.

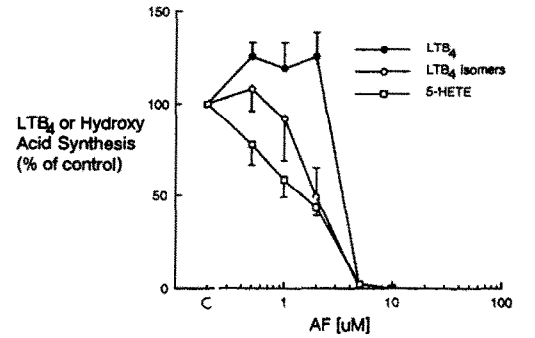

Fig. 3. Effect of increasing *in vitro* AF concentrations on 5-lipoxygenase pathway metabolite synthesis when PMN were activated with 4.0 μM fMLP plus 5.0 μM AA. Control levels for LTB_4, LTB_4 isomers and 5-HETE were 15.0 ± 1.4, 14.8 ± 1.0 and 147 ± 9 ng/10^6 PMN, respectively. Values are mean ± SD of four replicates.

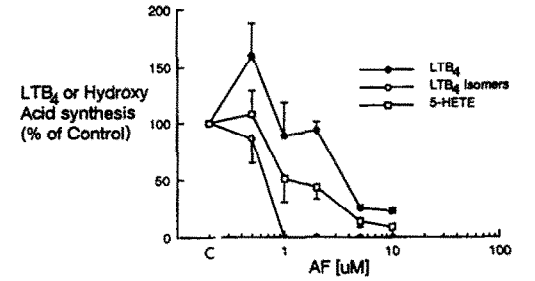

Fig. 4. Effect of increasing *in vitro* AF concentrations on 5-lipoxygenase pathway metabolite synthesis when PMN were activated with 5.0 μM A23187. Control levels for LTB_4, LTB_4 isomers and 5-HETE were 12.4 ± 2.3, 8.0 ± 1.5 and 51 ± 8 ng/10^6 PMN, respectively. Values are mean ± SD of four replicates.

$\approx 2\,\mu$M. In contrast, AF, over the same concentration range, caused only dose-dependent inhibition of the production of 5-HETE and the all-trans isomers of LTB_4 (Figs 2 and 3). Similar results were obtained when 5.0 μM A23187 without exogenous AA was used as the stimulus (Fig. 4), except that enhancement ($P < 0.03$) of LTB_4 synthesis was only seen at 0.5 μM, and not at 1–2 μM. Eighty nM PMA, a concentration that maximally activates protein kinase C (PKC) in PMN [8], also enhanced LTB_4 synthesis when 1 μM fMLP and 5 μM AA was the stimulus (Table 1). Comparison of the dose–response curves obtained with the agonists fMLP and A23187, shows that synthesis of leukotrienes stimulated by fMLP was more sensitive to inhibition by AF than synthesis stimulated by A23187 (Figs 2 and 3). Identical results were obtained using PMN from five patients with rheumatoid arthritis (not shown) indicating that PMN from these patients respond to AF in a similar manner to PMN from control individuals.

DISCUSSION

These results demonstrate that regardless of the stimulus used, AF caused an increase in LTB_4 synthesis at low concentrations of AF and complete inhibition at higher concentrations. Production of 5-HETE and the all-trans isomers of LTB_4 was not stimulated by low AF concentrations, and these were either unaffected or decreased over the concentration range where AF enhanced LTB_4 synthesis. These results suggest that AF at low concentrations does not affect the 5-lipoxygenase or LTA synthase enzymes, but increases the activity of LTA hydrolase. Enhancement of LTA hydrolase would be expected to reduce the amount of LTA_4 available for conversion to the all-trans isomers and also increase consumption of 5-HPETE resulting in the observed modest reduction in 5-HETE. Significant ($P < 0.05$) enhancement of LTB_4 synthesis by PMN was demonstrated in a previous report [6] where a 17% increase was obtained with 2.5 μM AF. Other investigators [2–4] may have also observed a similar enhancement if they had studied lower concentrations of AF.

Increased LTB_4 and decreased isomer production in the presence of PMA (Table 1) are also consistent with a stimulation of LTA hydrolase activity. There is evidence that this effect of PMA effect is mediated by phosphorylation of LTA hydrolase [11]. Low dose AF enhances protein phosphorylation in human PMN [8] and platelets [9] and might also increase LTA hydrolase activity by altering its phosphorylation state. The mechanism of the enhancing effect of AF on protein phosphorylation is unknown and does not appear to be mediated by a direct effect on PKC [12].

In the presence of higher concentrations of AF ($\geq 2\,\mu$M), synthesis of LTB_4, its isomers and 5-HETE falls rapidly, pointing to inhibition of either PLA_2 or 5-lipoxygenase. The addition of exogenous AA in the presence of either A23187 or fMLP made it possible to distinguish effects of AF on the activity of PLA_2, as exogenous AA is metabolized in preference to PLA_2-released AA. The finding that AF had biphasic effects in the presence of exogenous AA suggests that these effects of AF occur at some

Table 1. Effect of AF and PMA on PMN 5-lipoxygenase metabolite synthesis

No.	Experimental conditions		LTB_4	LTB_4 isomers (ng/10^6 PMN)	5-HETE
1	0.5 μM A23187 + 5 μM AA		13.7 ± 1.1	23.0 ± 2.3	126.0 ± 7
	0.5 μM A23187 + 5 μM AA	+2.0 μM AF	20.1 ± 1.4	15.1 ± 1.4	101.0 ± 9
2	5.0 μM A23187		12.3 ± 2.3	8.0 ± 1.5	51.0 ± 8
	5.0 μM A23187	+0.5 μM AF	19.7 ± 3.5	7.0 ± 1.7	55.0 ± 11
3	4.0 μM fMLP + 5 μM AA		7.5 ± 2.4	6.8 ± 2.4	66.0 ± 15
	4.0 μM fMLP + 5 μM AA	+2.0 μM AF	11.4 ± 4.0	4.2 ± 2.0	41.0 ± 9
4	1.0 μM fMLP + 5 μM AA		3.5 ± 0.3	5.4 ± 0.7	53.0 ± 5
	1.0 μM fMLP + 5 μM AA	+80 nM PMA	6.2 ± 0.5	2.9 ± 0.5	50.0 ± 1

Summary of data from four different experiments showing the effect of either low dose AF or PMA on the 5-lipoxygenase pathway. In Expt 4, PMN were treated with PMA for 10 min prior to addition of AA and fMLP.

point distal to PLA_2 in the 5-LO pathway. This is consistent with another report that AF does not inhibit PLA_2 [2], and suggests that AF alters the activity of the 5-lipoxygenase/LTA synthase enzyme complex. It has recently been demonstrated that these two enzymes reside on the same protein [13, 14], and thus inhibition of the activity of one enzyme by AF is likely to result in inhibition of the other. Our data are consistent with this hypothesis, since we observed equivalent decreases in LTB_4, its isomers and 5-HETE. It is possible that the strong inhibitory effect of AF on 5-lipoxygenase/LTA synthase could have masked inhibition of LTA hydrolase. However such an effect was ruled out by Honda *et al.* [3] who demonstrated that in homogenates of human PMN, 100 μM AF did not inhibit LTB_4 and LTC_4 synthesis when LTA_4 was used as a substrate, but inhibited both by more than 50% when AA and glutathione were the substrates.

This study has provided a comprehensive description of the effects of AF on the products of the 5-LO pathway, which has not been previously attempted. In general the results do not conflict with previous findings but add additional information. Stimulation with low AF concentrations may have been found had lower AF concentrations been studied. For example Honda *et al.* [3] may have found that AF stimulated LTB_4 synthesis with LTA_4 as substrate had AF concentrations less than 5 μM been used. The results of Herlin *et al.* [5], however, are different from ours in that they found no stimulation of LTB_4 synthesis with low AF concentrations, and differed from others in that they found no inhibition at 11 μM AF, when A23187 was used as stimulus, and these differences do require addressing. In their studies the PMN were only able to synthesize one tenth of the amounts of LTB_4 produced in this study and in others [3, 6], and unquantifiable amounts of 5-HETE, suggesting that their PMN preparations were compromised. This is supported by the evidence that their preparations produced 15-HETE and 12-HETE. 15-HETE is only synthesized by PMN that have been damaged [15]. 12-HETE is not produced by PMN but by platelets and its presence suggests platelet contamination of the PMN preparations. Their low LTB_4 levels may also be explained by an induction of metabolism to the 20-COOH and 20-OH metabolites of LTB_4 observed at PMN concentrations greater than 5×10^6/mL [16]. Similar reasons may also explain the lack of effect of AF on leukotriene synthesis in [4], because no information on PMN concentrations or the absolute amounts of leukotrienes synthesized, was given.

The mechanisms by which AF inhibits 5-lipoxygenase/LTA synthase are unclear. Possibilities include (i) a direct effect on the enzyme complex, or (ii) an indirect effect via inhibition of PKC-dependent phosphorylation of the enzyme complex. Support for the first possibility comes from a recent report of the primary structure of 5-lipoxygenase [17], which shows the presence of a number of amino acids residues, including methionine and histidine, at the putative catalytic site, with which AF could react. Support for the second possibility comes from two lines of evidence. Firstly, LTB_4 synthesis is inhibited by sphingosine (Ref. 18 and Betts, unpublished results), which is a specific inhibitor of PMN PKC [19], and secondly, AF inhibits protein phosphorylation in PMN and purified PKC [7–9, 11]. Thus AF may inhibit 5-lipoxygenase/LTA synthase via inhibition of PKC.

Auranofin is a relatively stable compound that partitions into cellular membranes rapidly via sequential thiol exchanges [20]. Thus delivery of AF to cells has been measured in terms of the uptake of Gold (I) into cells. There is no available data on the pharmacokinetics of the complete auranofin molecule. Additionally, its reactivity with cellular thiols may explain why it has different pharmacological properties to other therapeutic gold compounds. The maximum concentration of AF (10 μM) used in our experiments has been shown to lead to intracellular gold levels in PMN of up to 0.2 μg/10^6 PMN [20]. This is comparable to that found in PMN of patients (0.3 μg/10^6 cells) on chrysotherapy for up to 6 months [21]. Furthermore, the concentration of gold in whole blood of patients after 3 months of auranofin (6 mg/day) is 3.5 μM [22], and this is similar to the concentrations of AF, which both stimulated LTB_4 production and inhibited the 5-LO pathway of PMN in the *in vitro* experiments. Thus it is impossible to state whether an appropriate clinical response to AF is associated with an increase or decrease in LTB_4 production. The possibility that enhanced leukocyte functional responses brought about by penicillamine or gold, results in clinical

improvement of patients with rheumatoid arthritis has also been raised [23, 24].

In summary, these experiments demonstrate that *in vitro*, AF at low concentrations stimulates PMN LTA hydrolase (thus increasing LTB_4 synthesis), and at higher concentrations inhibits 5-lipoxygense/LTA synthase, but has no effect on the other enzymes of the 5-LO pathway.

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