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SELECTIVE INHIBITION OF LEUKOTRIENE C_4 SYNTHESIS IN HUMAN NEUTROPHILS BY ETHACRYNIC ACID

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Summary: Addition of glutathione S-transferase inhibitors, ethyacrynic acid (ET), caffeic acid (CA), and ferulic acid (FA) to human neutrophils led to inhibition of leukotriene C_4 (LTC_4) synthesis induced by calcium ionophore A23187. ET is the most specific of these inhibitors for it had little effect on LTB_4, PGE_2 and 5-HETE synthesis. The inhibition of LTC_4 was irreversible and time dependent. ET also had little effect on ^3H-AA release from A23187-stimulated neutrophils.

Leukotriene C_4 (LTC_4) is synthesized from arachidonic acid (AA) via 5-lipoxygenase and LTA_4 synthetase (1). The intermediate LTA_4 has been isolated from human polymorphonuclear leukocytes (PMNL) and shown to be transformed into LTC_4 enzymatically in PMNL by LTC_4 synthetase (LTA_4: glutathione S-transferase). This enzyme system is also found to be a class of enzymes called glutathione S-transferase (GST) (1,2). LTA_4 is also a precursor of LTB_4. LTB_4 is chemotactic for PMNL (3), and LTC_4 is a smooth muscle contractor (1).

A large number of 5-lipoxygenase inhibitors has been identified (4). Many of them are antioxidants and inhibit all leukotriene synthesis. Therefore, it is important to have inhibitors that are specific for leukotriene synthesis in order to understand the role of a specific leukotriene in a cellular process. Ethacrynic acid (ET), caffeic acid (CA), and ferulic acid (FA) have been shown to inhibit GST activity using chloronitrobenzenes as substrates (5,6). This paper was designed to study the effect of these GST inhibitors on PMNL to evaluate their potential as selective inhibitors of LTC_4 synthesis. Our data demonstrated that GST inhibitors, ET, CA, and FA, are capable of inhibiting leukotriene

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synthesis in PMNL induced by calcium ionophore A23187. ET is a relatively specific inhibitor of LTC_4 synthesis.

MATERIALS AND METHODS

Neutrophils Suspensions of human PMNL were prepared from heparinized blood obtained from healthy donors. The whole blood was mixed by gentle tube inversion then diluted five parts blood with one part 5% dextran 250 (Sigma, St. Louis, MO) in 0.9% NaCl. After 1 hr incubation at 37°C the plasma was layered over Ficoll-Paque (Pharmacia, Piscataway, NJ) and centrifuged at 400 x g for 20 min. The supernatant was decanted and the pellet was resuspended in 0.15M NH_4Cl to lyse any contaminating red blood cells. After centrifugation, the pellet was resuspended in Dulbecco's PBS. The neutrophil cell suspensions were more than 95% PMNL.

Incubations PMNL suspensions ($1-2 \times 10^6$) in 1 mL PBS were pipetted into 12 x 75mm polypropylene test tubes and were incubated at 37°C for 0 to 30 min with test agents or medium alone. In pretreatment experiments, the cells were washed twice with PBS. After incubation with A23187 for 5 to 30 min, the incubations were stopped by addition of 1 mL of ice-cold PBS. The samples were centrifuged at 4°C for 10 min and the supernatants were collected for radioimmunoassays (RIA).

Radioimmunoassays LTB_4 RIA kits were purchased form Amersham (Arlington Heights, Illinois). They showed little cross-reactivity with LTC_4, LTD_4, HETEs, PGE_2 and AA. LTC_4 RIA kits were purchased from DuPont NEN Research Products (Boston, MA) and shown 55% cross-reactivity with LTD_4 and little cross-reactivity with other AA metabolites. 5-HETE RIA kits were purchased from Seragen (Boston, MA) with 3-5% cross-reactivity with leukotrienes and little cross-reactivity with other AA metabolites. PGE_2 RIA kits were purchased from Seragen and shown 100% cross-reactivity with PGE_1 and little cross-reactivity with other prostaglangins. The information on cross-reactivity of each RIA kit was obtained from the suppliers.

Measurement of ^{3}H-arachidonic acid release Neutrophils (5 x 10 /mL) were labeled with ^{3}H-AA (100 Ci/mmol) for 2 hr. Cell suspensions at 1 x 10 /mL were stimulated with A23187 (1ug/mL) in the presence or absence of test agents for 15 min. Supernatants were collected for analysis of radioreactivity. Calcium Ionophore A23187 was purchased from Calbiochem-Behring (La Jolla, CA). ^{3}H-AA was purchased from DuPont NEN Research Products (Boston, MA). Caffeic acid, ethacrynic acid, ferulic acid and dextran 250 were obtained from Sigma (St. Louis, MO).

RESULTS

Inhibition of LTC_4 Production by Ethacrynic Acid. Neutrophils produced LTC_4 in a dose-dependent manner in response to 0.01 to 1 ug/mL A23187 (Fig. 1). The LTC_4 production was inhibited by ET also in a dose-dependent manner. The IC_{50} for ET was about 10 ug/mL (n=5). Time kinectics experiments showed that ET was inhibitory as early as 5 min after the A23187 stimulation (Fig. 2). To test the reversibility of LTC_4 inhibition by ET, PMNL were pretreated with ET for 0 to 30 min before the addition of A23187. As shown in Fig. 3, the LTC_4 inhibition by ET was irreversible and time dependent.

Selectivity of GST inhibitors on AA metabolism. To investigate the selectivity of ET, CA, and FA on AA metabolism, LTB_4, LTC_4, 5-HETE, and

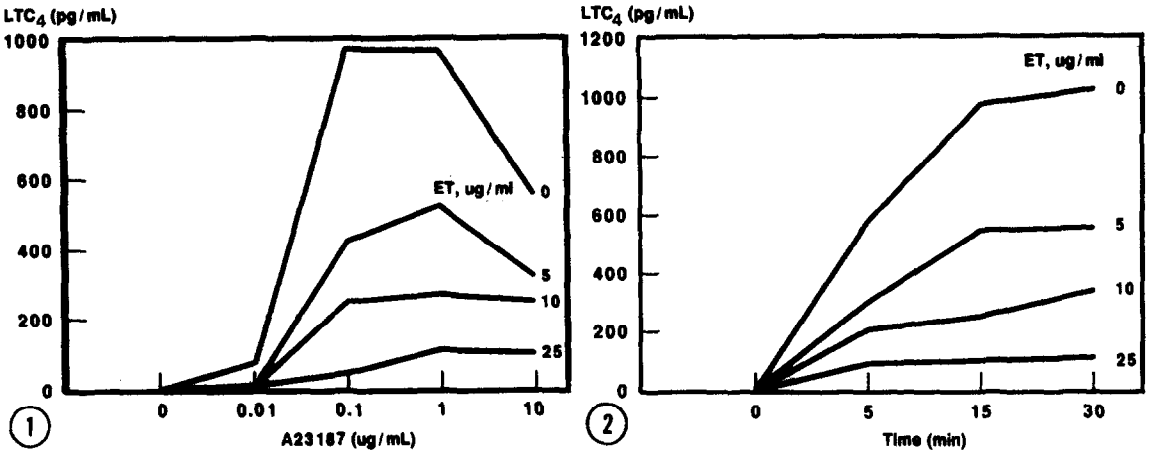

Figure 1: Inhibition of LTC_4 synthesis by ET in A23187-stimulated PMNL. PMNL (2×10^6 in 1 mL PBS) were stimulated with the different doses of A23187 in the presence or absence of ET for 15 min at 37°C.

Figure 2: Kinetics of inhibition of LTC_4 synthesis in PMNL by ET. PMNL (2×10^6 in 1 mL PBS) were stimulated with 1 ug/mL A23187 for 0, 5, 15 or 30 min at 37°C in the presence or absence of ET.

PGE_2 synthesis were analysed. As shown in Fig. 4, ET inhibited LTC_4 synthesis but had little effect on LTB_4 and PGE_2 synthesis. On the other hand, ET stimulated 5-HETE synthesis at low concentrations (5 or 10 ug/mL) and exerted a slight inhibitory effect at 25 ug/mL. CA (Fig. 5) and FA (Fig. 6) inhibited LTB_4, LTC_4, and 5-HETE synthesis in a dose-dependent

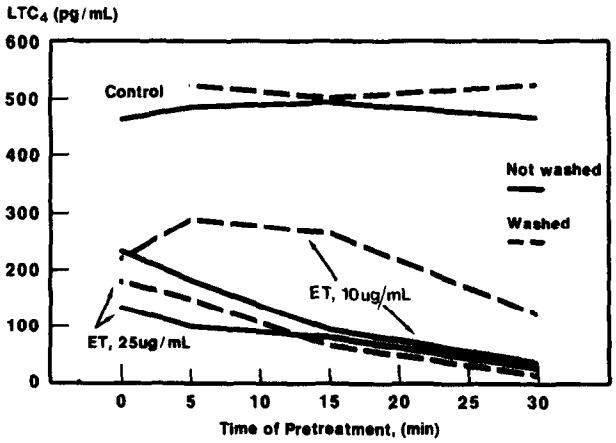

Figure 3: Time dependent and irreversible inhibition of LTC_4 synthesis by ET. PMNL (2×10^6/mL) were incubated with ET for 0, 5, 15, or 30 min at 37°C. Half of the identical samples were washed twice with PBS. A23187 (1ug/mL) was added to all samples which were then incubatd for 5 min at 37°C.

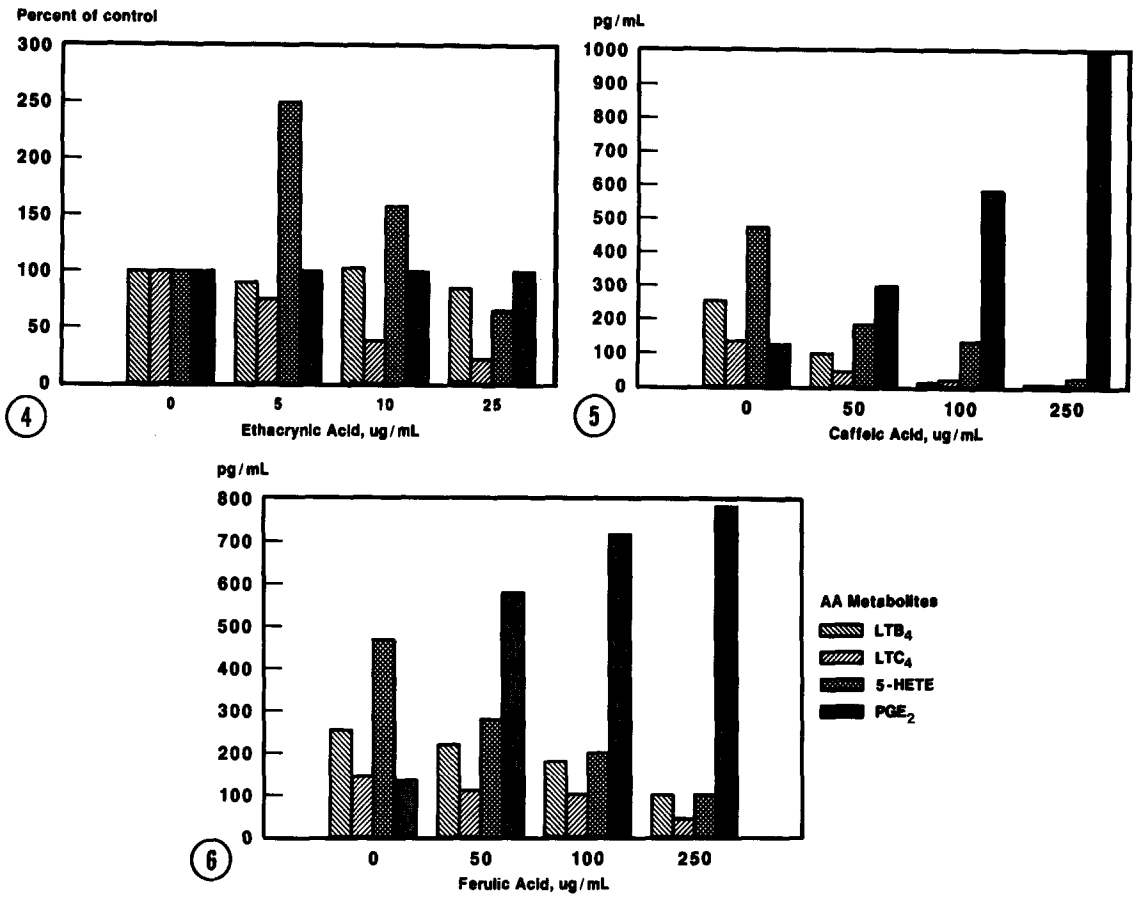

Figure 4: Selectivity of ET on AA metabolism. PMNL (1 x 10^6/mL) were stimulated with 1 ug/mL A23187 for 5 min at 37°C in the presence or absence of ET. Data are expressed as percent of control (n=4).

Figure 5: The effect of CA on AA metabolism. PMNL (1 x 10^6/mL) were stimulated with 1 ug/mL A23187 for 5 min at 37°C in the presence or absence of CA. Data are representative of 3 experiments.

Figure 6: The effect of FA on AA metabolism. PMNL (1 x 10^6/mL) were stimulated with 1 ug/mL A23187 for 5 min at 37°C in the presence or absence of FA. Data are representative of 3 experiments.

manner with IC_{50} of 50ug/mL and 100 ug/mL, respectively. However, both inhibitors stimulated PGE_2 synthesis.

Effect of GST inhibitors on ^{3}H-AA Release. Since AA release from neutrophils is an important step for AA metabolism, the effect of the GST inhibitors on AA release was assessed (Fig. 7). ET at effective doses that inhibited LTC_4 synthesis had little effect of ^{3}H-AA release from PMNL as stimulated by A23187. CA and FA showed inhibitory effect on ^{3}H-AA release at high concentrations.

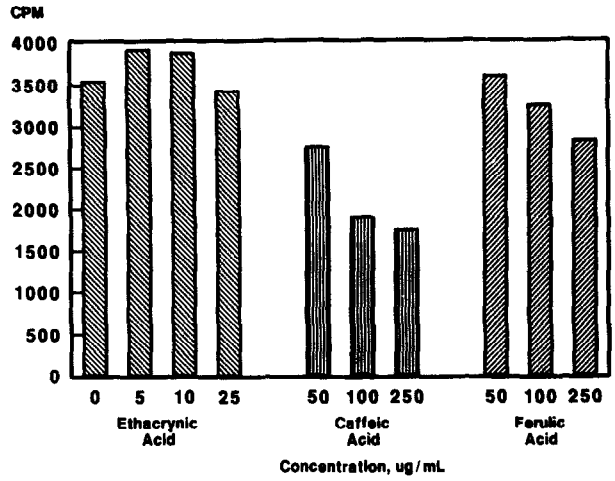

Figure 7: The effect of GST inhibitors on ^{3}H-AA release. PMNL were labeled with ^{3}H-AA for 3 hr. GST inhibitors and A23187 (11 ug/mL) were added to PMNL (1×10^{6}/mL). The samples were incubated for 15 min at 37°C and centrifuged. The supernatant were analyzed for radioactivity. Data are representative of 3 experiments.

DISCUSSION

One of the most important detoxicating enzyme systems in the liver is the GST system in its multiple forms. Although liver homogenates can convert LTA_4 into LTC_4, leukocytes are the major sources of LTC_4 (1). Our data show that inhibitors of the liver GST system, ET, CA, and FA are capable of inhibiting LTC_4 synthesis in PMNL suggesting that GST system is present in PMNL with the main function of formation of LTC_4.

Some relatively selective inhibitors of 5-lipoxygenase have been reported (4). These compounds, such as NDGA, have phenolic hydroxyl group(s) which may be essential for lipoxygenase inhibition. The GST inhibitors studied here, CA and FA contain phenolic hydroxyl groups while ET does not. Our results indicate that ET is the most specific of these three GST inhibitors of LTC_4 synthesis without affecting PGE_2 and LTB_4 synthesis in PMNL. The kinectic experiments on inhibition of LTC_4 synthesis support the observations by another laboratory that ET interacts with GST in two steps, first as a substrate and secondly binding covalently to GST (7). Our data show that ET inhibition of LTC_4 synthesis in PMNL is time dependent: the longer the pretreatment the stronger the inhibition (Fig. 3). The inhibition is also irreversible: the drug effect can not be abrogated upon

removal of the drug (Fig. 3). It is interesting to note that ET inhibition of LTC_4 synthesis is not reversed by AA or GSH (data not shown). These observations suggest that ET may be modifying the catalytic function of GST and not the binding of GST to its substrates. Because of the selectivity of ET it would be useful to study the role of LTC_4 in cellular processes and to design therapeutic treatment of diseases.

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