Biochemical Pharmacology, Vol. 31, No. 3, pp. 337–345, 1982.
Printed in Great Britain.

0006-2952/82/030337-09 $03.00/0
Pergamon Press Ltd.

EFFECT OF INHIBITORS OF ARACHIDONIC ACID METABOLISM ON α-AMINOISOBUTYRIC ACID TRANSPORT IN HUMAN LYMPHOCYTES*

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(*Received* 29 *September* 1980; *accepted* 19 *June* 1981)

Abstract—The role of arachidonic acid metabolism (or metabolites) in the modulation of α-aminoisobutyric acid transport in resting and concanavalin A-stimulated human peripheral blood lymphocytes was evaluated using previously characterized inhibitors of arachidonic acid metabolism. Nordihydroguairetic acid (a nonselective antioxidant), 5,8,11,14-eicosatetraynoic acid (an inhibitor of lipoxygenase and cyclooxygenase activities), indomethacin and acetylsalicylic acid (selective cyclooxygenase inhibitors), and 1-benzylimidazole, Ro-22-3581 and Ro-22-3582 (thromboxane synthetase inhibitors) proved to be potent inhibitors of amino acid transport activity in normal resting and lectin-activated lymphocytes at concentrations known to decrease thromboxane A_2 production. The rank order of effectiveness of these various inhibitors compared favorably with their relative potencies as inhibitors of thromboxane B_2 synthesis under the same conditions, as determined by radioimmunoassay. Inhibitory effects noted were not due to overt cytotoxicity and seemed to involve changes primarily in the V_{max} and not the K_m of the transport process. Drug-induced alterations in the magnitude of concanavalin A binding were not observed. These results suggest that the activity of amino acid transport systems can be influenced by certain arachidonic acid metabolites, probably thromboxanes, in both stimulated and unstimulated lymphocytes. In addition, these findings may provide a partial explanation for the observation that inhibitors of thromboxane formation prevent lymphocyte mitogenesis [J. P. Kelly, M. C. Johnson and C. W. Parker, *J. Immun.* **122**, 1563 (1979)].

Oxidative metabolism of polyunsaturated long chain fatty acids, particularly arachidonic (5,8,11,14-eicosatetraenoic) acid, to often unstable products with potent biologic activities has been demonstrated in multiple mammalian cell types [1]. In addition, roles for arachidonic acid metabolites as intracellular or intercellular modulators of cell function have been suggested [2]. Studies from the laboratories of others [3–12] and from this laboratory [13–17] have suggested that arachidonic acid metabolism may play an important role in normal immune responsiveness, particularly in lymphocyte proliferation. Radiolabeled arachidonic acid incorporated into resting human peripheral blood lymphocytes is released, primarily from the 2-position of phosphatidyl inositol, within minutes after stimulation by mitogenic lectins, reflecting increased endogenous phospholipase A_2 or lipase activities [17]. Arachidonic acid release is influenced by cytochalasins, microtubular agents, cyclic AMP agonists and cyclic nucleotides themselves in a way that approximately parallels the effects of these agents on lectin-induced lymphocyte mitogenesis (C. W. Parker and J. P. Kelly, unpublished observations). Furthermore, when mononuclear cells, and to a lesser extent purified lymphocytes, are preincubated with radiolabeled arachidonate and subsequently stimulated with mitogenic lectins, enhanced formation of hydroxylated derivatives of arachidonic acid (5- and 12-hydroxyeicosatetraenoic acids) and thromboxanes is observed [17]. It has also been demonstrated that low micromolar levels of exogenous arachidonic acid and certain other unsaturated fatty acids enhance lymphocyte responsiveness to mitogens [14]. Finally, both in the presence and absence of exogenously added fatty acids, various inhibitors of arachidonic acid metabolism (notably lipoxygenase, fatty acid cyclooxygenase and thromboxane synthetase inhibitors) effectively inhibit mitogenesis measured by tritiated thymidine incorporation or increases in cell number [15]. Since several of these inhibitors were shown to be most effective if added to stimulated cultures within a few hours of mitogen addition and were relatively ineffective if addition was delayed by 24 hr, it was of interest to ascertain their effects on an early indicator of lymphocyte activation and compare or contrast results obtained with those from the 3- to 4-day mitogenesis studies.

The activity of the neutral amino acid A, or alanine-preferring, transport system [18] is known to be elevated in growing cells relative to cells that are not cycling [19, 20]. Increases in the uptake of certain amino acids have been demonstrated in the

* This work was supported by NIH Research Service Award 5 T32 GMO 7200 and USPHS Research Grant 1 P50 AI 15322.

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lymphocytes of various species and tissues as a consequence of activation with a number of mitogens [21–24]. Measurements of α-aminoisobutyrate uptake made a few hours after stimulation with polyclonal activators appear to correlate directly with DNA synthetic activity determined days later [23]. Recently these observations have been confirmed in an antigen-driven *in vitro* secondary immune response [25].

Evidence that rates of amino acid transport are linked in some way to arachidonic acid metabolism will be presented. The possibility that continued thromboxane A_2 production is required for normal cellular metabolism in resting and activated human peripheral blood lymphocytes is discussed.

MATERIALS AND METHODS

Materials. Reagents and their sources were as follows: 1-[^{14}C]-α-aminoisobutyric acid (60 mCi/mmole) and Na^{125}I (carrier-free) (Amersham-Searle, Arlington Heights, IL); acetylsalicylic acid, α-aminoisobutyric acid (AIB)* bovine serum albumin, chloramine-T, imidazole, indomethacin, α-methyl-D-mannoside, and sodium metabisulfite (Sigma Chemical Co., St. Louis, MO); acetylene dicarboxylic acid, 1-benzylimidazole, 3-butynol, 3-octynol, nordihydroguaiaretic acid, and propiolic acid (Aldrich Chemical Co., Milwaukee, WI); dimethylsulfoxide and Scintiverse (Fisher Scientific Co., St. Louis, MO); and Con A (3 times crystallized) (Miles-Yeda, Elkhart, IN). ETYA (5,8,11,14-eicosatetraynoic acid), Ro-22-3581 [4-(1H-imidazol-1-yl)-acetophenone] and Ro-22-3582 [*p*-(1H-imidazol-1-yl)-phenol] were gifts from Hoffmann-LaRoche, Inc. (Nutley, NJ). Minimal essential media and RPMI-1640 were obtained from the Washington University School of Medicine Basic Cancer Research Center. Human sera obtained from AB-Rh$^+$ donors were heated to 56° for 30 min and stored at −20° prior to use. Lipid-poor human sera were prepared as described previously [14, 26]. Heat-inactivated fetal bovine serum was purchased from the Grand Island Biological Co., (Grand Island, NY).

Cell preparations. Human peripheral blood mononuclear cells were purified from the heparinized venous blood of normal volunteers (who had not ingested aspirin during the 2 weeks prior to donation) after removal of erythrocytes by dextran sedimentation and isopycnic centrifugation over Ficoll–Hypaque gradients as described previously [27]. The average lymphocyte recovery was 68%. Preparations obtained greater than 80 lymphocytes per 100 nucleated cells and less than 1 erythrocyte and 1 platelet per lymphocyte. The contaminating nucleated cells consisted of monocytes and polymorphonuclear leukocytes, especially the former. In selected experiments lymphocyte preparations were extensively depleted of platelets by differential centrifugation as previously described [16]. Results obtained with platelet-depleted lymphocytes were not different from those observed in experiments performed with the usual cell preparations.

Preparation of reagents. All non-radioactive reagent solutions were prepared fresh on the day of the experiment, neutralized if necessary, and diluted in 0.1 M NaCl. Controls for solvent effects were performed. Stock solutions were: ETYA and nordihydroguaiaretic acid at 10 mg/ml, and Ro-22-3581 and Ro-22-3582 at 0.5 M in dimethylsulfoxide, indomethacin and acetylsalicylic acid in 0.1 M Tris–HCl (pH 8.0), and imidazole (0.12 M) and 1-benzylimidazole (0.1 M) in 0.1 M NaCl. Con A was dissolved at 0.2 mg/ml in 0.1 M NaCl. α-Amino[^{14}C]isobutyric acid was purchased as a solid [or as a solution in 2% ethanol (v/v) in water which was lyophilized to solid form], dissolved to give a 1 mCi/ml solution in phosphate-buffered saline (0.01 M phosphate, 0.15 M NaCl; pH 7.4) which was stored at 4° and diluted to the desired specific activity with non-radioactive α-aminoisobutyrate in phosphate-buffered saline on the day of the experiment.

Determination of cell viability. Cell viability was quantitated by the Cetremid [28] or trypan blue due exclusion technique. Cytotoxicity, judged by decreased recovery of viable nuclei or in some instances by microscopic dye uptake, was not observed for any of the arachidonic acid metabolism inhibitors at the concentrations used.

Culture conditions and measurement of aminoisobutyric acid uptake. AIB uptake determinations were performed in triplicate using techniques that have been described previously [22]. Incubations were carried out at 37° under a 5% CO_2/95% O_2 atmosphere without shaking. Lymphocytes were suspended at 10.0 to 12.5 × 10^6 cells per ml in minimum essential media containing 2% human serum and added in 0.4-ml aliquots to 10 × 75 mm glass tubes containing 0.05 ml of inhibitor or 0.1 M NaCl. After 15 min, 0.05 ml of Con A or 0.1 M NaCl was added as indicated, and the incubation was continued for an additional 4 hr. A 30-min labeling period was initiated by adding 0.3 μCi of α-amino[^{14}C]isobutyric acid in 0.01 ml of phosphate-buffered saline to give a final AIB concentration of 0.12 mM. Uptake of α-amino[^{14}C]isobutyric acid was halted by adding 2 ml of ice-cold phosphate-buffered saline containing 0.01 M α-aminoisobutyrate (Buffer A) to each tube. Cells were sedimented at 4° by centrifugation at 1200 *g* for 7 min, washed twice with 2 ml volumes of ice-cold Buffer A and lysed in distilled water (0.5 ml); the lysates were stored at −20° for not more than 48 hr. Samples were thawed to room temperature, sonicated to disperse cellular debris, and quantitatively transferred to scintillation vials by vortexing with 2 × 5 ml portions of Scintiverse. Samples were assayed for radioactivity in an automatic liquid scintillation counter (Searle).

Con A binding studies. ^{125}I-labeled Con A was prepared using the method of Chang and Cuatrecasas [29], to yield a preparation with a specific activity of 19.6 μCi/μg. Binding studies were performed in duplicate or triplicate under conditions similar to those utilized in AIB uptake experiments with the following modifications. All volumes were reduced

* Abbreviations: AIB, α-aminoisobutyric acid; NDGHA, nordihydroguaiaretic acid; ASA, acetylsalicylic acid; Con A, concanavalin A; ETYA, 5,8,11,14-eicosatetraynoic acid; Ro-22-3581, 4-(1H-imidazol-1-yl)-acetophenone; and Ro-22-3582, *p*-(1H-imidazol-1-yl)-phenol.

by one-half and incubations were carried out in polypropylene tubes. Bovine serum albumin (0.125%, w/v) was substituted for the human serum supplement. Incubations were terminated after 60 min at 37° with the addition of 2 ml of ice-cold phosphate-buffered saline containing 0.1% (w/v) bovine serum albumin (Buffer B) to each tube. Cells were sedimented and washed twice with ice-cold Buffer B, and cell-associated radioactivity was determined in an automatic gamma counter (Searle).

Calculations of transport data. Daily variation in absolute amounts of α-aminoisobutyric acid internalized in the course of the amino acid transport assay was large enough to prohibit convenient combination of results obtained in separate experiments for statistical analysis. α-Aminoisobutyric acid transport activity measured in Con A-stimulated human lymphocytes in twenty-four experiments ranged from 182 to 759 pmoles·$(5 \times 10^6$ cells$)^{-1}$·30 min^{-1} (mean ± S.E.M. = 435 ± 31). Relative increases in transport activity in response to Con A (20 μg/ml) were much more precise. The stimulation ratio for twenty-four individual experiments was 2.08 ± 0.07 (mean ± S.E.M.). To allow results from experiments carried out with peripheral blood lymphocytes from different donors to be analyzed together, levels of transport activity were expressed relative to the highest mean value [in pmoles·5×10^6 lymphocytes$)^{-1}$ ·30 min^{-1}] observed in each experiment (as percentage of maximal response) before means were calculated and statistical analysis was carried out. In these experiments, 100% of maximal response corresponds to the AIB uptake measured in lymphocytes stimulated with Con A (20 μg/ml) in the absence of arachidonic acid metabolism inhibitors.

Radioimmunoassay measurements of thromboxane B_2 production. Thromboxane B_2 production was measured in lymphocyte cultures by radioimmunoassay. Cells were incubated under the usual conditions for measurement of AIB uptake in 2% AB serum at 37° for 4 hr in the presence and absence of Con A and inhibitors of thromboxane synthesis. The AB serum used for these studies was clotted and centrifuged at 4° to minimize formation of thromboxane B_2 during processing. After 4 hr the cell suspension was then diluted with 1.5 vol. of 95% ethanol at 4°, acidified with dilute HCl to a pH of 2.0 and extracted 2 times with 2 vol. ether; the ether extract was dried under nitrogen. On the day of the immunoassay, samples were reconstituted in 0.15 M NaCl, 0.01 M phosphate (pH 7.4) containing 0.1% gelatin, and several aliquots were analyzed for thromboxane B_2 by radioimmunoassay using a rabbit anti-thromboxane B_2 antibody (obtained from Dr. Brian Smith, Cardeza Foundation for Hematologic Research, Departments of Medicine and Pharmacology, Thomas Jefferson University, Philadelphia, PA). Free and bound antigen were separated by $(NH_4)_2SO_4$ precipitation in the presence of normal rabbit IgG carrier. Information on the preparation and specificity of the antibody and the immunoassay procedure has been given in detail previously [30]. The immunoassay marker was [5,6,8,9,11,12,14,15-^{3}H]thromboxane B_2 (New England Nuclear Corp., Boston, MA, sp. act. 125 Ci/mmole).

RESULTS

Effects of ETYA on AIB uptake. ETYA (5,8,11,14-eicosatetraynoic acid), a potent inhibitor of both lipoxygenase and cyclooxygenase enzymes [30], is, on a molar basis, the most effective inhibitor of lectin-induced lymphocyte mitogenesis of the various specific acid metabolism inhibitors presently available [14]. Data presented in Fig. 1 show that, as previously reported [22], Con A (20 μg/ml final

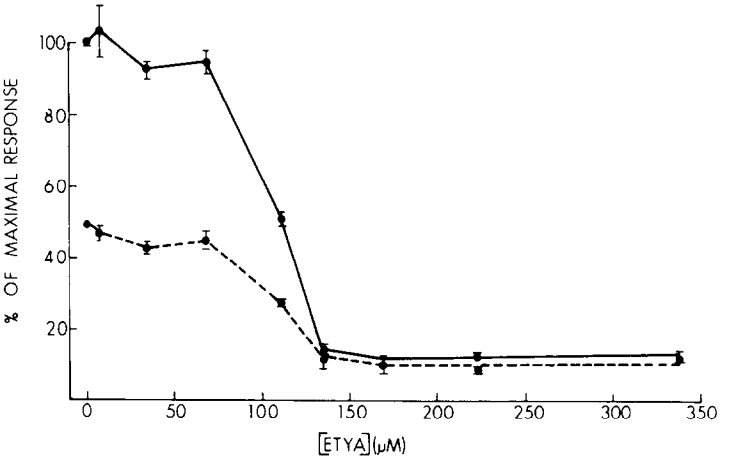

Fig. 1. Effects of 5,8,11,14-eicosatetraynoic acid (ETYA) on AIB uptake. Experiments were carried out under conditions described in Materials and Methods. Briefly, lymphocytes were incubated at 10×10^6/ml in MEM containing 2% AB with various concentrations of inhibitor for 15 min, Con A or 0.1 M NaCl was added and, beginning 4 hr later, [^{14}C]AIB accumulation was allowed to occur for 30 min. Cells were harvested and AIB uptake was calculated after determination of cell-associated radioactivity. Calculations were carried out as described in Materials and Methods. Data are expressed as the mean ± S.E.M. for two to eight experiments. Key: (●——●) ETYA + Con A (20 μg/ml); and (●---●) ETYA alone.

concentration) caused a 2-fold increase in the rate of α-aminoisobutyric acid uptake into human peripheral blood mononuclear cells when compared with the rate in cells treated with 0.1 M NaCl. Furthermore, ETYA was found to have a dramatic dose-dependent inhibitory effect on AIB accumulation in both stimulated and unstimulated lymphocytes over the concentration range 69–133 μM. The influence of ETYA was striking in that little or no effect was observed at a concentration equal to 60 μM, while virtually complete inhibition was seen at 133 μM. The 50% inhibitory concentration was found to be 112 μM. Since dimethylsulfoxide was used as a solvent in the preparation of ETYA stock solutions owing to limited solubility of the reagent in aqueous solutions, the contribution of dimethylsulfoxide to the inhibitory effects seen with ETYA was evaluated. Dimethylsulfoxide was found to cause a modest (15–20%) increase in the rate of amino acid transport in control or activated lymphocytes at concentrations equivalent to those present at the 50% inhibitory concentration for ETYA.

Short chain acetylenic compounds. In order to exclude non-specific influences of a triple-bond containing compound like ETYA on such things as sulfhydryl group reactivity as the primary cause of the inhibition of transport observed, a single experiment was performed with each of four commercially available short chain acetylenic compounds in parallel with ETYA (data not shown). Results obtained with acetylene dicarboxylic acid, propiolic acid and 3-butynol demonstrated that these agents had no effect on α-aminoisobutyrate accumulation in unstimulated or Con A-activated cells at levels adjusted to include the concentration of triple bonds present in up to 375 μM ETYA, a tetrayne. Although 3-octynol was modestly inhibitory, it was much less effective than ETYA.

Nordihydroguaiaretic acid. Nordihydroguaiaretic acid was described initially as a selective lipoxygenase inhibitor [31], but characterization of its effects in human lymphocytes revealed interference with arachidonic acid metabolism at multiple points, including fatty acid lipoxygenase and thromboxane synthetase [16]. The influence of NDGHA on α-aminoisobutyric acid transport is illustrated in Fig. 2. The inhibition observed can be characterized as log-linear over the effective range of the drug, with 50% inhibition seen at 100 μM.

Cyclooxygenase inhibitors. Since the experiments just discussed suggested that inhibition of lipoxygenase and/or cyclooxygenase could result in the inhibition of α-aminoisobutyrate accumulation in human lymphocytes, efforts were next directed at determining the impact of the cyclooxygenase inhibitors indomethacin and acetylsalicylic acid [30, 32] on amino acid uptake. Data presented in Fig. 3 demonstrate that indomethacin and acetylsalicylic acid (ASA) inhibited α-aminoisobutyric acid transport in both activated and resting lymphocytes in a dose-dependent fashion. For lectin-stimulated cells, the inhibition curves for indomethacin and acetylsalicylic acid approximated log-linearity and were essentially parallel. Indomethacin was found to be a much more effective inhibitor on a molar basis than acetylsalicylic acid, however, with 50% inhibitory concentrations (for stimulated cells) of 0.185 and 12 mM respectively.

Thromboxane synthetase inhibitors. To determine which of the known arachidonic acid metabolites produced via the cyclooxygenase pathway (prostaglandins, prostacyclin and thromboxane A_2) might modulate amino acid transport in human lymphocytes, the effects of the selective thromboxane synthetase inhibitors 1-benzylimidazole, Ro-22-3581 and Ro-22-3582 were studied. Recent work from this laboratory [15] has suggested a role for thromboxane A_2 in lymphocyte activation in that thromboxane synthetase inhibitors, in addition to inhibitors of the lipoxygenase and cyclooxygenase pathways, prevent lectin-induced mitogenesis. Figure 4 provides evidence that 1-benzylimidazole, at concentrations which prevent thromboxane A_2 production in lymphocyte rich mononuclear cells (see below), effectively reduced α-aminoisobutyrate uptake in the presence or absence of Con A. The drug concentration causing 50% inhibition approximates 1.3 mM.

Several more potent inhibitors of thromboxane synthetase have recently become available. The imidazole derivatives Ro-22-3581 and Ro-22-3582,

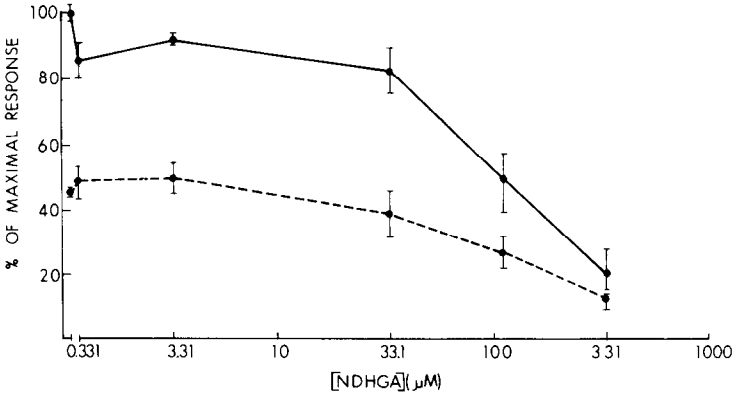

Fig. 2. Effect of nordihydroguaiaretic acid on AIB transport. Data shown are the mean ± S.E.M. for two to five experiments. Key: (●——●) nordihydroguaiaretic acid + Con A (20 μg/ml); and (●----●) nordihydroguaiaretic acid alone. For further details, see legend to Fig. 1.

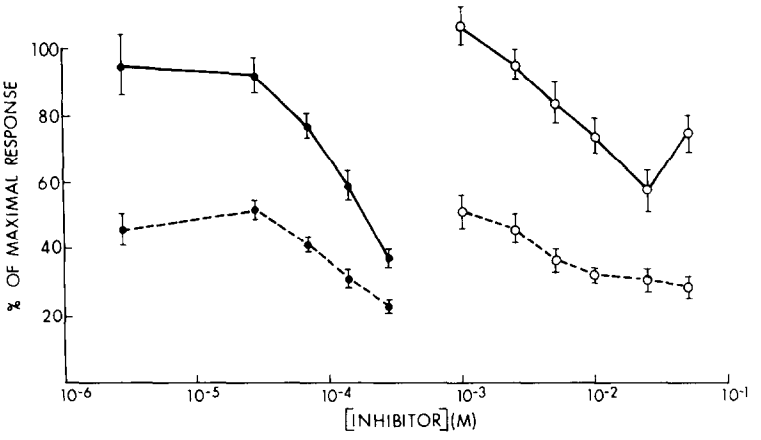

Fig. 3. Effects of cyclooxygenase inhibitors on AIB transport. Data shown above are the mean ± S.E.M. for two to seven experiments in the case of indomethacin and two to three experiments for ASA. Key: (●——●) indomethacin + Con A (20 μg/ml); (●----●) indomethacin alone; (○——○) ASA + Con A (20 μg/ml); and (○----○) ASA alone. For further details, see legend to Fig. 1.

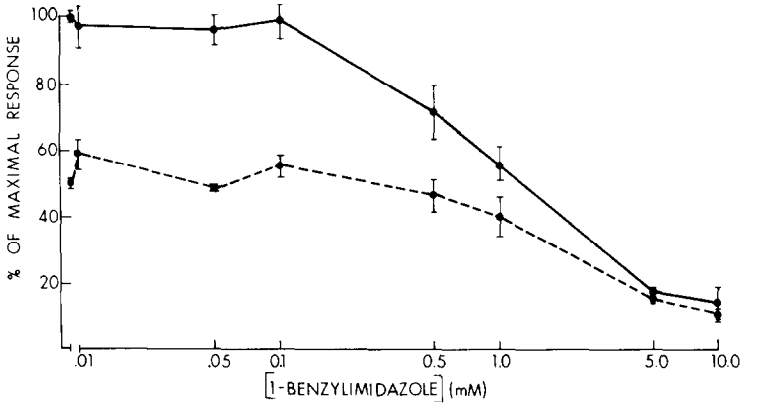

Fig. 4. Effect of 1-benzylimidazole in AIB uptake. Data presented were derived from two to six experiments and show the mean ± S.E.M. Key: (●——●) 1-benzylimidazole + Con A (20 μg/ml); and (●----●) 1-benzylimidazole alone. For further details, see legend to Fig. 1.

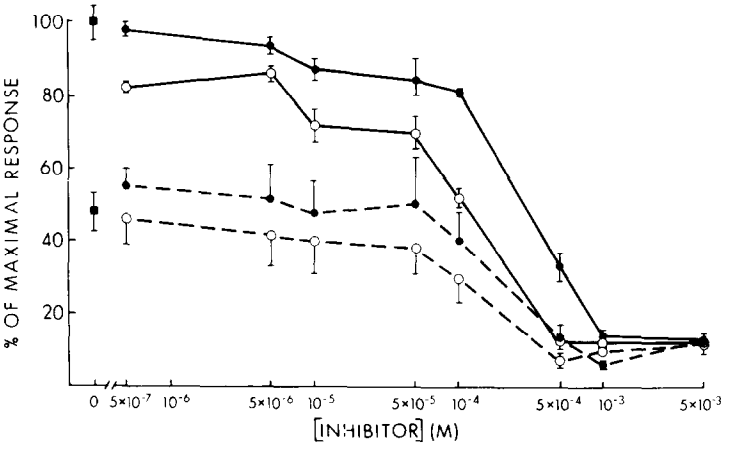

Fig. 5. Effect of the thromboxane synthetase inhibitors Ro-22-3581 and Ro-22-3582 on AIB transport in human lymphocytes. Data displayed are the mean ± S.E.M. for two experiments. Key: (●——●) Ro-22-3581 + Con A (20 μg/ml); (●----●) Ro-22-3581 alone; (○——○) Ro-22-3582 + Con A; and (○----○) Ro-22-3582 alone. For further details, see legend to Fig. 1.

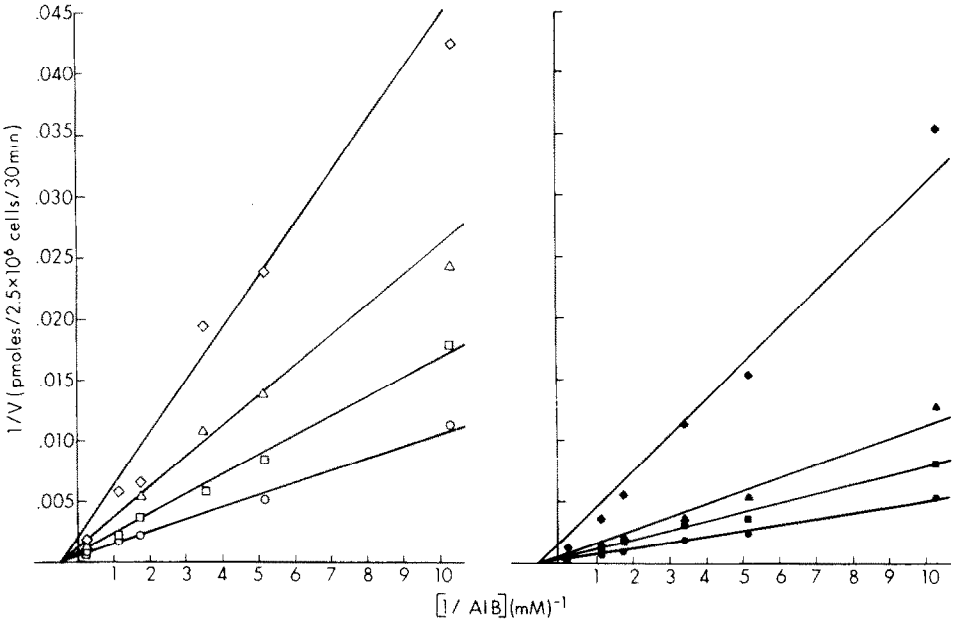

Fig. 6. Kinetic analysis of the influence of arachidonic acid metabolism inhibitors on AIB transport. Details of the experiment are discussed in Materials and Methods. Representative data from one of four experiments are shown. Left panel: (○) 0.1 M NaCl control; (□) 140 μM indomethacin: (△) 111 μM ETYA; and (◇) 5 mM 1-benzylimidazole. Right panel: (●) Con A (20 μg/ml); (■) 140 μM indomethacin + Con A; (▲) 111 μM ETYA + Con A; and (◆) 5 mM 1-benzylimidazole + Con A.

which have been demonstrated to inhibit thromboxane synthetase in broken cell preparations [33] at concentrations in the 10^{-6} to 10^{-5} M range (J. Hamilton, Hoffmann-La Roche, Inc., personal communication), were found to inhibit AIB uptake into resting and activated lymphocytes (Fig. 5) at concentrations known to prevent thromboxane A_2 production in human lymphocytes (C. W. Parker, unpublished observations). Ro-22-3582 proved to be a more effective inhibitor of amino acid transport than Ro-22-3581, with 50% inhibitory concentrations (for Con A-activated lymphocytes) of 0.12 and 0.28 mM, respectively, in agreement with the rank order of potency observed when the inhibitory effects of these agents on platelet thromboxane synthetase were evaluated (J. Hamilton, Hoffmann-La Roche, Inc., personal communication).

Excluding its 1-substituted derivatives, imidazole is perhaps the most selective of the thromboxane synthetase inhibitors available [34, 35]. Although the drug had been shown previously to prevent lymphocyte mitogenesis [14], it did not inhibit AIB accumulation in unstimulated or stimulated cells under the usual incubation conditions. Incubation of resting or Con A-stimulated lymphocytes with imidazole for longer time periods (e.g. 24 hr) did cause a reduction in AIB uptake without measurable cytotoxicity, however (data not shown). Convincing inhibition was observed only at the highest concentrations tested (8 and 12 mM) and was less dramatic than that seen in the mitogenesis system [14], never reaching the 50% level.

Studies with a cyclooxygenase and thromboxane synthetase inhibitor in combination. In two separate experiments (data not shown) it was found that indomethacin, a cyclooxygenase inhibitor, and 1-benzylimidazole, a thromboxane synthesis inhibitor, when used in combination (at levels giving intermediate inhibition when used separately), produced approximately additive inhibitory effects. The simplest interpretation of this result is that effects of thromboxane synthetase inhibitors on AIB transport were mediated by decreased thromboxane formation and not by increased prostaglandin production, since the thromboxane synthetase inhibitor retained its biologic potency in an experimental situation in which increased prostaglandin synthesis was prevented by including a cyclooxygenase inhibitor in the incubation mixture.

Influence of inhibitors of arachidonic acid metabolism on the kinetics of AIB uptake. Studies were carried out with single intermediate concentrations of various inhibitors at multiple substrate concentrations and double reciprocal plots were made (Fig. 6). As previously reported for human lymphocytes [21, 22], the K_m for the α-aminoisobutyrate transport mechanism was found to be approximately 2 mM, and Con A was found to increase amino acid uptake by changing the V_{max} without affecting the K_m. Data obtained with the various arachidonic acid metabolism inhibitors were interpreted as most consistent with the notion that the inhibition seen was due primarily to alterations in V_{max} and not K_m. Small changes in K_m, in the case of 1-benzylimidazole for example, cannot be discounted entirely.

Influence of inhibitors of arachidonic acid metabolism on Con A binding. None of the agents tested had a statistically significant influence on the extent of Con A binding to human lymphocytes (data not shown).

Measurements of thromboxane synthesis. We have reported previously on the effects of many of the inhibitors used in this study on incorporation of [^{14}C]arachidonic acid into thromboxane B_2, prostaglandins and hydroxy fatty acids in mononuclear cells and in preparations enriched in lymphocytes as determined by radio thin-layer chromatography and isotope counting of eluates of radioactive bands from the plates [15]. In the present study a number of these inhibitors of thromboxane biosynthesis have been studied by radioimmunoassay. As shown in Table 1, the rank order of effectiveness for inhibition of immunoassayable thromboxane formation (indomethacin, ETYA, NDGHA and Ro-22-3582 > benzylimidazole and Ro-22-3581 > aspirin > imidazole) is similar to their rank order of effectiveness in inhibiting Con A-stimulated AIB uptake and compares favorably with our earlier [^{14}C]arachidonic acid incorporation studies. The concentrations required for greater than 50% inhibition of AIB uptake inhibit AIB synthesis by 80% or more, however. While the concentrations required are somewhat higher than those in several other studies (particularly those in platelets), it should be kept in mind that 2% AB serum (which may bind the inhibitors) was present in the mononuclear cell cultures. Moreover, while residual platelets not removed by the low speed centrifugation were undoubtedly present, other cells, particularly monocytes, were almost certainly contributing to the thromboxane response. It is entirely possible that the enzyme(s) in monocytes was less susceptible to inhibition than the enzyme in platelets, or that monocytes synthesized new enzyme during the 4-hr period of the experiment.

Comparative studies in the usual mononuclear cell preparations used in the AIB uptake measurements obtained by Ficoll–Hypaque purification or filtered through nylon columns to remove nonlymphocytic cells revealed that little if any thromboxane B_2 was produced in the filtered cells. Since unpublished studies from our laboratory indicate that polymorphonuclear leukocytes make very little or no thromboxane, it appears that monocytes are the major source of thromboxane in ordinary mononuclear cell preparations, as suggested previously by Kennedy *et al.* [36].

Table 1. Relative potency of different agents as inhibitors of thromboxane synthesis in cultured mononuclear cells as determined by radioimmunoassay*

Inhibitor	Concentration producing 50% inhibition (μM)
Aspirin	50
Indomethacin	2,000
NDGHA	100
Benzylimidazole	500
Imidazole	>12,000†

* Samples were incubated in the presence of Con A under the usual conditions for AIB uptake studies, using 4×10^6 mononuclear cells suspended in 400 μl medium with 2% AB serum at 37° for 4 hr with and without preincubation for 15 min with one of the above inhibitors. Calculations are based on two or more experiments in duplicate at concentrations of each series of inhibitor selected from preliminary screening experiments. The mean production of thromboxane B_2 averaged 7500 (±310 S.E.M.) pmoles over a 3-hr period, whereas 2% AB serum alone contained 80 pmoles of thromboxane B_2. In the absence of Con A, cells produced an average of 5900 (±580) pmoles of thromboxane. In less extensive studies these inhibitors had similar effects on thromboxane synthesis in the absence of Con A.

† Twenty-eight per cent inhibition of 12 mM, which was the weight concentration used.

DISCUSSION

The results of studies presented here demonstrate that nordihydroguaiaretic acid (a relatively non-specific inhibitor of oxidative arachidonic acid metabolism), ETYA (an inhibitor of lipoxygenase and cyclooxygenase activities), indomethacin and acetylsalicylic acid (inhibitors of cyclooxygenase), and 1-benzylimidazole, Ro-22-3581 and Ro-22-3582 (thromboxane synthetase inhibitors) markedly reduced α-aminoisobutyric acid uptake into unstimulated and mitogen-activated human peripheral blood lymphocytes in a dose-related fashion. The inhibition was not associated with cytotoxicity or quantitative changes in the binding of Con A to cells. The effects observed appear to have been mediated, primarily, through changes in V_{max} of transport rather than in the K_m for the transport system. Another selective thromboxane synthetase inhibitor, imidazole, was much less effective. The relative ineffectiveness of imidazole as an inhibitor may have been related to limited drug permeability under the conditions employed or may have been due, entirely, to its decreased potency as a thromboxane synthetase inhibitor when compared with 1-benzylimidazole [34] and other structurally related drugs. Modulation of uptake via increased prostaglandin formation associated with the use of thromboxane synthetase inhibitors was ruled out through the use of indomethacin in combination with other inhibitors. These observations suggest that agents which are capable of reducing thromboxane A_2 production interfere with AIB transport.

Proper interpretation of the results presented in this study hinges on validation of the effects of the various inhibitors tested on arachidonic metabolism in human lymphocytes. Most of the agents used have been studied for their effect on radiolabeled ^{14}C in our laboratory in mononuclear cell preparations depleted of platelets, and to a lesser extent in enriched lymphocytes, and those results have been published elsewhere [16]. In the present study, thromboxane B_2 synthesis was measured under somewhat different conditions by radioimmunoassay, which provides a more quantitative and specific assay than the radiolabel incorporation experiments. The effects of the various inhibitors on lymphocyte arachidonate metabolism were similar to those seen in platelets although, in general, higher drug concentrations were required to achieve comparable levels of inhibition of thromboxane B_2 synthesis in mononuclear cells relative to platelets. Based on the present and previous studies [16], the inhibition of AIB uptake is marked at concentrations of inhibitors which reduce thromboxane synthesis by at least 80%. The drugs Ro-22-3581 and Ro-22-3582 were not available at the time of our earlier studies.

The relative inhibitory potencies of the 1-substituted imidazoles Ro-22-3581, Ro-22-3582, and benzylimidazole, and imidazole itself, in our AIB transport system are very similar to those found for the inhibition of thromboxane synthetase in other tissues (J. Hamilton, Hoffmann-La Roche, Inc., personal communication). Kennedy *et al.* [36] have recently reported that monocytes or macrophages are the principle source of thromboxane in human peripheral blood mononuclear cell preparations, although Morley *et al.* [37] reported the production of thromboxane B_2 (verified by gas chromatography–mass spectroscopy) by human thoracic duct lymphocytes. While we have not studied monocytes directly, our radioimmunoassay results with filtered lymphocytes (primarily T lymphocytes) indicate that monocytes are a much better source of thromboxanes than T cells. Despite this, it remains possible that human T lymphocytes do synthesize small amounts of these metabolites (especially in the presence of mitogens) which act as intracellular or local mediators in the response. Consistent with this notion is the observation that AIB transport is decreased similarily in mononuclear cell and T lymphocyte preparations by inhibitors of arachidonate metabolism. It seems likely, however, that the bulk of the arachidonic acid metabolites involved in the regulation of amino acid transport in mixed mononuclear cell preparations is ultimately derived from monocytes (or in part from platelets, if platelets have not been completely removed). Precedent exists for the interaction of different cell types through, or in, the production of arachidonate metabolites (reviewed in Ref. 38).

It is likely that each of the selective arachidonic acid metabolism inhibitors utilized has certain actions in intact cells that are unrelated to those on fatty acid metabolism. By employing a large number of agents whose structures differ markedly, the probability that the inhibition of AIB uptake by arachidonic acid metabolism inhibitors is related to decreased thromboxane formation has been markedly increased. The possibility that these inhibitors exert their effects on amino acid transport activity by altering the formation of arachidonate metabolites other than thromboxane A_2 cannot be completely ruled out. Cuatrecasas and coworkers have recently demonstrated that concentrations of indomethacin and acetylsalicylic acid similar to those reported in this study inhibit formation of hydroxylated arachidonic acid derivatives (subsequent to inhibition of peroxidase activities) in addition to their previously described effects on fatty acid cyclooxygenase [39, 40]. To date we have been unable to demonstrate convincing enhancement of transport activity in the AIB system by exogenous hydroxyeicosatetraenoic acids (namely 5-HETE, 12-HETE, and 5,12-di-HETE) (M. C. Udey, W. F. Stenson and C. W. Parker, unpublished observations).

When the effects of these various inhibitors on lymphocyte mitogenesis [14] and amino acid transport are compared, the agents studied in both systems show exactly the same *relative* inhibitory potencies. In general, however, higher drug concentrations are required to inhibit α-aminoisobutyric acid uptake than to reduce [^{3}H]thymidine incorporation [14]. A number of explanations may account individually, or in sum, for these discrepancies: (1) the acute nature of the transport experiments, with limited permeability of the agents tested; (2) differential drug binding to serum proteins in the various media; (3) different substrate concentrations, as influenced by media composition; of interest in this regard are the recent observations [41] that human sera contain more albumin and polyunsaturated fatty acids than fetal calf sera, which may contribute to the relative drug resistance observed in this study; and (4) a lesser amount of thromboxane A_2 formation may be required for the normal function of the amino acid A transport system than for the process of cellular activation and proliferation as a whole.

The mechanism by which thromboxane A_2 formation is involved in the regulation of amino acid transport activity and/or cell differentiation and proliferation is unknown. To date there are little data in the literature to suggest that any of the known arachidonic acid metabolites can influence cellular metabolism except through modulation of cyclic nucleotide metabolism, so cAMP- or cGMP-dependent protein phosphorylation must remain candidate effector mechanisms for thromboxane A_2. In addition, it is not possible to distinguish between a "signal-like" and a "permissive" role for increased thromboxane A_2 synthesis in the initiation of lymphocyte activation. Under certain circumstances, modest increases in thromboxane B_2 labeling by human mononuclear cells can be measured within a few hours of mitogen stimulation [Ref. 16 and present report (see Table 1)]. On the other hand, the present study clearly shows that arachidonic acid metabolism inhibitors can influence the biochemical behavior of both resting and stimulated cells. This action on unstimulated cells argues that the arachidonate metabolite is not acting by promoting cell interactions which favor activation. On the other hand, thromboxane A_2 has been reported to aggregate both platelets [1] and polymorphonuclear leukocytes [42], and there is considerable evidence to indicate that cell interactions are important in the response of lymphocytes to mitogens [43].

Roles for arachidonic acid metabolites as modulators of immune responsiveness have been suggested previously [44], although most considerations have been restricted to the actions of the prostaglandins. The importance of understanding lymphoid cell lipid metabolism has been re-emphasized by the recent discovery that at least certain of the lymphokines may consist of bioreactive lipids bound to inert carrier proteins [45, 46]. It seems likely that observations made in studies of lymphoid cell metabolism are applicable, in principle, to other systems where differentiation, proliferation and intercellular cooperation are important considerations.

Acknowledgements—We thank Ms. Beverly Raab for expert technical assistance, Dr. William F. Stenson for helpful discussions, and Ms. Debra K. Udey, Ms. Nancy Grimshaw and Ms. Mary Anne Blue for secretarial assistance in preparing this manuscript.

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