MK886, a Potent and Specific Leukotriene Biosynthesis Inhibitor Blocks and Reverses the Membrane Association of 5-Lipoxygenase in Ionophore-challenged Leukocytes*

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Recently, we have shown that ionophore activation of human leukocytes results in leukotriene synthesis and a translocation of 5-lipoxygenase from the cytosol to cellular membrane. This membrane translocation was postulated to be an important early activation step for the enzyme. 3-[1-(p-Chlorobenzyl)-5-(isopropyl)-3-tert-butylthiiodindol-2-yl]-2,2-dimethylpropanoic acid (MK886) is a potent and specific inhibitor of leukotriene biosynthesis in vivo and in intact cells, but has no direct effect on 5-lipoxygenase activity in cell-free systems. In this report, we show that MK886 can both prevent and reverse the membrane translocation of 5-lipoxygenase, in conjunction with the inhibition of leukotriene synthesis. Similar compounds of the indole class could also inhibit the membrane translocation of 5-lipoxygenase in a rank order of potency that correlated with their potencies for leukotriene synthesis inhibition. In contrast L-656,224, a direct 5-lipoxygenase inhibitor, had no effect on the translocation of the enzyme. Attempts to demonstrate the effects of MK886 on the association of 5-lipoxygenase with membrane in cell-free preparations failed due to a nonspecific Ca2+-dependent sedimentation of the enzyme.

The mechanism of action of MK-886 is therefore to block translocation, prevent subsequent activation of 5-lipoxygenase, and hence block cellular leukotriene biosynthesis.

Leukotrienes are a class of metabolites of arachidonic acid that are synthesized mainly by leukocytes in response to inflammatory and immunologic stimuli (1). The biologic effects of the leukotrienes, including leukocyte chemotaxis and aggregation, airway smooth muscle contraction, and edema formation, have implicated them as potential mediators of allergy and inflammation. Consequently, the regulation of leukotriene biosynthesis is of considerable interest with regard to the pathophysiology of these disease processes.

The first two steps of the leukotriene biosynthetic pathway are catalyzed by 5-lipoxygenase (2-5). These reactions involve the addition of molecular oxygen to carbon 5 of arachidonic acid to form 5(S)-hydroperoxy-6,8,11,14-eicosatetraenoic acid (5-HPETE),1 and the further conversion of 5-HPETE to 5,6-oxido-7,9,11,14-eicosatetraenoic acid (LTA,). LTA, is then further metabolized by other enzymes to LTE4 and LTC4, depending on the cell type. 5-Lipoxygenase activity in cell-free preparations is highly Ca2+-dependent, and is also stimulated by ATP, and phosphatidylcholine or cellular membranes (3-9). In addition, two soluble, partially purified protein fractions from human leukocytes have been shown to be stimulatory for the human 5-lipoxygenase, although the active components of these fractions remain unknown (10).

The exact mechanism for 5-lipoxygenase activation remains unclear, however, we recently reported that treatment of human leukocytes with ionophore A23187 results in leukotriene synthesis accompanied by the loss of 5-lipoxygenase protein and activity from the cell cytosol (100,000 x g supernatant) and the accumulation of inactive enzyme in cell membranes (100,000 x g pellet) (11). Since 5-lipoxygenase becomes irreversibly inactivated during its reaction with arachidonic acid, the fact that the membrane-associated enzyme is inactive suggests that this is the enzyme pool that has been utilized for leukotriene synthesis. This is further supported by the finding that the membrane association of the enzyme correlates temporally and quantitatively with leukotriene synthesis. Together, these results are consistent with the hypothesis that translocation of 5-lipoxygenase from the cytosol to a membrane site may be a critical early activation step for the enzyme.

In the present report, we describe the results of studies using a novel leukotriene biosynthesis inhibitor, MK886 (3-[1-(p-chlorobenzyl)-5-isopropyl-3-tert-butylthioindol-2-yl]-2,2-dimethylpropanoic acid) (12). This compound is a highly potent inhibitor of leukotriene formation in vivo, and in intact cells in vitro, but has no direct effect on the 5-lipoxygenase. However, as we show here, MK886 both prevents and reverses the membrane association of 5-lipoxygenase, and this effect on membrane association correlates with

1 The abbreviations used are: HPETE, hydroperoxyeicosatetraenoic acid; PBS, phosphate-buffered saline; HETE, hydroxyeicosatetraenoic acid; MK886, formerly designated L-656,538, 3-[1-(p-chlorobenzyl)-5-isopropyl-3-tert-butylthioindol-2-yl]-2,2-dimethylpropanoic acid; L-669,572, 3-[1-(p-chlorobenzyl)-5-isopropyl-3-cyclopentylmethylthioindole-2-yl]-2,2-dimethylpropanoic acid; L-683,511, 3-[1-(p-chlorobenzyl)-5-isopropyl-3-phenylsulfonylindol-2-yl]-2,2-dimethylpropanoic acid; L-654,639, 3-[1-(p-chlorobenzyl)-5-methoxy-3-methylindol-2-yl]-2,2-dimethylpropanoic acid; L-665,210, 3-[1-(p-chlorobenzyl)-5-isopropyl-3-phenylsulfinylindol-2-yl]-2,2-dimethylpropanoic acid; L-668,087, 3-[1-(p-chlorobenzyl)-5-isopropyl-3-tert-butylthioindol-2-yl]-2,2-dimethylpropanoic acid methyl ester; L-656,224, 3-[1-(p-chlorobenzyl)-5-isopropyl-3-phenylsulfinylindol-2-yl]-2,2-dimethylpropanoic acid; L-686,017, 7-chloro-2-[4-(methoxyphenyl)methyl]-3-methyl-5-propyl-4-benzofuranol; LT4, 5,6-oxido-7,9,11,14-eicosatetraenoic acid.

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the inhibition of leukotriene synthesis. Furthermore, our studies have shown that MK886 specifically interacts with an 18,000-dalton protein that has now been purified from leukocyte membranes (17). Together, these results suggest that the 18,000-dalton protein is involved in the translocation process. The mechanism of action of MK886, therefore, is through its interaction with the 18,000-dalton protein to prevent 5-lipoxygenase translocation, and thereby leukotriene synthesis.

**EXPERIMENTAL PROCEDURES**

**Human Leukocyte Suspensions**—Human leukocyte concentrates (buffy coat) were obtained from local blood collection centers. Contaminating erythrocytes were removed by dextran sedimentation and hypotonic lysis in a Tris-buffered ammonium chloride solution (10). The cells were washed twice in Dulbecco's phosphate-buffered saline (PBS, GIDICO) and then suspended in PBS at a final concentration of 4 x 10^6 cells/mL. The resulting suspension consisted of a mixture of granulocytes, monocytes, and lymphocytes in ratios comparable to those found in normal peripheral blood.

**Incubation Conditions**—For most experiments, 20-ml aliquots of the cell suspensions were prepared, and inhibitors were added as a stock solution (0.2 or 1.0 mM) in ethanol to give the desired final concentration. The samples were warmed to 37°C, and the cells were then stimulated by the addition of 2 or 4 μl of a solution (10 mM) of ionophore A23187 (Sigma) in dimethyl sulfoxide. The concentrations of ethanol and dimethyl sulfoxide were maintained constant for all samples in any given experiment and never exceeded 0.1%. After a 10-min incubation with ionophore, cell samples were placed on ice, and 900 μl of a solution (100 mM) of EDTA was added (4 mM final concentration). Samples (1 mL) were then removed and combined with 1 μl of a solution (1 μM) of proteinase inhibitor cocktail, 0.5 mM phenylmethylsulfonyl fluoride, and 60 μg/mL soybean trypsin inhibitor. The cells were homogenized by sonication for the analysis of 5-lipoxygenase activity by direct enzyme assay and immunoreactive 5-lipoxygenase protein by the immunoblot technique.

**Membrane Association of 5-Lipoxygenase in Leukocyte Supernatants**—Human leukocytes were prepared as described above, washed once in PBS, and resuspended at a concentration of 200 x 10^6/mL in homogenization buffer (containing 2 mM EDTA). Following homogenization by sonication (11), the homogenates were subjected to centrifugation at 10,000 x g for 15 min, followed by 100,000 x g for 60 min. The resulting 100,000 x g supernatants and pellets were then chromatographed on a column of Sephadex G-25 (PD-10, Pharmacia LKB Products, Inc.) equilibrated in 0.1 M NaCl, 2 mM EDTA, pH 7.4, containing 0.1 M NaCl, 2 mM EDTA, and 1 mM dithiothreitol. Aliquots (150 μl) of the resulting samples were then diluted to 980 μl in the same Tris buffer, and 1 μl of 1 mM MK886 in ethanol or ethanol alone was added. After warming to 37°C for 5 min, the samples were then incubated in a water bath at 37°C for 5 min and following centrifugation at 100,000 x g for 60 min, the tubes were then incubated in 200 μl of the sample buffer for gel electrophoresis (20 mM Tris-HCl, pH 6.8, containing 0.4% (w/v) sodium dodecyl sulfate, and a trace of bromophenol blue. Portions (75 μl) of the resulting samples were then analyzed for 5-lipoxygenase content by the immunoblot technique.

**Immunoblot Analysis of 5-Lipoxygenase**—Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed on 15 x 12 cm 10% gels overlaid with a 4% stacking gel by the method of Laemmli (11). Separated proteins in the gels were then transferred to nitrocellulose paper using a Trans-Blot electrophoretic transfer unit (LKB Produkter, Bromma, Sweden). The specificity of the anti-5-lipoxygenase antiserum used in these studies was verified on the basis that it recognizes purified 5-lipoxygenase (as an 80,000-dalton band) on immunoblot analysis. This immunoblot reaction is inhibited by preincubation of the antiserum with purified 5-lipoxygenase and the antisemum inhibits 5-lipoxygenase activity in cell-free system. Although we cannot rule out minor cross-reactivity between the polyclonal antibody and other lipoxygenases, only 5-lipoxygenase is present in any significant amount in these preparations and this has a molecular mass of approximately 75,000 daltons versus a molecular mass of approximately 80,000 daltons for 5-lipoxygenase and would clearly separate on these denaturing gels. There is no evidence for any major change in the structure of active versus inactive 5-lipoxygenase. In addition, since these studies were performed with a polyclonal antibody which recognizes multiple epitopes it is unlikely minor changes in structure would affect overall binding efficacy.

**RESULTS**

**Inhibition of the Membrane Translocation of 5-Lipoxygenase by MK886**—Leukocyte suspensions were incubated for 10 min in the presence and absence of 2 μM ionophore A23187. Leukotriene synthesis was measured, and the cells were homogenized for the preparation of 100,000 x g supernatants and pellets. The translocation of 5-lipoxygenase from cytosol to membrane was determined from the loss of enzyme activity in 100,000 x g supernatants and the accumulation of immunoreactive protein (measured by immunoblot) in the 100,000 x g pellets (11).

Ionophore challenge resulted in the synthesis of 5.2 ± 1.8 nmoles of leukotriene/10^6 cells (mean ± S.D., n = 13), whereas leukotriene synthesis by control cells (no ionophore) was undetectable. Consistent with previously reported data (11), the 5-lipoxygenase activity in 100,000 x g supernatants of ionophore-treated cells (5.3 ± 1.3 units/mg protein, mean ± S.D., n = 13) was lower than in supernatants from control cells (9.0 ± 1.5 units/mg protein, mean ± S.D., n = 13). The difference was statistically significant (p < 0.001). Concomitant with the loss of supernatant activity, there was an increase of 2.6 ± 0.8-fold (mean ± S.D., n = 13) in the quantity of immunoreactive but catalytically inactive 5-lipoxygenase protein in the 100,000 x g pellets. This difference was also statistically significant (p < 0.01).

**Fig. 1** shows the results obtained when ionophore challenge was performed in the presence of 0.5 or 1.0 μM MK886. Leukotriene synthesis by inhibitor-treated cells was undetectable (data not shown). Furthermore, the loss of supernatant enzymatic activity, and the appearance of enzyme protein in the pellets were almost completely eliminated. These results indicate that MK886 prevents the movement of 5-lipoxygenase from cytosol to membrane and protects the enzyme from the inactivation that routinely occurs in ino-
parameters. As shown in Fig. 2, MK886 caused a concentration-dependent inhibition of leukotriene synthesis by ionophore-treated cells. Under these conditions the IC50 for leukotriene biosynthesis inhibition was 102 ± 14 nM (mean ± S.D., n = 4). The effects of MK886 on the membrane translocation of 5-lipoxygenase were also concentration dependent as measured by the inhibition of the disappearance of supernatant enzymatic activity, or the inhibition of the appearance of membrane-associated enzyme protein. The inhibition of membrane translocation occurred over the same range of inhibitor concentrations as did the inhibition of leukotriene biosynthesis, indicating a correlation between these two effects of the inhibitor.

**Effect of Other Inhibitors of Leukotriene Synthesis on the Membrane Translocation of 5-Lipoxygenase**—During the course of the development of MK886, many other compounds of the indole class were synthesized and tested as leukotriene biosynthesis inhibitors. A series of five of these compounds were compared with MK886 for their capacity to inhibit leukotriene synthesis and block the membrane association of 5-lipoxygenase. In this experiment, leukocytes were challenged with ionophore in the presence of three different concentrations of each inhibitor. The results indicated that there was a linear correlation between the % inhibition of leukotriene synthesis by the cells and the amount of 5-lipoxygenase activity remaining in the cytosol (r = 0.83, Fig. 3A), and the amount of enzyme protein the cell membrane (r = −0.79, Fig. 3B). It appears, therefore, that the effect of these leukotriene synthesis inhibitors of the indole class, the structures of which are shown in Fig. 4, on the membrane association of 5-lipoxygenase is similar to that of MK886.

In contrast, an experiment was performed in which leukocytes were challenged with ionophore in the absence of concentrations of MK886 or L-656,224 that were sufficient to inhibit leukotriene synthesis by 100%. L-656,224 is a direct 5-lipoxygenase inhibitor (15). As seen in Fig. 5, L-
MK886 and 5-Lipoxygenase Translocation

FIG. 3. Effect of six compounds of the indole class on leukotriene synthesis and the translocation of 5-lipoxygenase. Leukocytes suspended in PBS were challenged with ionophore in the presence of three concentrations of six different leukotriene synthesis inhibitors of the indole class. Leukotriene synthesis was measured, and the cells were homogenized for the preparation of 100,000 x g supernatants and pellets. A, 5-Lipoxygenase activity was measured in the 100,000 x g supernatants and is expressed as percent of the activity in control supernatants (cells not challenged with ionophore). These values are plotted against the percent inhibition of leukotriene synthesis as compared with cells challenged with ionophore in the absence of inhibitor. B, the quantity of 5-lipoxygenase protein in the membrane was measured by immunoblot and is expressed as peak area/milligram membrane protein. Values are plotted against percent inhibition of leukotriene synthesis, as in A. Each point represents a single determination from cells incubated with the inhibitor indicated by the designated symbol. The numbers beside the symbols represent the concentration of inhibitor used in micromoles. The results are from a representative experiment that was carried out four times with similar findings (compound identities are given in the abbreviation footnote).

656,224 had no effect on the disappearance of cytosolic 5-lipoxygenase protein or the accumulation of the enzyme in the membrane of ionophore-challenged leukocytes. The quantity of enzyme activity in the cytosol could not be determined due to the presence of residual L-656,224 which interferes in the direct enzyme assay. From these data, we conclude that the inhibition of the membrane association of 5-lipoxygenase does not result from the effects of a direct enzyme inhibitor.

Reversal of the Membrane Association of 5-Lipoxygenase by MK886—The data presented in Figs. 1 and 2 indicate that preincubation of leukocytes with MK886 prevents the membrane translocation of 5-lipoxygenase. We next questioned whether addition of MK886 after the ionophore could reverse the membrane association of the enzyme after translocation had already occurred. Leukocytes were incubated in the presence or absence of ionophore for 10 min. At the end of the incubation period, MK886 (1 μM) was added to half of the ionophore-treated samples, whereas the remaining samples...
received ethanol as a control. The cells were then homogenized for the preparation of 100,000 × g supernatants and pellets to be assayed for 5-lipoxygenase activity and protein. The pellets of ionophore-treated cells, so that there was no significant change in the content of immunoreactive protein in the supernatants from ionophore-challenged cells. Inspection of the immunoreactive enzyme protein was determined for both supernatants and pellets. Data represent the mean ± S.D. from three separate experiments. Statistical significance was determined for immunoreactive 5-lipoxygenase by the immunoblot procedure. The results (Fig. 6) showed that, in the case of 10,000 × g supernatants, both Ca²⁺ and arachidonic acid caused an increase in the amount of 5-lipoxygenase protein associated with the membrane (100,000 × g pellet). This effect, which was augmented when Ca²⁺ and arachidonate were added together, was not inhibited by MK886. However, when 100,000 × g supernatants were used, it was discovered that Ca²⁺ and arachidonate caused a marked increase in the amount of 5-lipoxygenase that sedimented at 100,000 × g, even though no membrane was present. This sedimentation of the enzyme was also augmented in the presence of both Ca²⁺ and arachidonate. Again, MK886 had no inhibitory effects under any of these conditions (Fig. 7).

**DISCUSSION**

MK886 is a potent and specific inhibitor of leukotriene biosynthesis in intact leukocytes but does not inhibit 5-lipoxygenase activity in cell-free preparations. Because we could discern no effects of this compound on other aspects of...
buffer containing 2 mM EDTA and Ca++, concentrations in all were placed on ice and the EDTA and Ca++ concentrations in all were present in leukocyte membranes, a protein that diated by the 18,000-dalton protein. Binding of MK886 to the enzyme and a possible site of action for MK886. This hypoth-

protein in leukocyte membranes, a protein that inhibits its function and thereby blocks 5-lipoxygenase in leukocyte 10,000-g supernatants. A representative experiment that was performed three times. 100,000 g supernatants were incubated for 5 min at 37 °C in buffer containing 2 mM Ca++ and 50 μM arachidonic acid (204) as indicated. As shown, some samples also contained 1 μM MK886. The samples were then placed on ice and the EDTA and Ca++ concentrations in all were brought to 4 and 2 mM finally. After centrifugation at 100,000 × g, immunoblot analysis was performed on the resulting pellets. The data show the mean ± range for duplicate determination from a representative experiment that was performed three times.

MK886 and 5-Lipoxygenase Translocation

Fig. 7. Ca++ and arachidonic acid cause a sedimentation of 5-lipoxygenase in leukocyte 10,000 × g and 100,000 × g supernatants. Human leukocyte 10,000 × g supernatants (A) and 100,000 × g supernatants (B) were incubated for 5 min at 37 °C in buffer containing 2 mM EDTA in the absence (CON) or presence of 2 mM Ca++ and 50 μM arachidonic acid (204) as indicated. As shown, some samples also contained 1 μM MK886. The samples were then placed on ice and the EDTA and Ca++ concentrations in all were brought to 4 and 2 mM finally. After centrifugation at 100,000 × g, immunoblot analysis was performed on the resulting pellets. The data show the mean ± range for duplicate determination from a representative experiment that was performed three times.

1. If this phenomenon would not be expected to be inhibited by MK886. From these considerations, we conclude that, to date, we have only observed the specific, physiologically relevant translocation of 5-lipoxygenase in intact leukocytes. Further attempts to study this phenomenon in cell free preparations must be undertaken with extreme caution in order to avoid potential artifacts. We believe that inhibition of membrane translocation by MK886 may serve as a useful criterion to verify that the membrane association observed is specific and mediated by the 18,000-dalton protein.

It is important to note that our present results do not rule out the possibility that MK886 actually interferes with an enzyme activation step prior to the membrane association of 5-lipoxygenase, rather than the actual translocation step itself. In fact, we cannot completely dismiss the possibility that the membrane association of 5-lipoxygenase is not an enzyme activation step but occurs after leukotriene synthesis and enzyme inactivation have been completed. However, regardless of the role of translocation in the enzyme activation process, our studies with MK886 strongly suggest that the 18,000-dalton protein is important for the function of 5-lipoxygenase in the intact leukocyte.

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