Modulation of Polymorphonuclear Leukocyte Function by Cetiedil

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Cetiedil citrate monohydrate inhibits sickling of red cells and aggregation of platelets. We assessed its ability to attenuate polymorphonuclear leukocyte (PMN) function. PMN aggregation in response to 2 × 10^{-7} M formyl-met-leu-phe (FMLP) was inhibited in a dose-dependent fashion by cetiedil concentrations ranging from 60 to 250 μM. Additionally, 125 μM cetiedil inhibited PMN aggregation in response to 2 × 10^{-7} M FMLP, 20 ng/ml phorbol myristate acetate (PMA), and 1 × 10^{-4} M A23187 by 69% ± 18%, 72% ± 20%, and 65% ± 4%, respectively. Inhibition of FMLP-induced aggregation was provided by only 5 min of incubation of the drug with the cells and was partially reversible. Cell viability was unaffected by exposure of PMN to the drug. Correspondingly, 125 μM cetiedil prevented the translocation of calcium from the PMN membrane as assessed by chlorotetracycline fluorescence. Parallelizing the effect of the drug on PMN aggregation, 125 μM cetiedil inhibited release of superoxide by 55% and decreased the number of available 3H-FMLP receptors. However, its effect on release of the primary granule constituent, myeloperoxidase, was minimal (4.5% inhibition), while the effect on release of the specific granule product, lactoferrin (27% inhibition), was modest. These studies indicate that cetiedil affects PMN aggregation and superoxide release to a much greater extent than PMN degranulation. Thus, cetiedil may have potential uses in modulating inflammatory response in vivo.

Polymorphonuclear leukocytes (PMN) are one of the principal effector cells observed in numerous inflammatory diseases. In response to a variety of stimuli, PMN are activated to release oxygen byproducts as part of the metabolic burst and to discharge the contents of their cytoplasmic granules to extracellular spaces. When activation within the vascular space is uncontrolled, PMN aggregate, which sometimes leads to pulmonary vascular leukostasis associated with lung dysfunction and peripheral neutropenia. Many antiinflammatory agents are thought to exert their activity by blunting PMN activation. Thus, corticosteroids are able to reduce PMN aggregation and improve lung dysfunction in septic shock. Because 30 mg/kg of the steroid methylprednisolone is required for this effort, we have been evaluating other drugs with potentially lesser side effects. We evaluated cetiedil [2-cyclohexyl-3-thiophenacetic acid 2-(hexahydro-1H-azepin-l-yl) ester], a drug that is being currently used in Europe as a drug that is being currently used in Europe as an antisickling agent.

The antisickling property of cetiedil at concentrations from 50 to 500 μM appears to be related to its effect on the erythrocyte membrane. Schmidt and coworkers reported that cetiedil can alter cation transport in red cells by promoting changes in cell water, sodium, and potassium. The increases in net transport of both sodium and potassium were diminished by the presence of extracellular calcium, suggesting that cetiedil may affect a common membrane site for the action of calcium, leading to alternations in monovalent flux in the red cell. Our results show that cetiedil can block PMN degranulation and aggregation and that its effect, like that on the red cell, may be mediated through inhibition of calcium translocation.

MATERIALS AND METHODS

PMN Isolation

Human leukocytes (98% PMN with less than 1 platelet/1,000 cells) were isolated from heparinized venous blood drawn from consenting normal human volunteers. Prior to dextran sedimentation, 1 mM adenosine diphosphate (ADP) was added to enhance removal of platelets, and then the leukocyte-enriched plasma was layered on Ficoll-Hypaque for preparation of purified PMN.

PMN Aggregation

Aggregometry was performed by a previously described method. PMN suspensions of 10⁶ cells/ml were incubated at 37°C in a shaking water bath for varying lengths of time, with cetiedil at concentrations ranging between 60 and 250 μM. The PMN were incubated with 5 μg/ml cytochalasin-B (Sigma Chemical Co., St. Louis, MO) for 5 min at room temperature prior to the addition of FMLP. Control samples were incubated with Krebs-Ringer-phosphate (KRP) alone. Quantitation of the PMN aggregation during the initial 3 min was done using a Leitz compensating polar planimeter.

PMN Degranulation

PMN suspensions (10⁶ cells/ml) were incubated for 30 min at 37°C with cetiedil, and during the last 5 min of incubation, 5 μg/ml of cytochalasin-B was added to enhance granule content release.
from the cell. The suspensions were then stimulated in a shaking water bath with $2 \times 10^{-17} M$ FMLP or 0.01% DMSO at 37°C for 5 min and centrifuged at 400 g for 5 min at 4°C. The cell-free supernatant fluids were assayed for the specific granule constituent lactoferrin, the primary granule constituent myeloperoxidase, and for the cytoplasmic enzyme lactate dehydrogenase.

**Release of Membrane-Bound Calcium**

Fluorescent measurements were made using a temperature-controlled cuvette holder and automatic stirrer. Excitation was at 390 nm and emission at 530 nm. Chlorotetracycline (CTC), final concentration 10 μM, was added to 3 x 10⁶ PMN/ml suspended in Hepes buffer (10 mM Hepes, pH 7.35, with 130 mM NaCl, 5 mM KCl, and 5.5 mM glucose). Exogenous calcium chloride, 1 mM final concentration, was added just before the addition of CTC. The cell suspensions were incubated for 30 min at 37°C in the presence of CTC and cetiedil. Cetiedil by itself did not affect CTC loading or the CTC signal.

**Superoxide Release**

Superoxide anion was measured by the superoxide dismutase (SOD) sensitive reduction of cytochrome-C, as described by Babior et al. After incubating the PMN for 30 min with varying concentrations of cetiedil at 37°C, the cells were treated for 5 min with 5 μg/ml cytochalasin-B, then stimulated by the addition of $2 \times 10^{-7} M$ FMLP.

**FMLP Receptors**

To quantitate PMN receptors, 5 x 10⁶ cells were incubated with 125 μM of cetiedil or saline solution for 5 min at room temperature. Then, PMN were placed in the ice bath, and nonradioactive FMLP (10⁻¹⁵ M) or DMSO-saline-bovine serum albumin (BSA), as well as ³H-FMLP at varying final concentration (5-180 nM), were added. The PMN were kept in an ice bath for 2 hr or at 37°C for 15 min, with constant shaking. Specific ³H-FMLP binding was examined by Sips analysis and by the method of Scatchard. In other experiments PMN were incubated for 5 min at room temperature with varying concentrations of cetiedil or saline containing the solvent employed for dissolving cetiedil. Nonradioactive FMLP (10⁻¹⁵ M) or DMSO (0.1% in saline-BSA) were added for determination of nonspecific and total binding, respectively, at ice bath temperatures.

**RESULTS**

**PMN Aggregation**

Aggregation of cytochalasin-B-treated PMN was induced by $2 \times 10^{-17} M$ FMLP. When PMN were incubated with 125 μM cetiedil for 30 min and then stimulated with FMLP, aggregation was inhibited by 69.3% ± 18.1%. Aggregation in response to 20 ng/ml PMA and 10⁻⁴ M A23187 were inhibited by 71.5% ± 19.5%, 64.8% ± 3.8%, respectively. Cetiedil inhibited aggregation of cytochalasin-treated PMN in a dose-dependent manner (Table 1). The attenuating effect of cetiedil on PMN aggregation upon stimulation with A23187 was lost by washing of the treated PMN (3.4% ± 5.1% of control). On the other hand, the addition of either 5% BSA or 20% fetal calf serum (FCS) to the PMN suspension did not prevent the marked inhibitory effects of cetiedil (data not shown).

**PMN Degranulation and Superoxide Anion Release**

Treatment with cetiedil also led to an attenuation of the release of granule products from cytochalasin-B-treated PMN following stimulation with $2 \times 10^{-17} M$ FMLP. Cetiedil inhibited, in a dose-dependent fashion, the release of both the primary granule constituent myeloperoxidase and the specific granule constituent lactoferrin (Table 2). However, the effect of cetiedil was more profound on release of the latter. Cetiedil failed to promote the release of the cytoplasmic marker, lactate dehydrogenase, beyond 5% of total, indicating that the cell permeability remained unchanged; furthermore, trypan blue exclusion, another indicator of cell viability, was 98% in the presence of all concentrations of cetiedil employed. Appropriate experiments

<table>
<thead>
<tr>
<th>Incubation Mixture</th>
<th>Myeloperoxidase (% of Total)</th>
<th>Lactoferrin (% of Total)</th>
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<tbody>
<tr>
<td>No drug</td>
<td>42.2 ± 3.7</td>
<td>46.9 ± 7.9</td>
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<tr>
<td>60 μM cetiedil</td>
<td>49.8 ± 4.5</td>
<td>35.8 ± 6.2</td>
</tr>
<tr>
<td>125 μM cetiedil</td>
<td>40.3 ± 3.8</td>
<td>34.2 ± 5.1</td>
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<tr>
<td>250 μM cetiedil</td>
<td>34.1 ± 3.2</td>
<td>21.1 ± 0.9*</td>
</tr>
</tbody>
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Table 2. Effect of Cetiedil on Degranulation of Cytochalasin-B-Treated Polymorphonuclear Leukocytes Stimulated by FMLP

Human PMN, 10⁶ cells/ml, suspended in KRP from 3 different donors were incubated at 37°C for 30 min with cetiedil. During the last 5 min of incubation, 5 μg/ml cytochalasin-B was added. The supernatant fluids were assayed after 5 min for myeloperoxidase or lactoferrin content. Each experiment was performed in triplicate.

*The p values were calculated by comparing suspensions containing drugs to those without drugs, and all were nonsignificant except for the effect of 250 μM cetiedil on lactoferrin release (p < 0.01).
indicated that cetiedil did not influence the enzyme assays, the radioimmunoassay for lactoferrin, or the recovery of enzymes from the PMN themselves.

As indicated in Fig. 1, release of superoxide from cytochalasin-B-treated PMN upon stimulation with $2 \times 10^{-7} \text{M} \text{FMLP}$ was inhibited by cetiedil in a dose-dependent fashion. The extent of inhibition paralleled the results for FMLP-induced aggregation.

**FMLP Receptors**

As shown in Fig. 2, the binding ability of FMLP to the PMN surface was inhibited by cetiedil. The inhibition was dose-dependent and was linear between concentrations of 50 and 200 μM. The kinetic behavior of FMLP binding in the presence and absence of 125 μM cetiedil was examined at different concentrations of $^3\text{H-FMLP}$. A representative Scatchard plot for this study is shown in Fig. 3, where the best fit lines were derived from linear regression analysis. Data from three different experiments are summarized in Table 3. The available FMLP binding sites per PMN were calculated from the corresponding Scatchard plots. As indicated in Table 3, the number of surface FMLP receptors was decreased by 33% in the presence of 125 μM cetiedil, as compared to the control cells ($p = 0.02$, paired sample t test). The $K_v$ values were calculated to be 1.8 and $2.2 \times 10^{-8}/M$, respectively, in the absence and presence of 125 μM cetiedil at 37°C. Greater $^3\text{H-FMLP}$ binding occurred at 37°C than at 0°C, but the association constants for $^3\text{H-FMLP}$ were not altered by temperature.

**Calcium Translocation by PMN**

Chlorotetracycline (CTC) has been employed to monitor translocation of calcium from membrane locations in mammalian cells. We have employed CTC-labeled human PMN in an effort to determine whether cetiedil affected the release of membrane-associated calcium in the PMN. As indicated in Fig. 4, cetiedil was affected by the translocation of calcium upon stimulation with $2 \times 10^{-7} \text{M} \text{FMLP}$ in a dose-dependent fashion. Concentrations of 125–250 μM cetiedil markedly impaired CTC fluorescence, whereas the lowest concentration enhanced the FMLP-dependent decrease in the CTC signal. This effect was far greater than the drug’s effect on FMLP receptor binding, suggesting that the effect of cetiedil...
on impairing PMN granule and metabolic function was at the level of the plasma membrane.

**DISCUSSION**

Chemotactic factors profoundly modify the permeability of the plasma membrane of human PMN. Among the most dramatic of these changes are rapid increases in the rates of uptake of sodium and calcium. These events are thought to be intimately involved in the sequence of events that is initiated by the binding of chemotactic factors to the plasma membrane receptors, eventually leading to PMN degranulation and aggregation. The involvement of cations in PMN chemotaxis, superoxide anion release, lysosomal exocytosis, and aggregation has been well established. There appears to be an absolute requirement for calcium and magnesium for optimal PMN aggregation. Previous studies indicated that verapamil could block the transport of calcium into PMN and impair release of superoxide following FMLP stimulation of the cells. Additionally, we previously reported that the absence of divalent cations would blunt human PMN aggregation upon exposure to FMLP, implicating a role of membrane-associated calcium in this response.

We found that cetiedil, like dexamethasone, inhibited the release of both superoxide and the specific granule product lactoferrin by activated PMN. The concentration of cetiedil required to produce measurable changes in PMN activation compares favorably with the drug concentration required for changes in red cell membrane permeability to sodium and potassium associated with inhibition of sickling in vitro.
Approximately 2 mg cetiedil/kg body weight would be required to achieve intravascular concentrations of 100 μM in vivo. As a single intravenous dose, this would be tenfold more drug than is usually given to humans. However, little is known about the metabolism and excretion of cetiedil, and blood levels have not been reported. On the other hand, intravenous injections of 3.75 mg cetiedil/kg body weight have been administered successfully to animals. We carried out several investigations to examine the mechanism by which cetiedil blocked PMN activation. Two separate lines of evidence suggest that cetiedil may bind to a site on the PMN membrane involved in calcium metabolism. Functionally, cetiedil blocked PMN aggregation in response to the calcium ionophore A23187. Furthermore, 125 μM cetiedil completely prevented the translocation of membrane-bound calcium. The effect of cetiedil on calcium translocation could readily explain cetiedil’s ability to block PMN activation. Although cetiedil has been reported to inhibit phosphodiesterase, other studies have shown that cyclic adenosine monophosphate (cAMP) agonists, which raise intracellular levels of cAMP in PMN, only minimally affect PMN aggregation.

Recent studies indicate that cetiedil binds to erythrocyte membranes, resulting in an alteration of sodium flux in the red blood cells. Our studies would suggest that cetiedil likely binds to PMN membranes altering not only calcium translocation, but also affecting FMLP receptor numbers as well. Unlike studies previously reported with corticosteroids, which failed to affect receptor number but did affect a decrease in association rate constant, cetiedil diminished FMLP receptors without decreasing the association rate constant. Although cetiedil diminished the number of available binding sites at equivalent concentrations of 125 μM, its effect was much more profound on preventing calcium translocation than on diminishing binding sites. Furthermore, its ability to block not only FMLP-induced aggregation, but also responses to PMA and A23187, suggests that cetiedil has a more generalized effect on the membrane properties of the PMN. Unlike gold compounds, which also suppress PMN aggregation and granule extrusion, cetiedil activity was maintained in the presence of plasma proteins in vitro. Whether cetiedil may prove to be an effective agent in dampening PMN activation in vivo remains to be determined.

REFERENCES


5. Craddock PR, Hammerschmidt DC, Moldow CF, Yamada P, Jacob HS: Granulocyte aggregation as a manifestation of membrane interactions with complement: Possible role in leukocyte margination, microvascular occlusion, and endothelial damage. Semin Hematol 16:140, 1979


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