Bafilomycin A₁, a Specific Inhibitor of Vacuolar-type H⁺-ATPase, Blocks Lysosomal Cholesterol Trafficking in Macrophages*

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Takemitsu Furuchi, Kazuhiro Aikawa, Hiroyuki Arai, and Keizo Inoue

From the Department of Health Chemistry, Faculty of Pharmaceutical Sciences, The University of Tokyo, Tokyo 113, Japan

Certain steroids having an oxo group at the C-17 or C-20 position such as pregnenolone and dehydroisoandrosterone inhibit the cholesterol transport from lysosomes to other cellular sites. Taking advantage of the fact that the inhibition is reversed upon removal of the steroids, we studied the factors that control the cholesterol transport from lysosomes to other cellular sites in macrophages. Macrophages that accumulated unesterified cholesterol in their lysosomes were prepared by incubating cells with liposomes containing cholesterol and phosphatidylserine in the presence of a steroid inhibitor. These cells were chased by means of steroid washout, and then the effects of various pharmacological agents on the subsequent metabolism of cholesterol were examined. When the cells were chased in the absence of the agents, some of the cholesterol was converted to cholesteryl esters in the cells, and others were desorbed into the medium as unesterified forms, suggesting recovery of lysosomal cholesterol trafficking. Among the agents tested, bafilomycin A₁, a specific inhibitor of vacuolar-type H⁺-ATPase, completely blocked both cholesterol esterification and cholesterol desorption at 10 nM. Moreover, agents that neutralize the lysosomal proton gradient, such as ammonium chloride and chloroquine, also reduced both of the processes. Fluorescent microscopic examination of bafilomycin A₁-treated cells revealed extensive filipin-cholesterol staining of perinuclear lysosomes. From these data, we conclude that acidic pH is required for the efflux of cholesterol from lysosomes to other cellular sites.

Although the overall pathway for the accumulation of cholesteryl esters in macrophages is understood, the mechanisms underlying the intracellular transport of endocytosed cholesterol from lysosomes to other cellular sites are not fully understood. Liscum and Faust (6) were the first to demonstrate that U18666A (3-β-[2-diethylamino]ethoxy)androst-5-en-17-one suppresses the intracellular transport of low density lipoprotein-derived cholesterol from lysosomes to plasma membranes in cultured Chinese hamster ovary cells. Very recently, Butler et al. (7) demonstrated that progesterone and its precursor, pregnenolone, restrict the cellular processing of lysosomal cholesterol in cultured fibroblasts. We also observed that a series of structurally related steroids having an oxo group at the C-17 or C-20 position, such as pregnenolone, progesterone, androstenedione, and dehydroisoandrosterone, specifically inhibit lysosomal cholesterol trafficking in macrophages. In the present study, taking advantage of the fact that steroid inhibition of lysosomal cholesterol trafficking is reversible in macrophages, we elucidated intracellular factors that control the transport of cholesterol from lysosomes to other cellular sites.

MATERIALS AND METHODS

Chemicals—[1α,2α-2H]Cholesterol (45.1 Ci/mmol) was purchased from Amersham, United Kingdom. Cholesterol, pregnenolone, progesterone, androstenedione, dehydroisoandrosterone, cytochalasin B, colchicine, and chloroquine were obtained from Sigma. Bafilomycin A₁, a kind gift from Dr. Moriyama, Osaka University. Filipin was obtained from Polysciences, Inc.

Cells—Mouse peritoneal macrophages were prepared as described (8). Briefly, peritoneal cells were harvested from unstimulated ICR mice using Hanks' balanced salt solution and then suspended at 2 x 10⁶ cells/ml. Aliquots (0.5 ml) were dispensed into 24-well plastic microplates and then incubated under a humidified CO₂ (5%) atmosphere at 37 °C. After 2 h, each plate was washed three times with Hanks' balanced salt solution. The medium was immediately replaced with 0.5 ml of Dulbecco's modified Eagle's medium containing 5% lipoprotein-deficient serum, penicillin (100 units/ml), and streptomycin (100 µg/ml) (hereafter referred to as medium A).

Preparation of Liposomes—Multilamellar liposomes were prepared as described previously (8). Briefly, phosphatidylcholine (1 mol%), phosphatidylserine (1 mol%), digalactosylceramide (0.2 mol%), cholesterol (1.5 mol%), and a trace amount of [1α,2α-2H]cholesterol (20 µCi) were dried and then suspended in 1 ml of 0.3 M sucrose.

Metabolism of Liposomal Cholesterol by Cultured Macrophages—To macrophage cultures, 25 µl of liposomes containing [2H]cholesterol (17H-FC) liposomes and 5 µl of 1 µM pregnenolone (dissolved in ethanol) were added. Cells were harvested at the indicated times, and then cellular [2H]cholesterol ester and [2H]unesterified cholesterol were quantitated as described previously (8). To determine the metabolism of lysosomal cholesterol, cells were incubated with 25 µl of [1H-FC] liposomes and 5 µl of 1 mM pregnenolone for 12 h. Then the cells were washed twice with 0.5 ml of...

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† To whom correspondence should be addressed: Dept. of Health Chemistry, Faculty of Pharmaceutical Sciences, The University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo 113, Japan. Tel.: 03-3812-2111 (ext. 4720); Fax: 03-3818-0173.


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cultured macrophages and that the liposomal components unesterified [3H]cholesterol level decreased with a concomitant increase in the [3H]cholesteryl ester level (Fig. 1B). In contrast, in cells without steroid washout, the cellular unesterified cholesterol level remained almost constant, and no appreciable amount of cholesteryl esters was formed. Fig. 2 shows the total radioactivities of the cellular and medium fractions during the chase incubation. The radioactivity in the cellular fraction (unesterified plus esterified [3H]cholesterols) decreased gradually, with a concomitant increase in that of the medium fraction when cells were chased in the absence of the steroid. Most of the radioactivity in the medium was found to be due to unesterified cholesterol. At 10 h, control cells had desorbed about 25% of the cellular cholesterol, whereas only 5% had been desorbed by pregnenolone-treated cells. These data indicated that the inhibition of lysosomal cholesterol transport by pregnenolone is reversed on removal of the steroid.

Effects of Various Drugs on Lysosomal Cholesterol Transport—With the system described above, it became possible to examine the effects of various pharmacological agents on the postlysosomal process of cholesterol metabolism. Macrophage monolayers were pulsed with [3H-FC]liposomes for 12 h in the presence of 10 μM pregnenolone and then chased for 5 h in the absence or presence of various pharmacological agents. First, we examined the effects of agents that disrupt the cytoskeleton, such as cytochalasin B and colchicine. As shown in Table I, cytochalasin B (1 μM) and colchicine (50 μM) had appreciably no effect on either cholesteryl ester formation or cholesterol desorption. In a separate experiment, cytochalasin B caused marked inhibition of the cholesteryl ester formation on concomitant incubation with liposomes (8), indicating that cytochalasin B only inhibits the prelysosomal process of cholesterol metabolism, probably endocytosis of the liposomes. Next, we examined the effect of bafilomycin A1, a specific inhibitor of V-type H+-ATPase, on lysosomal cholesterol metabolism. Surprisingly, both cholesteryl ester formation and cholesterol efflux into the medium were effectively inhibited by a low concentration of bafilomycin A1 (10 nM). Fig. 3 shows the dose-response curves for the inhibition of both cellular [3H]cholesteryl ester formation and [3H]cholesterol release into the medium. Complete inhibition of both of the processes was achieved at 10 nM. The dose-response curve for the inhibition was essentially the same as that for the inhibi-
Port. To confirm that low pH plays an important role in lysosomal cholesterol transport, we determined the ability of lysosomal cholesterol transport. After being incubated with pregnenolone for 12 h. The monolayers were then washed and refed 0.5 ml of medium A without or with various inhibitors. After 5 h incubation, the amounts of cellular [^3H]cholesterol (it is not necessarily consistent with the data previously reported by Liscum (15). She demonstrated that cholesterol transport from lysosomes to the plasma membrane was not significantly altered by agents that affect the lysosomal pH, significantly reduced the lysosomal cholesterol efflux. From these results, we concluded that the efflux of cholesterol from lysosomes to the cytoplasm requires a low pH in lysosomes. The acidic pH generated in these compartments by V-type H^+-ATPases appears to play a crucial role in the regulation of several important functions, including receptor-ligand dissociation, vesicular and protein trafficking, transport of solutes, and intracellular hydrolysis of macromolecules (14). This paper presents the first evidence that V-type H^+-ATPase (thus acidic pH) plays an important role in lysosomal cholesterol transport. However, this notion is not necessarily consistent with the data previously reported by Liscum (15). She demonstrated that cholesterol transport from lysosomes to the plasma membrane was not significantly altered by agents that affect the lysosomal function or energy poisons in cultured Chinese hamster ovary cells, although weak inhibitory effects of chloroquine and NH4Cl on the desorption of lysosomal cholesterol into the medium were mentioned in the article. Different mechanisms of postlysosomal cholesterol transport may function between macrophages and fibroblasts. The difference in the experimental conditions such as the cholesterol-loading conditions may also be responsible for the observed difference.

The mechanisms that regulate the transport of cholesterol from lysosomes to other cellular sites are now being characterized (16, 17). The fact that genetic mutants (Niemann-Pick type C, etc.) exist that affect the transport of cholesterol (18-23) indicates that the efflux of cholesterol from lysosomes is not a spontaneous diffusion process but rather is highly

### TABLE I

**Effects of various inhibitors on lysosomal cholesterol transport**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Cholesteryl ester formation (cpm × 10^3)</th>
<th>Cholesterol in medium (cpm × 10^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.17</td>
<td>10.4</td>
</tr>
<tr>
<td>Pregnenolone</td>
<td>0.2</td>
<td>2.3</td>
</tr>
<tr>
<td>Cytochalasin B</td>
<td>10.8</td>
<td>10.0</td>
</tr>
<tr>
<td>Colchicine</td>
<td>10.2</td>
<td>9.1</td>
</tr>
<tr>
<td>Bafilomycin A1</td>
<td>0.2</td>
<td>2.4</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>0.3</td>
<td>2.5</td>
</tr>
<tr>
<td>NH4Cl</td>
<td>0.6</td>
<td>3.2</td>
</tr>
</tbody>
</table>

**DISCUSSION**

In the present study, we demonstrated that the cholesterol efflux from lysosomes to other cellular sites in macrophages was completely inhibited by bafilomycin A1, a specific inhibitor of V-type H^+-ATPase. The dose response of the inhibition by this drug exactly coincides with that of the inhibition of V-type H^+-ATPase (11), indicating that the inhibition of the lysosomal cholesterol efflux is due to the suppression of V-type H^+-ATPase present in the lysosomal membranes. In addition to bafilomycin A1, NH4Cl and chloroquine, which increase the lysosomal pH, significantly reduced the lysosomal cholesterol efflux. From these results, we concluded that the efflux of cholesterol from lysosomes to the cytoplasm requires a low pH in lysosomes. The acidic pH generated in these compartments by V-type H^+-ATPases appears to play a crucial role in the regulation of several important functions, including receptor-ligand dissociation, vesicular and protein trafficking, transport of solutes, and intracellular hydrolysis of macromolecules (14). This paper presents the first evidence that V-type H^+-ATPase (thus acidic pH) plays an important role in lysosomal cholesterol transport. However, this notion is not necessarily consistent with the data previously reported by Liscum (15). She demonstrated that cholesterol transport from lysosomes to the plasma membrane was not significantly altered by agents that affect the lysosomal function or energy poisons in cultured Chinese hamster ovary cells, although weak inhibitory effects of chloroquine and NH4Cl on the desorption of lysosomal cholesterol into the medium were mentioned in the article. Different mechanisms of postlysosomal cholesterol transport may function between macrophages and fibroblasts. The difference in the experimental conditions such as the cholesterol-loading conditions may also be responsible for the observed difference.

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controlled. However, the molecular bases of these genetic disorders or the machinery for the lysosomal cholesterol transport is totally unknown. In our preliminary experiments, a group of structurally related steroids characterized as pregnenolone and dehydroisoandrosterone, exhibited the ability to block lysosomal cholesterol trafficking. Moreover, it was revealed that the replacement of an oxo group at the C-17 position with a hydroxy group resulted in marked reduction of the inhibitory effect on the lysosomal cholesterol transport. This putative cellular machinery may require the acidic pH in the interior of lysosomes for its proper function.

REFERENCES