Effect of Cupric Ion on Cholesteryl Ester Hydrolysis in Rat Peritoneal Macrophages

Mitsuo Tanaka

Showa College of Pharmaceutical Sciences, 3-3165, Higashi-tamagawagakuen, Machida, Tokyo 194, Japan.

Received August 11, 1992

The inhibitory effects of Cu\(^{2+}\) on the hydrolysis of cholesteryl [\(^{4-14}\)C]oleate droplets in rat peritoneal macrophages were studied. Macrophages rapidly incorporated cholesteryl [\(^{4-14}\)C]oleate droplets and hydrolyzed at a linear rate over 9 h.

When macrophages were preincubated in the medium containing Cu\(^{2+}\), the hydrolysis of cholesteryl oleate within macrophages was markedly inhibited by Cu\(^{2+}\) and eventually led to the accumulation of cholesteryl oleate in macrophages. The inhibitory effect on the hydrolysis of cholesteryl oleate was dependent on the concentration of Cu\(^{2+}\). Also, the activity of acid cholesteryl ester hydrolase (acid CEH) in lysosomal fractions isolated from rat peritoneal macrophages with Cu\(^{2+}\) showed a marked decrease. This decrease also occurred in a dose-dependent manner.

These results suggest that Cu\(^{2+}\) inhibits the hydrolysis of cholesteryl ester in macrophages, and that the accumulation of cholesteryl ester in macrophages of the early atherosclerotic lesion is responsible for the decrease of lysosomal acid CEH activity.

Keywords: cholesterol metabolism; acid cholesteryl ester hydrolase; lysosome; macrophage; cupric ion

It is well known that the accumulation of cholesteryl ester inclusions within smooth muscle cells and macrophages develops into foam cells and has been established as one of the early events in the development of atherosclerosis.\(^1\)\(^-\)\(^6\) Therefore, the hydrolysis of cholesteryl ester represents an important reaction in intracellular cholesterol metabolism.

It is reported that acid cholesteryl ester hydrolase (acid CEH) plays an important role in the metabolism of exogenous cholesteryl ester introduced into cells as lipoproteins.\(^7\) Recently, Mahlberg et al.\(^8\) reported that J774 macrophages rapidly incorporated cholesteryl ester droplets by a non-saturating phagocytic process, and that the cholesteryl oleate incorporated was hydrolyzed in the lysosomes. However, the mechanism of cholesteryl ester accumulation in the cells has not yet been clarified.

On the other hand, we recently reported that the acid CEH activity in liver lysosome of rats treated with cupric ions showed a marked decrease with increasing cupric ion concentration both in vivo and in vitro, and that excessive cupric ion concentrations could cause various disorders in lipid metabolism.\(^9\)\(^-\)\(^10\) In addition, Kuzuya et al.\(^11\) reported that transition metals such as cupric or ferrous ions are required for the induction of oxidized low density lipoprotein (LDL) toxicity. Therefore, we have demonstrated the effects of cupric ions on cholesteryl ester hydrolysis in rat peritoneal macrophages.

Materials and Methods

Materials: Cholesteryl [\(^{4-14}\)C]oleate was purchased from New England Nuclear. Cholesteryl oleate, egg phosphatidylcholine, bovine brain phosphatidylserine, penicillin, streptomycin, Dulbecco modified eagles medium (DMEM) and heat-inactivated fetal calf serum (FCS) were purchased from Sigma Co., Ltd. (Tokyo, Japan).

Cell Culture: Rat peritoneal macrophages were stimulated by an intraperitoneal injection of 4% thioglycolate medium into the rat (Young male Sprague Dawley rats weighing 150—180 g). The cells were harvested 4 d after the injection. The cells were suspended in DMEM supplemented with 10% FCS and penicillin (100 units/ml) and streptomycin (100 \(\mu\)g/ml). The cells were plated in Falcon tissue culture wells (50 mm wells), at a density of \(2 \times 10^6\) cells/ml, using 2.0 ml/well and were incubated for 2 h at 37°C in a CO\(_2\) (5%) incubator. Prior to the initiation of the experiments, the cells were washed twice with DMEM to remove contaminating nonadherent cells. Cholesteryl [\(^{4-14}\)C]oleate droplets were added in the above medium containing 10% FCS together with any other conditions as described in the figure legends. The cells were incubated at 37°C for 4 h.

To measure the effect of cupric ions on the hydrolysis of cholesteryl ester, the loaded cells were further preincubated in DMEM and various concentrations of CuCl\(_2\) for the indicated period of time.

Preparation of Lysosomal Fractions: For the preparation of lysosomal fraction from rat peritoneal macrophages, the method of Brecher et al.\(^12\) was followed. The macrophages were homogenized in ice-cold 0.25M sucrose/1 mM EDTA/0.01M Tris-HCl buffer (pH 7.5). The homogenate was centrifuged at 1000 \(\times\) g for 10 min and the resulting supernatant was centrifuged at 3300 \(\times\) g for 15 min. The 3300 \(\times\) g supernatant solution was centrifuged at 12000 \(\times\) g for 35 min. The resulting pellet was used for enzymatic study.

Acid Cholesteryl Ester Hydrolase Assay: Acid CEH activity was measured by the method of Brecher et al.\(^12\) The substrate was prepared as described previously.\(^13\)

Extraction of Lipids in Macrophages: At the end of the experiments, cell monolayers were rapidly washed three times with 2.0 ml cold phosphate buffer—saline and cells were treated with 0.05% sodium dodecyl-sulfate and then cell lipids were extracted with n-hexane—iso-propanol (3:1). The lipid extracts obtained were applied to silica Sep Pak cartridge column for separation of cholesterol and cholesteryl ester. The Sep Pak cartridges were preconditioned by washing with 10.0 ml n-hexane. Lipid extracts taken up in 1.0 ml n-hexane were applied to the preconditioned cartridges, and each cartridge was washed once with 2.0 ml n-hexane. Cholesteryl esters were eluted from the cartridge with an additional 3.0 ml n-hexane-diethyl ether (9:1), and cholesterol was eluted for the cartridge with an additional 3.0 ml diethyl ether. More than 95% of the total cholesteryl [\(^{4-14}\)C]oleate and [\(^{4-14}\)C]cholesterol were eluted.

Preparation of Lipid Droplets: For the preparation of lipid droplets, cholesteryl [\(^{4-14}\)C]oleate (2.5 \(\mu\)Ci), 1.0 mg cholesterol, 2.5 mg phosphatidylcholine and 0.76 mg phosphatidylethanolamine were dissolved in chloroform. A mixture of the lipids was placed in a tube and the chloroform was removed in a rotary evaporator under reduced pressure. The dried lipids were usually dispersed with a vortex mixture in 0.3 M sucrose solution, and the solution was then sonicated for 1.0 min using a Branson sonifier cell disrupter.

Protein Determination: Protein was determined by the method of Lowry et al.\(^14\) using crystalline bovine serum albumin as a standard.

Results and Discussion: The uptake of cholesteryl oleate droplets by rat peritoneal macrophages was determined by monitoring cholesteryl metabolism...
[4-14C]oleate radioactivity in cells. As shown in Fig. 1a, the incorporation of cholesteryl [4-14C]oleate droplets by macrophages was time-dependent during the first 9 h, proportional to the increasing amount of cholesteryl oleate (Fig. 1b).

To determine the hydrolysis of the incorporated cholesteryl oleate in the macrophages during the incubation period, free [4-14C]cholesterol liberated by the hydrolysis of cholesteryl [4-14C]oleate was measured. As shown in Fig. 2a, the cholesteryl oleate hydrolysis was liner over a 9 h incubation period. The rate of incorporation and hydrolysis of cholesteryl oleate droplets by rat peritoneal macrophages in this study is similar to that of J 774 macrophages reported by Mahlberg et al.\(^8\) and that of smooth muscle cells reported by Minor et al.\(^15\)

Next, macrophages were preincubated with increasing concentrations of Cu\(^{2+}\). As shown in Fig. 3, the hydrolysis of cholesteryl oleate within macrophages was markedly inhibited by the addition of Cu\(^{2+}\). The inhibitory effect on the hydrolysis of cholesteryl oleate was dependent on the concentration of Cu\(^{2+}\). In contrast, the cholesteryl oleate content in macrophages increased with the addition of Cu\(^{2+}\) and this increase occurred in a dose dependent manner. Therefore, the uptake of cholesteryl [4-14C]oleate by the macrophages were compared in the presence or absence of Cu\(^{2+}\) in the preincubation medium. There are no significant differences in cholesteryl [4-14C]oleate droplets incorporated in both conditions in the presence of concentrations ranging between 10 and 50 \(\mu\)M Cu\(^{2+}\) per dish (data not shown). This result suggests that Cu\(^{2+}\) did not affect the incorporation of cholesteryl oleate droplets by macrophages. Next, when macrophages were preincubated with 25 \(\mu\)M Cu\(^{2+}\) for a 0.5—3.0 h period, the hydrolysis of cholesteryl oleate was inhibited by Cu\(^{2+}\) at all the time points studied, including the earliest one of 0.5 h (Fig. 4). In contrast, the inhibitory effects of hydrolysis of cholesteryl oleate droplets by Zn\(^{2+}\) was much lower than that of Cu\(^{2+}\), and not that by Fe\(^{2+}\), Co\(^{2+}\) and Mn\(^{2+}\) at the same conditions (data not shown). These results
suggest that the Cu$^{2+}$-dependent inhibitory effect on hydrolysis of cholesteryl ester in macrophages may be a specific characteristic of Cu$^{2+}$.

On the other hand, Minor et al. recently reported that the hydrolysis of cholesteryl ester droplets taken up by J774 macrophages occurred within the lysosomal site, and that the esterification of lysosomally generated free cholesterol by acyl-CoA cholesterol acyltransferase was low in the macrophages. In addition, Adelman et al. and Glick et al. reported that in Fu5AH rat hepatoma cells, the hydrolysis of the endogenous cholesteryl ester occurred within the cytosolic fraction by the neutral CEH. Moreover, we recently reported that Cu$^{2+}$ inhibited the hydrolysis of the cholesteryl ester by hepatic lysosomal acid CEH both in vivo and in vitro. In this paper, the direct effect of Cu$^{2+}$ on lysosomal acid CEH activity was investigated by incubation with lysosomal fractions isolated from rat peritoneal macrophages. As shown in Fig. 5, acid CEH activity was markedly inhibited by the addition of an increasing amount of Cu$^{2+}$.

These results suggest that the Cu$^{2+}$-dependent inhibitory effect of the hydrolysis of cholesteryl ester within macrophages may be directly related to a decrease of lysosomal acid CEH activity, and the accumulation of cholesteryl esters in macrophages of the early atherosclerotic lesion is responsible for the decrease of lysosomal acid CEH activity.

References