Supplemental Data

Methods.

Determination of cholesterol synthesis in P388D1.

Cholesterol synthesis was evaluated at 0, 6, 24, 48 and 72 hours after diluting cells at 2 x 10^5/ml (exponential growth) up to the concentration of about 10^6/ml (stationary growth). To determine the rate of cholesterol synthesis, cells at the concentration of 10^6/ml were incubated for 3h with 37 KBq/ml of sodium [^{14}C]-acetate. After incubation cells were separated by centrifugation and collected. Lipids were extracted with cold acetone and neutral lipids separated by thin layer chromatography (TLC) Kiesegel plates using a solvent system containing heptane/isopropylether/formic acid (60:40:2 v/v/v). Free cholesterol and cholesterol ester bands were identified by means of comparison with standards running simultaneously with samples, and visualized using iodine vapor (1). For counting, bands were excised and added directly to counting vials containing 10 ml Ultima Gold. All incubations were carried out in triplicate and the results of individual experiments are given as mean values, variation between triplicates was less than 10%. All data are expressed as the rate of [^{14}C] acetate incorporation into cholesterol for μg of protein (2).

Dil-LDL and Dil-HDL binding kinetics.

LDL and HDL bound to 1,1'-dioctadecyl-3,3,3',3'-tetramethyllindocarbocyanine perchlorate (Dil) were used in order to evaluate exogenous cholesterol uptake in P388D1 macrophages. Dil is a fluorescent dye bound to lipid component which, when excited with wavelengths around 514nm, features an emission peak in the range of 550nm, suitable for viewing with a common filter for rhodamine. Kinetic experiments have been performed in order to investigate lipoprotein binding and internalization. Kinetics of LDL have already been well described (3-5). The kinetic of LDL uptake is strongly influenced by the temperature: at 4°C the lipoprotein binds to its receptor, at 18°C it is internalized (3), while the full lysis of the molecules is obtained at 37°C. Conversely, less is known about membrane binding and CE uptake from HDL. It has been described that after HDL binding to SR-B1 protein, only the CE component passes through the cell membrane of specialized organs (6-8). Thus, in order to investigate these aspects, preliminary experiments were carried out and a modified protocol by Stephan and Yurachek was applied (5). In particular, cells were seeded at a concentration of 2.0x10^5 cells/ml in growth medium and incubated at 37°C and 5% CO2 for 72h. Subsequently, cells were washed twice with PBS, once with medium 199 supplemented with 10mM HEPES pH 7.3, and incubated with Dil-LDL or Dil-HDL 10μg/ml for 2h at 4, 18 and 37°C respectively. Afterward, cells were washed in PBS and fixed with 4% PFA for 10 minutes at room temperature, once more washed with PBS before being observed with an Olympus IX71 inverted microscope (Olympus, Tokyo, Japan) fitted with a 20x/0.7 planapochromatic objective. Twelve bit-images were captured using a cooled CCD camera (PCO Sensicam, Kelheim, Germany), electronically coupled to a mechanical shutter interposed between the 100W Hg lamp and the microscope to limit photo bleaching. Excitation light was attenuated with a 6%

density neutral density filter. The nominal resolution of images was 0.3 microns/pixel. Image processing was performed with the Image Pro Plus package (Media Cybernetics, Silver Springs, MD).

Results and discussion.

As shown in **figure I**, P388D1 cells synthesize very low amounts of cholesterol throughout 72 hours growth.

Dil-LDL and Dil-HDL binding kinetics.

Figure II shows the fluorescent signal in P388D1 macrophages after 2h incubation with Dil bound to LDL or HDL at different temperatures. As depicted in the upper panel, macrophages incubated with Dil-LDL showed a preponderant peri-membrane fluorescence, associated with very low, or absent intra-cytoplasmatic signal at 18 and 37°C, respectively. When P388D1 macrophages were incubated with HDL (lower panel), the fluorescence signal was evident within the cytoplasm already at 4°C and was further increased at 18 and 37°C.

These results suggest that at 4°C the binding of LDL to their receptors, with the typical crown shape, is evidenced as peri-membrane fluorescence. The subsequent internalization is denoted by the increased sub-membrane fluorescence at 18°C. The lack of signal in the cytoplasm at 37°C suggests that lipoprotein digestion by endo-lysosomal pathway induces the lysis of Dil from LDL, avoiding its diffusion in cytoplasm and organelles.

Peri-membrane fluorescent spots were also evident at 4°C in cells incubated with Dil-HDL, probably related to the lipoprotein binding to SR-B1. However, the presence of fluorescence at sub-membrane level is already evident at this temperature, then progressively increases at 18 and 37°C, suggesting an early diffusion of Dil bound to the lipid component of HDL. It can be concluded that CE uptake is already present at 4°C and that the DIl component undergoes to a different fate than DIl-LDL.

References.

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Figures legends

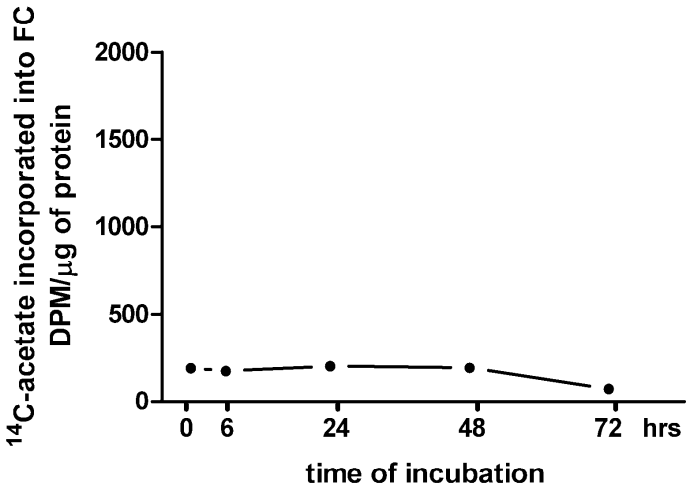
Figure I. Rate of cholesterol synthesis in P388D1

To determine the rate of cholesterol synthesis 10^6/ml cells were incubated for 3h with 185 kBq/ml of sodium [14C]-acetate. After incubation they were separated by centrifugation, collected and processed as described in the Methods section. All incubations were carried out in triplicate and the results of individual experiments are given as mean values; variation between triplicates was less than 10%. All data are expressed as the rate of [^{14}C] acetate incorporation into cholesterol for μg of protein.

Figure II. LDL and HDL temperature-dipendent kinetic in P388D1

P388D1 cells were seeded at a concentration of $2.0x10^5$/ml and incubated at 4°C for 2h with 10 μg/ml of Dil-LDL or Dil-HDL at 4, 18 and 37°C respectively. Cells were observed using an Olympus IX 71 inverted microscope fitted with a 20x/0.7 planapochromatic objective. 12 bit-images were captured using a cooled CCD camera (for details see Methods section).

Figures I


^{14}C-acetate incorporated into FC
DPM/μg of protein
2000
1500
1000
500
0
0
6
24
48
72
hrs
time of incubation

Figures II