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CHLOROQUINE REDUCES THE NUMBER OF ASIALO-GLYCOPROTEIN RECEPTORS IN THE HEPATOCYTE PLASMA MEMBRANE

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Abstract—Rat hepatocytes which had been incubated with chloroquine in concentrations ranging from 0.2 to 1.0 mM showed markedly reduced ability to take up asialo-glycoproteins. Chloroquine had no effect on the actual binding of asialo-orosomucoid to the receptor on hepatocytes. Chloroquine caused a progressive reduction in the binding capacity of the plasma membrane for asialo-glycoproteins; in hepatocytes that had been exposed to 1.0 mM chloroquine for 30 min at 37°C, the binding capacity of the plasma membrane was reduced to 15 per cent of controls. The results support the idea that the asialo-glycoprotein receptors of hepatocytes are internalized regardless of binding of ligand.

We have studied the mechanism of inhibition by chloroquine of the uptake of asialo-orosomucoid in isolated rat hepatocytes. Asialo-glycoproteins are bound to hepatocytes by a receptor which is specific for the β-galactoside residues which are exposed when sialic acid is removed from the carbohydrate side chains [1]. In the liver, the receptor is found exclusively on parenchymal cells [2].

Chloroquine is an anti-malarial drug which accumulates in the lysosomes [3]. Cells exposed to chloroquine show reduced degradation of proteins and other substances which are normally degraded in the lysosomes [4], including asialo-glycoproteins which have been taken into hepatocytes by adsorbtive endocytosis [5, 6]. The mechanism of inhibition of degradative processes may be increased in intralysosomal pH [7] and/or inhibition of lysosomal proteases [7]. It is also possible that chloroquine in hepatocytes slows down degradation of endocytosed macromolecules by preventing fusion between endocytic vesicles and lysosomes [5, 6].

It has been shown by Wiesman *et al.* that chloroquine inhibits the uptake of lysosomal enzymes in fibroblasts [8]. This uptake is likely to be receptor-mediated [9, 10]. It was proposed by Wiesman *et al.* [8] that chloroquine might compete with the enzyme for the binding sites.

METHODS

Hepatocytes were isolated from rat liver by the collagenase perfusion method [11, 12] and purified by centrifugation [2]. They were incubated in a minimal salt medium containing 1% of charcoal-treated bovine serum albumin [2]. Viability of the cells was checked by the trypan blue exclusion method. Cells were separated from the medium by placing a 250 μl aliquot on top of the same volume of dibutyl phthalate and centrifuging. The amount of radioactivity in the cell pellet was determined in a gamma counter [2].

At 10°C, the amount of internalized asialo-glycoproteins into hepatocytes is negligible in experiments lasting for 30 min or so (H. Tolleshaug, unpublished results). Accordingly, the amount of cell-associated asialo-glycoprotein determined in experiments at this temperature will be referred to as 'plasma-membrane bound' or simply 'bound' asialo-glycoprotein. 10°C was preferred to lower temperatures because the equilibrium between free and receptor-bound asialo-glycoprotein is established only very slowly at low temperatures.

Chloroquine diphosphate was dissolved in distilled water to a concentration of 0.1 M. If the solution of chloroquine diphosphate was added directly to the incubating medium to make the concentration of the drug 1 mM, the decrease in pH of the medium was 0.1 unit, which is within the range of variation normally experienced with incubations of hepatocyte cell suspensions. It made no difference to the results whether the pH of the stock solution was adjusted to 7.4 or not.

Orosomucoid was isolated from human plasma by the method of Weimer *et al.* [13]. Sialic acid was removed by treatment with neuraminidase from *Clostridium perfringens* (E.C.3.2.1.18) (Type VIII, Sigma Chemical Co.). 2 mU were added per mg of protein and the incubation was for 2 hr at pH 5.0. The desialylated protein was labelled with ^{125}I by the lactoperoxidase method of Frantz and Turkington [14] to a specific activity of 0.4 μCi/μg = 0.7 MBq/nmole, corresponing to one ^{125}I-atom per 100 molecules of asialo-orosomucoid. The resulting preparation was taken up by hepatocytes to an extent of 90–100 per cent within 20 min (see, for instance, Figs. 1 and 2 in Results). No effort was made to remove neuraminidase, as the final activity in the cell suspension would be less than 2 μU/ml, which is far below the minimum activity required to influence the uptake of asialo-glycoproteins into hepatocytes (H. Tolleshaug, unpublished observations).

RESULTS

Pre-incubating hepatocytes with 1 mM of chloroquine for various times up to 1 hr before the addition of ^{125}I-asialo-orosomucoid resulted in a reduced uptake,

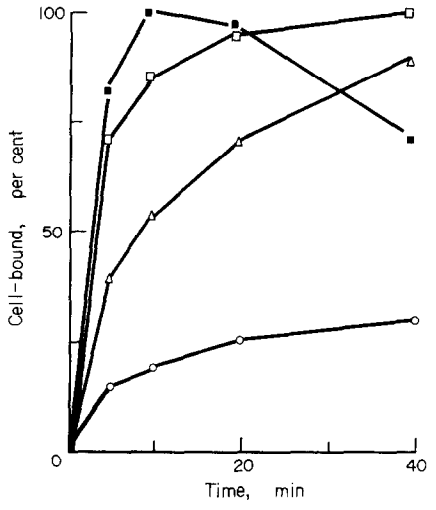

Fig. 1. The effect of various times of pre-incubation with 1 mM chloroquine on the uptake of asialo-orosomucoid by isolated rat hepatocytes at 37°. The cell density was 8 million cells per ml and the initial concentration of labelled asialo-orosomucoid was 10 nM in all flasks. ■, control (no chloroquine); □, asialo-orosomucoid and chloroquine added simultaneously; △, 30 min pre-incubation with chloroquine; ○ 60 min pre-incubation.

the reduction being progressively more marked with increasing times of pre-incubation (Fig. 1). About 10 min of incubation in the presence of 1 mM chloroquine was necessary for a definite effect on uptake at 37°. In subsequent studies, 30 min of pre-incubation with chloroquine at 37° was adopted as a standard experimental condition. Chloroquine was present throughout the incubation (except as noted). Cells exposed to 1 mM chloroquine at 37° had a 'granular' appearance which was clearly distinguishable in the microscope even at low (250 ×) magnification.

Figure 2 shows the variation with time of the amount of cell-associated ^{125}I-asialo-orosomucoid at 37° with several different concentrations of chloroquine, from 0.2 to 1.0 mM. 0.1 mM had no effect on uptake (not shown). With the concentrations of asialo-orosomucoid used in the uptake experiments, the control cells removed about 90 per cent of the labelled protein from the medium in 10–20 min. In the control cells, the amount of cell-associated radioactivity decreased after 20 min of incubation, because labelled degradation products were released from the cells [2]. Chloroquine inhibited degradation of endocytosed asialo-glycoprotein. At the same time, the uptake continued for as long as any asialo-glycoprotein was left in the medium. These two circumstances explain why the uptake curves continued to rise for cells incubated with chloroquine, even though the uptake rate was reduced compared to control cells.

The effect of pre-incubation with chloroquine on the binding of asialo-orosomucoid to hepatocytes at 10° depended strongly on the concentration of the drug (Fig. 3). Under identical experimental conditions, including 30 min pre-incubation at 37°, 10 mM procaine had the same effect as 1 mM chloroquine on binding at 10° (not shown). In order to gain some insight into the mechanism of inhibition of uptake by chloroquine, the binding capacity of the plasma membrane of control cells was compared to cells treated with chloroquine. Cells were pre-incubated at 37° for 30 min in the absence of asialo-glycoprotein, with or without 1 mM chloroquine. Measurements of binding of ^{125}I-asialo-orosomucoid were subsequently carried out at 10°, and the results were plotted according to Scatchard [15] (Fig. 4). The binding capacity of the cells treated with 1 mM chloroquine was reduced to 15 per cent of controls. 0.1 mM chloroquine had no effect on the binding capacity of hepatocytes for asialo-orosomucoid under the conditions just described (not shown).

If a cell suspension kept at 10° was made 1 mM or

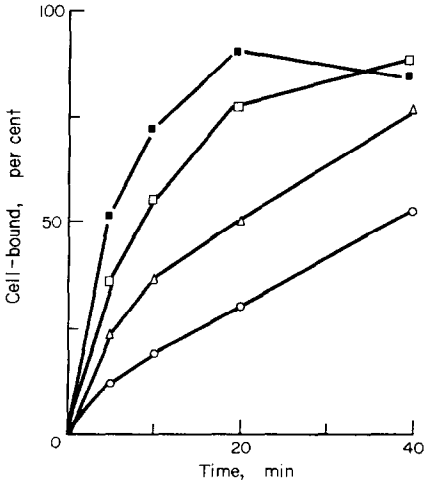

Fig. 2. The effect of different concentrations of chloroquine on the uptake of asialo-orosomucoid by isolated rat hepatocytes at 37°. Cells were incubated for 30 min with chloroquine before the addition of labelled asialo-orosomucoid (6 nM). ■, control (no chloroquine); □, 0.2 mM; △, 0.5 mM; and ○, 1 mM chloroquine.

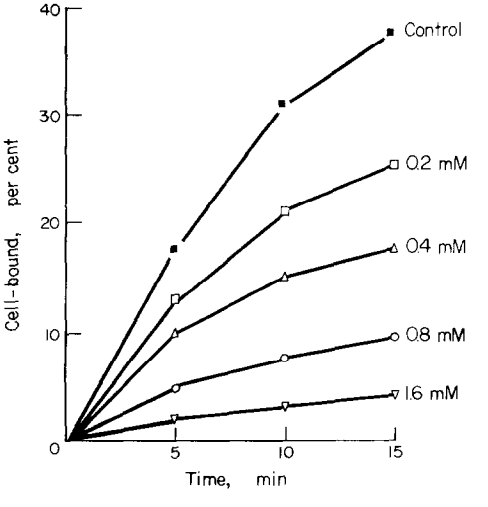

Fig. 3. Binding of ^{125}I asialo-orosomucoid by isolated rat hepatocytes at 10° following 30 min pre-incubation with different concentrations of chloroquine at 37°. The initial concentration of asialo-orosomucoid was 20 nM in all flasks.

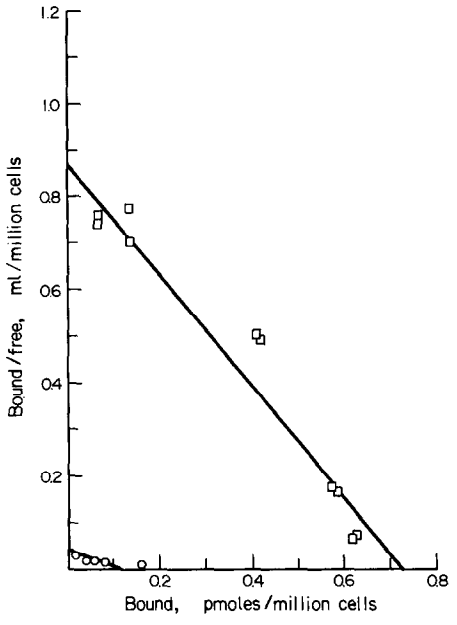

Fig. 4. Scatchard plots of binding of asialo-orosomucoid at 10° to control (□) cells and to cells pre-incubated with 1 mM chloroquine (○) for 30 min at 37°. Initial concentrations of labelled asialo-orosomucoid ranged from 1 to 20 nM. Cells were incubated for 3 hr at 10°. Surface-bound asialo-orosomucoid was determined by releasing it by treatment of the cells with the chelating agent EGTA, which binds calcium ions [10].

2 mM in chloroquine simultaneously with the addition of ^{125}I-asialo-orosomucoid, then no effect on the binding reaction was observed during a 60-min incubation at 10°, showing that the observed effect on the binding capacity of the hepatocyte plasma membrane is not due to competition by chloroquine with asialo-glycoprotein for the receptor.

In order to test the reversibility of the effect of chloroquine, duplicate cell suspensions were made 1 mM in chloroquine and incubated at 37° for 30 min. The cells in both flasks were thoroughly washed and resuspended in fresh medium, and the incubation was continued at 37°. Both suspensions were made 20 nM in asialo-orosomucoid, and one of them was also made 1 mM in chloroquine. The uptake rates in both suspensions were indistinguishable. For 60 min, they remained at less than 25 per cent of the rate in control cells (identical treatment, but no chloroquine). Exposure of hepatocytes to concentrations of chloroquine in excess of 1 mM at 37° for more than 2 hr lead to reduced viability of the cells.

DISCUSSION

0.1 mM of chloroquine has a marked effect on the degradation of endocytosed asialo-glycoprotein by hepatocytes [5, 6]. However, this concentration has no effect on uptake, a concentration of 0.2 mM being required for an effect on this process. The reduction of uptake of asialo-orosomucoid could be fully explained by a reduction of the binding capacity of the plasma membrane. This followed from the results of the measurements of the rate of binding at 10°, as the initial rate of binding is directly proportional to the number of receptors on the cell membrane when the concentration of cells as well as the initial concentration of asialo-glycoprotein are kept constant.

The assumption that exposure of the cells to chloroquine reduced the binding capacity of the plasma membrane was corroborated by comparison of the binding capacities of control cells and chloroquine-treated cells. (Although it has not been possible to determine with certainty how many moles of asialo-orosomucoid are bound per mole of receptor [16], it is assumed here that the binding capacity of the plasma membrane is proportional to the number of receptors.)

The effect of chloroquine on uptake, as presented in this paper, is strongly time-dependent (as well as concentration dependent), and the effect is irreversible in our experiments. Hence a quantitative model based on simple principles cannot be presented. Chloroquine does seem to have a selective effect, as fluid pinocytosis (marker: ^{125}I-polyvinylpyrrolidone) and uptake of high density lipoprotein are not affected by 1 mM chloroquine (L. Ose and T. Berg, unpublished results). These observations show that the hepatocytes maintained the structural integrity and functions necessary for endocytosis.

Recycling of plasma membrane components has been the subject of several recent studies [17, 18]. It has been proposed that plasma membrane receptors involved in adsorbtive endocytosis of ligands such as low-density lipoproteins [19] are recycled [20]. This may also be the case for asialo-glycoprotein receptors of hepatocytes, as the uptake is not affected by inhibitors of protein synthesis (H. Tolleshaug, unpublished results). The reduction of binding capacity of the plasma membrane may be the effect of derangement of the recycling of receptors. Colchicine, an inhibitor of the microtubular system, also reduces the binding capacity of the plasma membrane, although the effect is smaller [21].

It cannot be decided from our data at what point in this recycling it is that chloroquine may interfere with. As the receptors are made unavailable for combination with the ligand, it is likely that they are internalized. The intracellular transport of the receptor seems to be stopped at some later step. The recycling of plasma membrane components may involve fusion between intracellular membranes of various types, such as the fusion between endocytic vesicles and lysosomes [5, 6]. For that reason, the suggestion that chloroquine interferes with membrane fusion is an interesting one. Evidence has been presented that chloroquine reduces the transport of endocytized asialo-fetuin into lysosomes of liver cells [5, 6]. At concentrations of chloroquine up to 0.1 mM, this is the only apparent effect on the uptake and degradation of asialo-glycoproteins. It is possible that at this concentration, essentially all of the chloroquine that enters the cell is concentrated within the lysosomes so that other organelles escape the effect of the drug.

Chloroquine not only accumulates in lysosomes [3], it also binds to cell membranes. At pH 7.4, some 40 per cent of the total uptake of chloroquine in fibroblasts represent binding to the cell surface [22]. The proposition that binding of chloroquine to membrane systems is involved, is supported by the similarity of the action of chloroquine on uptake to the action of procaine. The

latter drug, which is a local anesthetic of the tertiary amine type, has a definite effect on cell membranes [23] and it influences the mobility and distribution of receptors for concanavalin A on cell surfaces [24, 25]. As the effect of chloroquine only appears after some 10 min of exposure to the drug, and as the effect is reversed only slowly (see also [8]), the effect of chloroquine might involve binding of chloroquine to membrane systems wthin the cell.

It has not been possible to determine with certainty whether only occupied asialo-glycoprotein receptors are internalized, or *all* receptors on the plasma membrane are internalized at the same rate [26]. However, the experiments presented here favor the assumption that all receptors are internalized, as the binding capacity of the cell membrane is reduced following incubation with chloroquine in the absence of asialo-orosomucoid.

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