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Ursodeoxycholic acid increases low-density lipoprotein binding, uptake and degradation in isolated hamster hepatocytes

Bernard BOUSCAREL,* Hans FROMM, Susan CERYAK and Marie M. CASSIDY
Division of Gastroenterology and Nutrition, Department of Medicine and Department of Physiology, The George Washington University Medical Center, Washington, DC 20037, U.S.A.

Ursodeoxycholic acid (UDCA), in contrast to both chenodeoxycholic acid (CDCA), its 7α-epimer, and lithocholic acid, enhanced receptor-dependent low-density lipoprotein (LDL) uptake and degradation in isolated hamster hepatocytes. The increase in cell-associated LDL was time- and concentration-dependent, with a maximum effect observed at approx. 60 min with 1 mM-UDCA. This increase was not associated with a detergent effect of UDCA, as no significant modifications were observed either in the cellular release of lactate dehydrogenase or in Trypan Blue exclusion. The effect of UDCA was not due to a modification of the LDL particle, but rather was receptor-related. UDCA (1 mM) maximally increased the number of ^{125}I-LDL-binding sites ($B_{max.}$) by 35%, from 176 to 240 ng/mg of protein, without a significant modification of the binding affinity. Furthermore, following proteolytic degradation of the LDL receptor with Pronase, specific LDL binding decreased to the level of non-specific binding, and the effect of UDCA was abolished. Conversely, the trihydroxy 7β-hydroxy bile acid ursocholic acid and its 7α-epimer, cholic acid, induced a significant decrease in LDL binding by approx. 15%. The C_{23} analogue of UDCA (nor-UDCA) and CDCA did not affect LDL binding. On the other hand, UDCA conjugated with either glycine (GUDCA) or taurine (TUDCA), increased LDL binding to the same extent as did the free bile acid. The half maximum time ($t_{\frac{1}{2}}$) to reach the full effect was 1–2 min for UDCA and TUDCA, while GUDCA had a much slower $t_{\frac{1}{2}}$ of 8.3 min. Ketoconazole (50 μM), an antifungal agent, increased LDL binding, but this effect was not additive when tested in the presence of 0.7 mM-UDCA. The results of the studies indicate that, in isolated hamster hepatocytes, the UDCA-induced increase in receptor-dependent LDL binding and uptake represents a direct effect of this bile acid. The action of the bile acid is closely related to its specific structural conformation, since UDCA and its conjugates are the only bile acids shown to express this ability thus far. However, certain agents other than bile acids, such as ketoconazole, have a similar effect. Finally, the studies suggest that the recruitment of LDL receptors from a latent pool in the hepatocellular membrane may be the mechanism by which UDCA exerts its direct effect.

INTRODUCTION

For more than a decade, the low-density lipoprotein (LDL) receptor has been known to play a central role in the regulation of cholesterol homoeostasis [1–3]. In a number of species, including the hamster [4,5], approx. 75% of the receptor-mediated clearance of LDL occurs in the liver. The LDL receptor binds the apolipoprotein-B_{100}-containing LDL particle, and the LDL–LDL-receptor complex formed is rapidly internalized [6–8]. Once in the cell the LDL particle is delivered to lysosomes, where it is degraded to cholesterol and amino acids for use by the cell [9,10]. Within 10 min following internalization, the LDL receptor is generally cycled back to the membrane. In addition, a receptor-independent pathway is involved in the removal of LDL from the circulation [11,12], for review, see [1,13].

The activity of the LDL receptor has been shown to be tightly regulated. Hamster hepatic LDL receptor activity is suppressed when the animals are fed on a high cholesterol diet [3,14,15]. Conversely, when the animals are treated with drugs such as mevinolin, compactin, ketoconazole or bile-acid-binding resins, the number of LDL receptors increases [16–20]. The changes in cellular cholesterol demand and/or the rate of bile acid synthesis, which are induced by these agents, modulate the production of mRNA and thus either decrease or increase the synthesis of the LDL receptor [16,21]. This indirect adaptive process is slow and requires hours or days. On the other hand, there is also evidence that LDL receptor activity can be directly altered. Such a direct action may be one of the pathways by which triacylglycols and ketoconazole modulate LDL receptor function [18,22,23]. Bihain *et al.* [23] have reported that unesterified fatty acids directly decrease the number of LDL receptors in human fibroblasts. Ketoconazole has been shown to increase the number of LDL-binding sites in the human hepatoma cell line HepG2. This finding has been interpreted to reflect a change in the conformation of the LDL receptor [18].

In previous studies, ursodeoxycholic acid (UDCA), a bile acid used to dissolve cholesterol gallstones in man [24–26], has been reported to modulate the hepatic uptake of LDL. Hamsters fed UDCA showed a significant increase in receptor-dependent LDL uptake in the liver, in spite of a marked suppression of bile acid synthesis [27]. These results are consistent with the finding by Singhal *et al.* [28] that UDCA significantly decreased serum cholesterol levels in the hypercholesterolaemic hamster model. However, the mechanism of action of this bile acid in the regulation of LDL metabolism is not known.

The purpose of the present study was to investigate the effect of UDCA on the receptor-dependent and -independent uptake of LDL in isolated hamster hepatocytes. Both the LDL particle

Abbreviations used: LDL, low-density lipoprotein; LDH, lactate dehydrogenase; CDCA, chenodeoxycholic acid (3α,7α-dihydroxy-5β-cholan-24-oic acid); UDCA, ursodeoxycholic acid (3α,7β-dihydroxy-5β-cholan-24-oic acid); LCA, lithocholic acid (3α-hydroxy-5β-cholan-24-oic acid); CA, cholic acid (3α,7α,12α-trihydroxy-5β-cholan-24-oic acid); UCA, ursocholic acid (3α,7β,12α-trihydroxy-5β-cholan-24-oic acid); TUDCA, tauroursodeoxycholic acid; GUDCA, glycoursodeoxycholic acid; NUDCA, nor-ursodeoxycholic acid; EC_{50}, concn. causing 50% of maximum effect.

* To whom correspondence should be addressed, at Division of Gastroenterology and Nutrition, Department of Medicine, The George Washington University Medical Center, 2300 I Street, N.W., 523 Ross Hall, Washington, DC 20037, U.S.A.

and the LDL receptor were studied as possible sites of action of the bile acid. The structural requirements for the effects of UDCA on LDL binding and uptake were assessed by comparing the action of this bile acid with those of different 7β-hydroxy UDCA analogues and ketoconazole, an imidazole derivative.

MATERIALS AND METHODS

Rationale for the model

Freshly isolated hepatocytes as well as those in primary culture have been proposed as a model to study the binding, uptake and degradation of LDL [29–34]. However, in both models LDL receptor cycling has been suggested to be slower than in the perfused organ [35]. We have used hepatocytes, either isolated or in primary culture, to study different membrane receptors and their respective biological responses [36–38]. However, freshly isolated hepatocytes have been selected as the most suitable model for the present study for two reasons. (1) The previously observed stimulation of receptor-dependent uptake of LDL by UDCA in the hamster liver *in vivo* may be linked to the cellular uptake of this bile acid. While freshly isolated hepatocytes express active bile acid transport, they may lose this ability with prolonged culture following attachment to the dish [39,40]. (2) The observation has been made that the number of membrane proteins and receptors decreases with increasing length of time in culture [41–44]. As far as the LDL receptor is concerned, Salter *et al.* [31,32] have shown it to be up-regulated during a 48 h culture of rat liver cells, an effect the authors attributed to the presence of either high-density lipoprotein or fetal calf serum in the culture media, rather than to the regeneration of receptors damaged during the isolation of the hepatocytes. Nevertheless, there are no published data concerning the comparative number of LDL receptors in freshly isolated hepatocytes and those in primary culture.

Isolation and incubation of hepatocytes

Male Golden Syrian hamsters (100–130 g body weight), fed on a 0.027% cholesterol rodent chow diet, were used. Hepatocytes were isolated by perfusion of the liver with collagenase, as described by Exton [45]. The cells were suspended at a final concentration of 40–50 mg wet wt./ml of Krebs–Henseleit bicarbonate buffer containing 118 mM-NaCl, 5 mM-KCl, 2.5 mM-$CaCl_2$, 1.2 mM-KH_2PO_4, 1.2 mM-$MgSO_4$ and 25 mM-$NaHCO_3$, pH 7.4. Prior to each experiment the hepatocytes were incubated for 20–30 min at 37 °C under constant agitation and gassing (O_2/CO_2, 19:1), to allow the cell to reach steady state. For binding experiments the hepatocytes were washed five times with ice-cold buffer and kept on ice until proceeding with an experiment.

LDL preparation and labelling

Blood was collected from human subjects and from hamsters into EDTA-containing vacuum collection tubes. The plasma was separated by centrifugation at 300 *g* for 40 min at 4 °C. Plasma lipoproteins were separated by density gradient ultracentrifugation, as previously described [27]. Briefly, 4 ml plasma fractions were adjusted to a density of 1.21 g/ml with solid KBr. A discontinuous density gradient was prepared as described by Redgrave *et al.* [46]. Ultracentrifugation was carried out at 14 °C for 24 h at 286000 *g* using an SW41 swinging-bucket rotor (Beckman Inst Corp., Palo Alto, CA, U.S.A.). The fraction containing LDL (density 1.020–1.060 g/ml) was adjusted to a density of 1.063 g/ml and concentrated by centrifugation at 289000 *g* for 20 h in a 70.1 Ti fixed-angle rotor (Beckman). The purity of the LDL fraction was confirmed by SDS/PAGE. The human LDL fraction was reductively methylated as described by Weisgraber *et al.* [47], in order to block its binding to the LDL receptor. Hamster and human LDL particles were labelled with ^{125}I to a specific radioactivity of 80–200 c.p.m./ng using the iodine monochloride technique [48] as modified for lipoproteins [49], and were dialysed overnight against 0.9% NaCl containing 0.01% EDTA. ^{125}I-LDL was filtered through a 0.45 μm cellulose nitrate membrane filter (Millipore) and used within 2 days of preparation.

Binding, uptake and degradation of ^{125}I-LDL

For binding experiments the hepatocytes were incubated at 4 °C in bicarbonate buffer with increasing concentrations of ^{125}I-labelled hamster LDL (1–70 μg/ml) in the presence and the absence of the indicated bile acid. Rudling *et al.* [50,51], using tissue homogenates, found the same LDL specific binding in the presence of either heparin or an excess of unlabelled LDL. However, since Edge *et al.* [29] have reported that heparin is toxic to intact hepatocytes, non-specific binding was determined by incubating the cells under the same conditions as previously described, but with an excess of unlabelled native human LDL (1.6 mg/ml). The results were not significantly different regardless of whether native human or hamster LDL was used to determine non-specific binding (results not shown). Specific binding was determined by subtracting non-specific binding from total binding. Binding of LDL to isolated hamster hepatocytes reached equilibrium in 20–30 min and remained stable for over 1 h (results not shown). In all experiments, unless otherwise mentioned, the period of incubation selected was 60 min. The reaction was stopped by diluting the cells in 2 ml of ice-cold bicarbonate buffer. The cell suspension was successively centrifuged at 50 *g* for 2 min and washed three times. Cells were finally suspended in 0.2 M-NaOH to measure LDL binding. In the kinetic studies the cells were incubated at 4 °C with ^{125}I-labelled hamster LDL for 60 min to reach the steady-state binding equilibrium prior to the addition of the bile acids. Due to the necessity of rapidly stopping the reaction, 200 μl portions of cells were layered on top of 300 μl of a mixture of dibutylphthalate/bis(2-ethylhexyl)phthalate (3:2, v/v) (Kodak, Rochester, NY, U.S.A.) at fixed periods of time up to 60 min. The tubes were rapidly centrifuged at 500 *g* for 1.5 min in a Beckman Microfuge 12 table-top centrifuge. In the absence of cells the radioactivity remained in the aqueous layer, and there was no contamination of the oil with radioactivity. In addition, after washing the cells three times with ice-cold buffer, specific ^{125}I-labelled hamster LDL binding was identical to that measured after centrifugation with oil. The effect of UDCA on LDL binding was also tested after proteolytic degradation of the LDL receptor, which was carried out by preincubating the hepatocytes with 30 μg of Pronase/ml for 10 min at 37 °C according to the method of Rudling *et al.* [50].

To measure cell-associated LDL at 37 °C, isolated hepatocytes were incubated in bicarbonate buffer containing either ^{125}I-labelled hamster LDL or ^{125}I-labelled methylated human LDL, unless otherwise stated, with and without the addition of the respective bile acid. Internalized LDL was distinguished from that specifically bound by further incubating the hepatocytes with 30 μg of Pronase/ml for 1 h at 4 °C. At the indicated times duplicate aliquots of 50 μl of cell suspension were diluted in 2 ml of ice-cold bicarbonate buffer and centrifuged at 50 *g* for 2 min. The cells were then washed and centrifuged four times. Finally, the cells were resuspended in 0.2 M-NaOH and the radioactivity was measured in a Beckman model 4000 γ-radiation counter.

LDL degradation was assessed by quantifying the radioactivity present in free iodotyrosine residues in the incubation media, as described by Goldstein *et al.* [52] and Edge *et al.* [29]. Briefly,

150 μl of cell suspension was mixed with 150 μl of fetal bovine serum and 300 μl of 20% trichloroacetic acid. The samples were incubated for 20–30 min at room temperature and centrifuged at 800 *g* for 15 min. To 200 μl of supernatant were added sequentially 20 μl of 20% KI, 40 μl of 30% H_2O_2 and 1 ml of chloroform. The solution was vortex-mixed and centrifuged at 50 *g* for 15 min. A 50 μl sample of the aqueous portion containing the iodotyrosine was counted for radioactivity. The radioactive counts were corrected for blank values, which were obtained in parallel experiments in the absence of hepatocytes.

Lactate dehydrogenase (LDH) activity

The release of the cytosolic enzyme LDH into the medium was measured 60 min after the addition of increasing concentrations of the indicated bile acids. Following centrifugation at 50 *g* for 4 min, LDH activity was measured in the supernatant using a standard technique (Boehringer Mannheim, Indianapolis, IN, U.S.A.). The activity was expressed as a percentage of the total LDH activity obtained after cell homogenization with a Tissumizer (Tekmar, Cincinnati, OH, U.S.A.).

Bile acid stock solutions and protein assay

Bile acid stock solutions were prepared by dissolving the sodium salt of the appropriate bile acid in 0.9% NaCl to give a final concentration of 100 mM. The pH was adjusted to 7.5 with 1 M-HCl. In all experiments the effect of the bile acid was compared with that of 0.9% NaCl as a control. Protein was assayed by a modification of the method of Lowry *et al.* [53], using the Pierce protein assay kit (Pierce, Rockford, IL, U.S.A.) with BSA as standard. However, in the experiments performed at 37 °C, the cells were incubated in bicarbonate buffer containing 1.5% gelatin to protect the cells against proteolytic degradation. Gelatin, in contrast to albumin, did not affect bile acid binding and uptake (results not shown). However, since it is impossible to completely remove the gelatin in these experiments, the results were expressed per mg wet wt. of cells.

Materials

$Na^{125}I$ (specific radioactivity 16–20 mCi/μg) was purchased from Amersham. Gelatin, BSA (fraction V), protease XXV (Pronase) and ketoconazole were purchased from Sigma. Lithocholic acid (LCA) and cholic acid (CA) were obtained from Steraloids (Wilton, NH, U.S.A.). Chenodeoxycholic acid (CDCA) was supplied by Dr. Falk GmbH. and Co. (Freiburg, Germany) and UDCA was from Tokyo Tanabe Co. (Tokyo, Japan). LCA, CDCA and UDCA were 98–99% pure, as judged by g.l.c. Ursocholic acid (UCA) and nor-UDCA (NUDCA) were generously supplied by Dr. A. Roda (University of Bologna, Italy) and Dr. E. Mosbach (Beth Israel Hospital, New York, NY, U.S.A.) respectively. Other chemicals were of the highest purity available.

RESULTS

Concentration- and time-dependent effect of UDCA on cell-associated LDL at 37 °C

It has previously been shown by different investigators [5,27,47] that the hamster LDL receptor does not recognize either methylated hamster LDL or methylated human LDL. Therefore, human LDL was isolated and methylated in order to measure the receptor-independent uptake of LDL. To determine whether UDCA affected either receptor-dependent or receptor-independent LDL uptake, isolated hepatocytes were incubated for 60 min at 37 °C with increasing concentrations of either ^{125}I-(hamster LDL) (2–70 μg/ml) or ^{125}I-(methylated human LDL) (2–90 μg/ml) with and without the addition of 1 mM-UDCA. In both the presence and the absence of 1 mM-UDCA the cell-associated ^{125}I-(hamster LDL) reached a maximum at around 50 μg of LDL/ml (Fig. 1*a*). However, the maximum binding and internalization of ^{125}I-(hamster LDL) increased from 14 ± 3 ng/mg of cells for the control to 23 ± 4 ng/mg of cells for UDCA-treated cells ($P < 0.05$, paired *t* test). Thus UDCA induced a 60% increase in cell-associated ^{125}I-(hamster LDL). In contrast, cell-associated ^{125}I-(methylated human LDL), which is mediated by a receptor-independent mechanism, was not affected by the addition of 1 mM-UDCA (Fig. 1*b*).

Fig. 2 shows the concentration-dependent effect of UDCA on the cell-associated ^{125}I-(hamster LDL) after a 60 min incubation at 37 °C. The cells were incubated with 20 ± 3 μg of ^{125}I-LDL/ml and increasing concentrations (0–2 mM) of UDCA. Treatment with 0.1 mM-UDCA did not significantly affect the binding and internalization of ^{125}I-LDL. However, 1 mM-UDCA maximally increased cell-associated LDL by 23% above control ($P < 0.05$, paired *t* test). The concentration of UDCA required to produce a half-maximum effect was calculated to be 0.60 ± 0.13 mM. Under the same conditions neither CDCA, the 7α-epimer of UDCA, nor LCA, the product of intestinal bacterial 7-dehydroxylation of CDCA and UDCA, had an effect (results not shown).

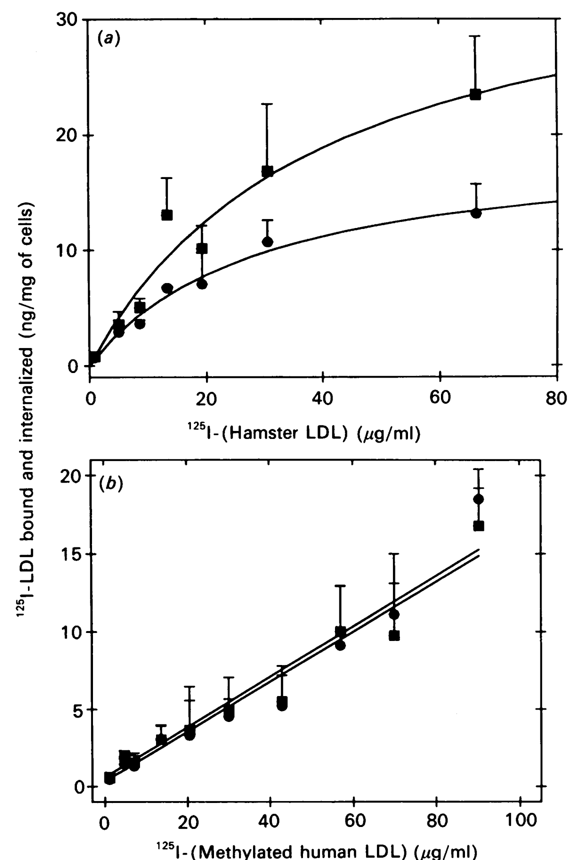

Fig. 1. Effect of UDCA on binding and internalization of ^{125}I-(human LDL) and ^{125}I-(hamster LDL) in isolated hamster hepatocytes

Cells were isolated from Golden Syrian hamsters and incubated at 37 °C with either ^{125}I-(hamster LDL) (*a*) or ^{125}I-(methylated human LDL) (*b*) with (■) or without (●) 1 mM-UDCA. After 60 min, two aliquots were removed, centrifuged at 50 *g* and washed four times with ice-cold bicarbonate buffer. The pellet was then counted for radioactivity to determine the amount of cell-associated ^{125}I-LDL, which was expressed as ng/mg of cells in the 60 min period. Results are means ± S.E.M. of five separate experiments performed in duplicate.

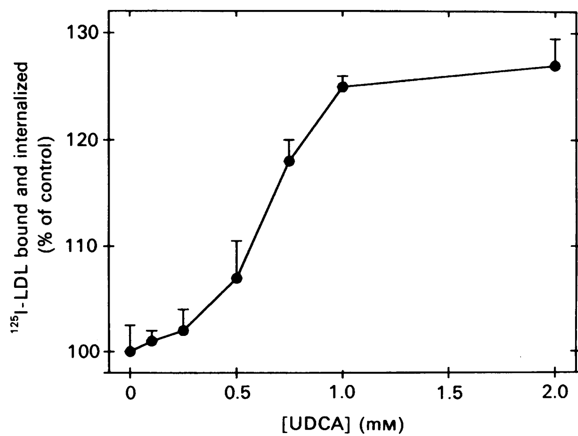

Fig. 2. Effect of increasing concentrations of UDCA on cell-associated ^{125}I-(hamster LDL)

For experimental details, see the legend to Fig. 1 and the Materials and methods section. Isolated hamster hepatocytes were incubated at 37 °C for 60 min with ^{125}I-(hamster LDL) (20±3 μg/ml) and increasing concentrations of UDCA (0–2 mM). The results are expressed as a percentage of control, which represents cell-associated LDL in the absence of bile acid. Results are means±S.E.M. of three separate experiments performed in duplicate.

Table 1. Effect of increasing concentrations of UDCA and CDCA on LDH release

For experimental details, see the legend to Fig. 1. Isolated hepatocytes were incubated for 60 min at 37 °C with increasing concentrations (0–8 mM) of UDCA or CDCA. Duplicate samples of 100 μl were removed and centrifuged at 50 g for 10 min. LDH activity was measured in the supernatant using a standard assay (Boehringer Mannheim). The results are expressed as a percentage of the total LDH activity obtained after cell homogenization with a Tissumizer. Results represent means±S.E.M. of four separate experiments performed in triplicate. * Significantly different from control, $P < 0.05$.

[UDCA] (mM)	LDH activity (% of total)	[CDCA] (mM)	LDH activity (% of total)
0	6.6±0.9	0	6.6±0.9
0.1	6.3±0.6	0.1	7.4±1.0
0.25	6.8±0.8	0.25	8.7±2.0
0.5	7.4±0.7	0.5	11.0±1.3
1	8.1±1.1	1	31.0±3.5*
2	11.7±2.0	2	90.9±4.3*
4	34.0±9.0*	4	93.7±8.8*
8	61.5±8.7*	8	99.6±4.6*

The time-dependence of the effect of UDCA on cell-associated LDL and LDL degradation was examined by incubating isolated hepatocytes at 37 °C in bicarbonate buffer, with or without 1 mM-UDCA, in a final volume of 5 ml containing 25.0±2.5 μg of ^{125}I-LDL/ml. In the control experiment the binding and internalization of LDL was significantly increased after a 120 min incubation ($P < 0.05$, paired t-test). Cells incubated in the presence of UDCA displayed a significant increase in cell-associated LDL at each time point, with increases of 23 % above control after 60 min and of 37 % after 120 min (Fig. 3*a*). Under the same conditions (Fig. 3*b*) a time-dependent increase in the cellular degradation of LDL paralleled that of LDL uptake. LDL degradation at 60 min and 120 min was increased about 2 and 3.5 times respectively above the control level.

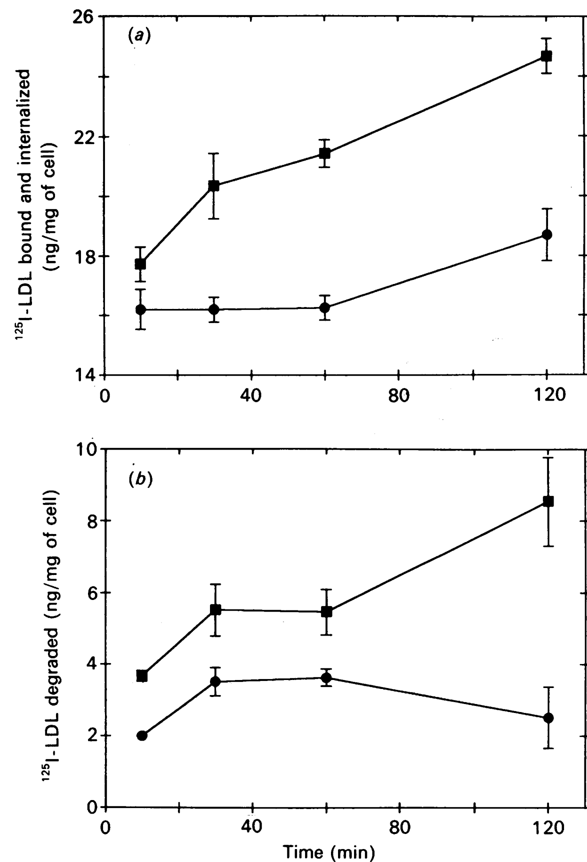

Fig. 3. Time-dependent association and degradation of ^{125}I-(hamster LDL) in the presence or absence of UDCA

For experimental details, see the legend of Fig. 1 and the Materials and methods section. Isolated hamster hepatocytes were incubated at 37 °C with 25±2.5 μg of ^{125}I-(hamster LDL)/ml with (■) or without (●) the addition of 1 mM-UDCA. At the indicated times, cell-associated ^{125}I-LDL was determined (*a*). The radioactivity remaining in the supernatant after chloroform extraction was counted to determine the amount of ^{125}I-(hamster LDL) degraded (*b*). Results are means±S.E.M. of three different experiments performed in duplicate.

Effect of UDCA on the release of the cytosolic enzyme LDH

To exclude a possible detergent effect of UDCA, the cells were incubated with increasing concentrations (0.1–8 mM) of either UDCA or CDCA (Table 1). After 60 min incubation at 37 °C, portions of cells were centrifuged at 50 g for 4 min and the cytosolic LDH activity was measured in the supernatant. In the absence of bile acid, the LDH activity in the supernatant was measured to be 6.6±0.9 % of the total. UDCA, at concentrations ranging from 0.1 to 2.0 mM, did not lead to a significant release of LDH into the medium. However, 4 and 8 mM-UDCA were cytotoxic, as indicated by the respective 5- and 10-fold increases in LDH release. Using the same lot of cells, CDCA appeared to be cytotoxic at much lower concentrations. There were 4.6- and 13.5-fold increases in LDH activity in the medium when the cells were incubated with 1 and 2 mM-CDCA respectively. Similar results were obtained when the toxic effect of the bile acids was assessed by measuring cell viability by Trypan Blue exclusion (results not shown).

Effect of UDCA on specific ^{125}I-(hamster LDL) binding at 4 °C

The specific binding of ^{125}I-(hamster LDL) to intact hepatocytes was measured at 4 °C after a 60 min incubation with

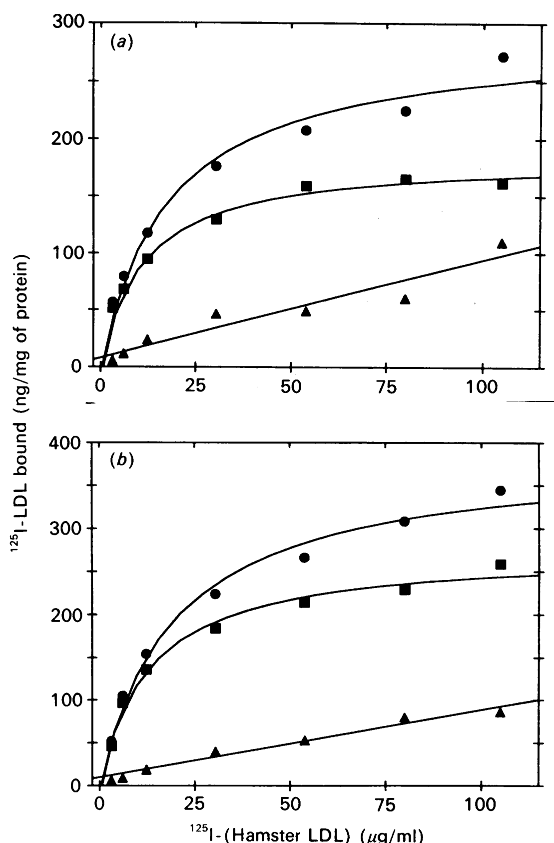

Fig. 4. Effect of UDCA on the binding of ^{125}I-(hamster LDL) to isolated hepatocytes

Isolated hamster hepatocytes (150–300 μg of cell protein) were incubated at 4 °C for 60 min with the indicated concentration of ^{125}I-(hamster LDL) without (*a*) or with (*b*) the addition of 1 mM-UDCA. The unbound ^{125}I-(hamster LDL) was separated from the bound by rapid centrifugation of the cells (50 *g* for 2 min) and by washing them three times. Non-specific binding (▲) was determined in a parallel set of experiments by incubating the cells under the same conditions but with an excess of unlabelled native human LDL (1.6 mg/ml). Specific binding (■) represents the difference between total binding (●) and non-specific binding. Each curve is representative of four separate experiments performed in duplicate.

Table 2. Effect of UDCA on LDL receptor number and affinity in isolated hepatocytes

The dissociation constant, K_d, and the maximum number of binding sites, $B_{max.}$, were calculated from Fig. 4. Results are means ± S.E.M. * Significantly different from control, $P < 0.05$.

Treatment	K_d (μg/ml)	$B_{max.}$ (ng/mg of protein)	$B_{max.}$ (ng/mg of cells)
Control	19.3±2.7	176±16	23.4±2.1
UDCA	15.5±1.8	240±11*	31.9±1.5*

increasing concentrations of LDL, with and without the addition of 1 mM-UDCA (Figs. 4*a* and 4*b*). Non-specific binding, measured by incubating the cells with 1.6 mg of unlabelled native human LDL/ml, represented 30±5% of total binding. Saturation of specific binding was reached at approx. 50 μg of ^{125}I-(hamster LDL)/ml. Analysis of the saturation curve (Fig.4*a* and

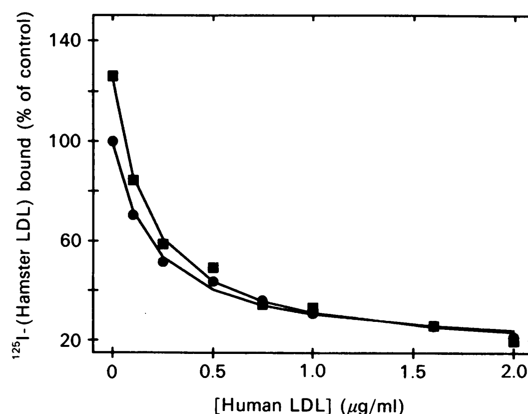

Fig. 5. Effect of increasing concentrations of human LDL on the binding of ^{125}I-(hamster LDL) in the presence or absence of 700 μM-UDCA

For experimental details, see legend of Fig. 4 and the Materials and methods section. Isolated hamster hepatocytes were incubated at 4 °C with 50 μg of ^{125}I-(hamster LDL)/ml and increasing concentrations (0.1–2 mg/ml) of human LDL in the presence (■) or the absence (●) of 0.7 mM-UDCA. The results, which are expressed as percentages of the ^{125}I-(hamster LDL) bound in the absence of both unlabelled native human LDL and UDCA, are representative of three experiments performed in duplicate.

Table 2) gave a maximum number of binding sites ($B_{max.}$) of 23.4±2.1 ng/mg of cells with a binding affinity (K_d) of 19.3±2.7 μg/ml. When binding was measured in the presence of 1 mM-UDCA (Fig. 4*b* and Table 2) the $B_{max.}$ was increased by 36% to 31.9±1.5 ng/mg of cells without significant modification of the K_d, which was 15.5±1.8 μg/ml. In addition, the effect of UDCA on specific LDL binding was transient and reversible. Following a 60 min preincubation of hepatocytes at 37 °C with 1 mM-UDCA, 80–90% of the bile acid was removed by extensive washing of the cells with ice-cold buffer. The binding of ^{125}I-LDL at 4 °C to the UDCA-preincubated cells was indistinguishable from that to control cells. However, ^{125}I-LDL binding increased to the same extent in control and UDCA-pretreated cells in the presence of 1 mM-UDCA (results not shown).

Effect of UDCA on both the LDL particle and the LDL receptor

Both the LDL particle and the LDL receptor were studied as potential sites for the effect of UDCA on LDL binding. To determine whether UDCA affects the LDL particle itself, hamster plasma was incubated at 4 °C for 2 h with or without the addition of 1 mM-UDCA before LDL isolation. The incubations of LDL with UDCA were carried out in whole plasma rather than with the isolated LDL fraction for two reasons: (1) the yield of LDL from hamster plasma is very low due to its low plasma concentration, and (2) the binding of bile acids to LDL incubated in whole plasma is similar to that with the isolated LDL fraction [54]. After isolation of the LDL fraction, 0.15 nmol of UDCA/μg of LDL remained associated with the LDL fraction. The binding of LDL to isolated hepatocytes was measured as a function of the LDL concentration, using either LDL from the UDCA-preincubated plasma or native hamster LDL (control). Analysis of the two saturation curves indicated that neither the affinity nor the maximum number of binding sites was affected by UDCA preincubation. In addition, preincubation with UDCA did not affect the UDCA-induced increase in the maximum number of LDL-binding sites (results not shown). In the presence of 0.7 mM-UDCA, LDL binding increased from 23±1 ng/mg of cells to 30±2 ng/mg of cells ($P < 0.05$, paired *t* test) in the control

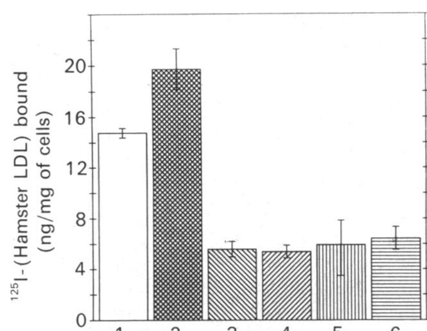

Fig. 6. Effect of Pronase pretreatment on the binding of ^{125}I-(hamster LDL) in the presence and the absence of UDCA

Hepatocytes were preincubated for 10 min at 37 °C without (control) or with 30 μg of Pronase/ml. The study of the binding of ^{125}I-(hamster LDL) to isolated hepatocytes was performed as described in the legend of Fig. 4 and in the Materials and methods section, using 20±2 μg of ^{125}I-LDL/ml with and without the addition of 0.7 mM-UDCA. Non-specific binding was determined in the presence of 1.6 mg of native human LDL/ml. The results represent the means±S.E.M. of four separate experiments performed in duplicate. 1, Control, total binding; 2, +UDCA, total binding; 3, +Pronase; 4, +Pronase+UDCA; 5, control, non-specific binding; 6, +UDCA, non-specific binding.

Table 3. Effect of different bile acids on the binding of ^{125}I-(hamster LDL) to isolated hepatocytes

Hepatocytes (43–48 mg wet wt./ml) were incubated at 4 °C for 60 min with 15–20 μg of ^{125}I-(hamster LDL)/ml without bile acids (control) or with increasing concentrations (0.1–1 mM) of UDCA, TUDCA, GUDCA, UCA, CA, NUDCA or CDCA. The maximum effect is expressed as a percentage of the ^{125}I-(hamster LDL) specifically bound in the absence of bile acids (control). The EC_{50} represents the concentration of the respective bile acid required to either increase or decrease the binding to 50% of the maximum level. The results are the mean±S.E.M. of four separate experiments performed in duplicate. All results shown are significantly different from the control ($P < 0.05$). NUDCA and CDCA had no effect on LDL binding.

Bile acid	Maximum effect (% of control)	EC_{50} (mM)
UDCA	141.4±5.6	0.4±0.1
TUDCA	134.7±4.4	0.3±0.1
GUDCA	138.8±2.2	0.3±0.1
UCA	86.4±1.4	0.1±0.1
CA	82.7±2.9	0.2±0.1

experiment and from 22±2 ng/mg of cells to 26±1 ng/mg of cells ($P < 0.05$, paired t test) using UDCA-pretreated LDL.

To determine whether UDCA was specifically affecting the LDL receptor, hepatocytes were incubated with 50 μg of ^{125}I-(hamster LDL)/ml and increasing concentrations (0.1–2 mg/ml) of unlabelled native human LDL in the presence or the absence of 0.7 mM-UDCA (Fig. 5). In the control experiment the maximum LDL binding was designated as 100% binding. All results were expressed as a percentage of this concentration. The ^{125}I-(hamster LDL) was displaced from its binding sites by increasing concentrations of unlabelled native human LDL, with a maximum displacement of 75–80% with 1–2 mg of LDL/ml. The residual binding represents the non-specific binding. In the presence of 0.7 mM-UDCA, total binding was increased by 26%, whereas non-specific binding was not different from that in the control experiment.

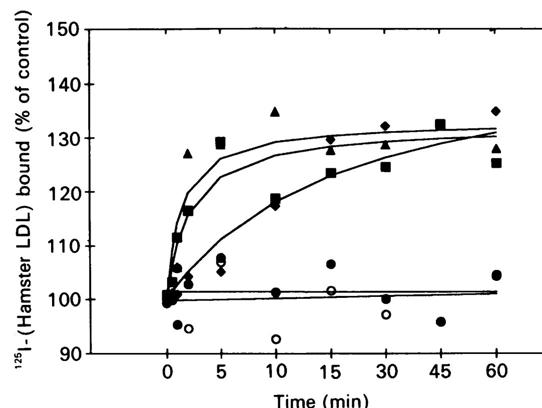

Fig. 7. Time-dependent effect of UDCA analogues on the binding of ^{125}I-(hamster LDL) to isolated hepatocytes

Hepatocytes were incubated with 15–20 μg of ^{125}I-(hamster LDL)/ml for 60 min at 40 °C. At the end of this period, 0.6 mM-UDCA (■), -TUDCA (▲), -GUDCA (◆), -NUDCA (○) or 0.9% NaCl as control (●), was added and, at the indicated times, aliquots were washed and counted for radioactivity. The results are expressed as percentages of the control. The time required to increase the binding to 50% of maximum level ($t_{\frac{1}{2}}$) was calculated for UDCA, TUDCA and GUDCA to be 2.1±1.8, 1.3±1.6 and 8.3±2.6 min respectively. The data are representative of five experiments performed in duplicate. The S.E.M. values were less than 15% of the means and were omitted for clarity of presentation.

To further investigate the specific effect of UDCA on the LDL receptor, we studied the effect of UDCA on the binding of hamster LDL (20±2 μg/ml) after pretreatment of the cells with Pronase (Fig. 6). In the control experiment 0.7 mM-UDCA significantly increased ^{125}I-(hamster LDL) binding at 4 °C from 15.5 ng/mg of cells to 19 ng/mg of cells. When the cells were treated for 10 min at 37 °C with 30 μg of Pronase/ml, the specific binding of hamster LDL was abolished, whereas non-specific binding remained unaffected. Under these conditions the UDCA-induced increase in LDL binding was also abolished.

Structural requirements for bile acids to affect ^{125}I-(hamster LDL) binding at 4 °C

The effect of UDCA on ^{125}I-(hamster LDL) binding was compared with that of UCA and NUDCA to determine whether 7β-hydroxy analogues of UDCA were able to affect the specific binding of LDL to isolated hepatocytes. In these experiments cells were incubated for 60 min at 4 °C with 15–20 μg of ^{125}I-(hamster LDL)/ml, with and without the addition of increasing concentrations (0.1–1 mM) of bile acid. The maximum increases or decreases in specific LDL binding induced by the bile acids are reported in Table 3. Non-specific binding, obtained by incubating the cells with an excess of unlabelled native human LDL (1.6 g/ml), was not significantly affected by the presence of the different bile acids, and ranged from 25 to 30% of total LDL binding (results not shown). Conversely, UDCA increased the specific binding of LDL in a concentration-dependent manner, with a maximum increase of 41±6% at 0.8–1 mM. In contrast to UDCA, its trihydroxy analogue UCA which, in addition to the 3α- and 7β-hydroxy groups possesses a 12α-hydroxy group, inhibited LDL binding with a maximum effect of 13.6±1.4% at concentrations of 0.1–0.2 mM. NUDCA, a C_{23} analogue of UDCA, did not affect the binding of ^{125}I-(hamster LDL) to isolated hamster hepatocytes. The possible hydrophilic action of a bile acid on LDL binding was tested using CA, which has a degree of hydrophilicity close to that of UDCA [25]. CA, in contrast to UDCA, inhibited LDL binding with a maximum

Table 4. Effect of ketoconazole with and without the addition of UDCA on the binding of ^{125}I-(hamster LDL) to isolated hamster hepatocytes

The binding of ^{125}I-(hamster LDL) (30–35 μg/ml) to isolated hamster hepatocytes was measured at 4 °C, as described in the legend to Fig. 4 and in the Materials and methods section, in the presence and the absence of 0.7 mM-UDCA and/or 50 μM-ketoconazole. The results are means ± S.E.M. of three separate experiments performed in triplicate. * Significantly different from control (−UDCA), $P < 0.05$.

Treatment	^{125}I-(Hamster LDL) specific binding (ng/mg of cells) −UDCA	+UDCA
Control	22.1 ± 1.1	27.5 ± 0.3*
Ketoconazole	30.2 ± 2.1*	27.0 ± 1.8*

inhibitory effect of 17.3 ± 2.9 % at around 0.2 mM. The effects of taurine- (TUDCA) and glycine- (GUDCA) conjugated UDCA on LDL binding were also compared with that of the free bile acid. TUDCA and GUDCA increased the specific binding of LDL to isolated hamster hepatocytes by 34.7 ± 4.4 % and 38.8 ± 2.2 % respectively. The effect of the conjugated bile acids was not significantly different from that of the free UDCA. The EC_{50} represents the concentration of a bile acid required to produce 50 % of the maximum effect. According to the EC_{50} values (Table 3), the order of efficacy of the different bile acids to increase LDL binding was as follows: UDCA ⩾ TUDCA ⩾ GUDCA ≫ NUDCA ⩾ CDCA.

The time-dependence of the effects of UDCA, TUDCA, GUDCA and NUDCA on LDL binding was also investigated (Fig. 7). In these experiments, hepatocytes were initially incubated with 15–20 μg of ^{125}I-LDL/ml at 4 °C for 60 min to reach the steady-state binding equilibrium. NaCl (0.9 %, control) or 0.7 mM of one of the bile acids was then added to the cell suspension. Thereafter, at indicated times, portions of cells were removed and the radioactivity was counted as described in the Materials and methods section. UDCA, GUDCA and TUDCA were all able to increase LDL binding by approx. 30 %, which is consistent with the results shown in Table 3. However, GUDCA was significantly slower than UDCA and TUDCA in eliciting this effect. The time required to half-maximally stimulate LDL binding ($t_{\frac{1}{2}}$) was 8.3 ± 2.6 min for GUDCA, but 2.1 ± 1.8 and 1.3 ± 1.6 min for UDCA and TUDCA respectively. The C_{23} analogue of UDCA, NUDCA, did not significantly affect the binding of LDL in the 60 min period.

Comparative effects of ketoconazole and UDCA on LDL binding

Studies were conducted to determine whether, in addition to specific bile acids, certain non-steroidogenic agents could affect LDL binding. It has been shown previously that ketoconazole increases the binding and uptake of LDL [18,19]. This effect was thought to be due to a suppression of cholesterol synthesis [19,55]. However, Kempen *et al.* [18] suggested that, in addition, this imidazole derivative could directly affect the LDL receptor. To define whether ketoconazole affects LDL binding and to compare its effect to that of UDCA, 50 μM-ketoconazole, 0.7 mM-UDCA or 0.9 % NaCl (control) was added to the cells immediately prior to the measurement of LDL binding at 4 °C. Both UDCA and ketoconazole increased LDL binding to the same extent, around 25–30 % above control (Table 4). However, when ketoconazole and UDCA were added together, the effect

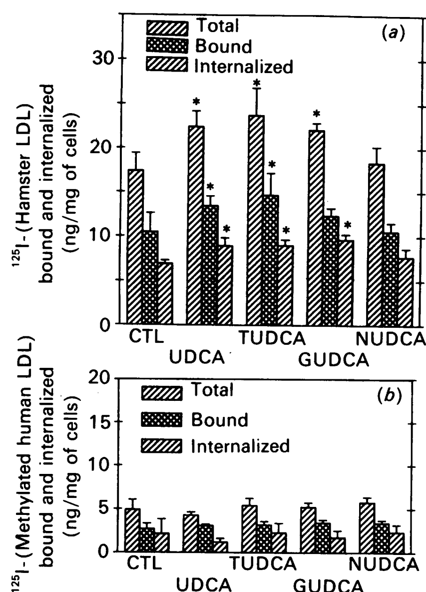

Fig. 8. Effect of UDCA analogues on the binding and internalization of hamster and human ^{125}I-LDL to isolated hepatocytes at 37 °C

Isolated hamster hepatocytes were incubated for 1 h at 37 °C with 30–35 μg of either ^{125}I-(hamster LDL) (*a*) or ^{125}I-(human LDL) (*b*)/ml and 0.7 mM-UDCA, -TUDCA, -GUDCA, -NUDCA or 0.9 % NaCl (CTL). The specifically bound LDL was removed from the cell surface by further incubating the hepatocytes with 30 μg of Pronase/ml for 1 h at 4 °C. The remaining cell-associated LDL represented internalized LDL. Non-specific binding was determined by two different methods. In one study, cell-associated LDL was measured in the presence of 1.6 mg of unlabelled native human LDL/ml and before and after Pronase treatment. In the other study, cell-associated LDL was measured at 4 °C under the same conditions as described previously. The two methods gave a similar level of non-specific LDL binding of approx. 25 % and 55 % of the total binding for ^{125}I-(hamster LDL) and ^{125}I-(human LDL) respectively. All results have been corrected for non-specific binding. The total cell-associated LDL represents specific LDL binding plus internalization. The results represent the means ± S.E.M. of three separate experiments performed in triplicate. * Significantly different from control, $P < 0.05$.

was similar to that produced by each agent individually, which suggests a similar action for both agents. In addition, in a parallel experiment, both agents were added to the cells at 37 °C for 5 min prior to the performance of the binding experiment. UDCA and ketoconazole had similar effects either when preincubated with the cells at 37 °C or when added to the cells at 4 °C (results not shown).

Effect of UDCA analogues on the binding and internalization of hamster LDL and human methylated LDL at 37 °C

Studies were conducted to determine whether the structural requirements for a bile acid molecule to stimulate the binding and internalization of LDL at 37 °C are similar to those for a bile acid to stimulate LDL binding at 4 °C. The effects of both the conjugation with taurine or glycine and a shortening of the side chain on the binding and internalization of hamster LDL and of human methylated LDL were studied. The difference between bound and internalized LDL was determined as described in the Materials and methods section.

When hamster LDL (30–35 μg/ml) was incubated with 0.7 mM-UDCA, -TUDCA or -GUDCA for 60 min at 37 °C, LDL binding increased from 10.5 ± 2.1 ng/mg of cells in the control experiment to 13.4 ± 1.1 ng/mg of cells ($P < 0.05$, paired *t* test), 14.7 ± 2.5 ng/mg of cells ($P < 0.05$) and 12.4 ± 0.9 ng/mg

of cells respectively (Fig. 8*a*). In contrast, when hamster LDL was incubated with 0.7 mM-NUDCA, LDL binding (10.6 ± 0.95 ng/mg of cells) was not significantly different from the control, and was significantly lower than that induced by UDCA, TUDCA and GUDCA. UDCA, TUDCA and GUDCA also stimulated hamster LDL internalization, which was increased by 28 %, 31 % and 40.6 % respectively. Conversely, the effect of NUDCA was not different from that of control. When ^{125}I-(human methylated LDL) was used to measure binding and internalization of LDL, 50–55 % of the total cell-associated LDL represented non-specific LDL binding (results not shown). Specifically bound and internalized human methylated LDL represented 10–15 % and 27–32 % of that of hamster LDL respectively. Under these conditions, none of the bile acids tested affected the binding or the internalization of human methylated LDL (Fig. 8*b*).

DISCUSSION

The present studies with isolated hamster hepatocytes demonstrate that UDCA, in contrast to its 7α-epimer CDCA, has the ability to directly modulate LDL metabolism. The effect of UDCA was shown to be specific for the receptor-dependent pathway, since it did not affect receptor-independent LDL uptake. These findings are in agreement with those reported by Malavolti *et al.* [27] in the hamster model *in vivo*. In addition, although one has to be cautious in relating findings *in vitro* to the *in vivo* situation, it is of interest that the concentration at which UDCA began to significantly affect cell-associated LDL was within the range of intrahepatic bile acid concentrations observed by Strange [56].

Several findings indicate that the increase in cell-associated LDL stimulated by UDCA was not related to a detergent or toxic effect of this bile acid on the membrane. First, the absence of cellular damage by UDCA was shown by the lack of change in the release of LDH into the medium. Secondly, if the effects of UDCA were the result of cytotoxicity, then cytotoxic bile acids such as CDCA and LCA [57] should exhibit a similar effect on LDL uptake, but they do not. Finally, several authors, including Leuschner *et al.* [58] and Galle *et al.* [59] have shown that, even at very high concentrations, UDCA not only is non-toxic but also shows evidence for both improving certain chronic liver diseases and preventing the cytotoxicity induced by other bile acids, such as CDCA and LCA.

Other mechanisms which could explain increased LDL uptake include a UDCA-induced increase in intrahepatic cholesterol requirements and direct modification of the LDL receptor. In man, the ingestion of UDCA has been reported to be associated with either no change or a slight increase in bile acid synthesis [60,61]. The latter would lead to an increased demand for the bile acid precursor cholesterol. However, in the hamster, in contrast to man, a large proportion of UDCA is biotransformed into CDCA, which suppresses bile acid synthesis [27]. Therefore the results of the present study, in conjunction with the observation by Malavolti *et al.* [27] that UDCA stimulates receptor-dependent LDL uptake, in spite of an inhibition of bile acid synthesis, support the hypothesis of a direct modulatory effect of UDCA on the LDL receptor.

The effect of UDCA was not due to either an alteration in the LDL particle or a change in non-specific LDL binding, and thus appeared to be specific for the LDL receptor. Evidence for a receptor-specific action of UDCA was also provided by other experiments. Preincubation of hepatocytes with Pronase abolished their ability to specifically bind LDL, both under control conditions and after exposure to UDCA. This effect of Pronase is in keeping with the reported effect of this agent on the LDL receptor described by Rudling *et al.* [50]. The enhancement of LDL binding by UDCA was not due to an increase in the affinity of the receptor for the LDL particle. The K_d of 15.5 ± 2 μg/ml, which was not significantly different from the control value, is of the same order of magnitude as that reported in rat and human hepatocytes [29,31]. However, UDCA was found to induce an increase in the maximum number of LDL-binding sites by approx. 36 %, from 176 to 240 ng/mg protein.

Ketoconazole is able to mimic the effect of UDCA in increasing LDL binding. Ketoconazole is an imidazole derivative which is known to inhibit cytochrome *P*-450-dependent enzymes [62] and to decrease cholesterol synthesis [19,55]. Consequently, LDL receptor gene expression and LDL receptor synthesis are stimulated [55]. However, Kempen *et al.* [18] observed an induction in the number of LDL-binding sites by ketoconazole in the presence of cycloheximide, an inhibitor of protein synthesis. These authors suggested that the observed increase may be due to conformational changes in the LDL receptor molecules induced by ketoconazole. The present results support this hypothesis. Van Den Bossche *et al.* [63] have shown that the orientation of the ketoconazole molecule changes in a lipid matrix, with the piperazine moiety inserted into the hydrophobic region of the bilayer while the dichlorophenyl group is inserted into the hydrophilic phase. This effect may be specific and of importance, since it was not observed with another dichlorophenyl imidazole derivative, miconazole. Therefore it could be hypothesized that the molecular conformation of ketoconazole in the plasma membrane may position the two chloride groups in a fashion similar to that of the two hydroxyl groups of the hydrophilic bile acid UDCA. This would result in the rearrangement of certain phospholipids and proteins such as the LDL receptor.

Previous studies [64–68] have shown that certain agents or hormones may change the number of binding sites without affecting the number of receptors at the plasma membrane level, supporting the hypothesis proposed by Triggle [69] that cryptic receptors or receptor reserve recruitment may act as modulators of cell sensitivity. Our finding that UDCA and ketoconazole uncover receptors present at the membrane surface, which are normally not able to bind the LDL particle, is in agreement with these observations. These agents may either induce conformational allosteric changes leading to exposure of latent binding sites or make accessible receptors which are sequestered in the plasma membrane. Finally, the binding of a certain number of LDL particles may block further LDL binding. UDCA and ketoconazole may facilitate increased LDL binding by unblocking these binding sites.

The effect of UDCA on LDL binding correlated well with that on LDL uptake, with a maximum effect for both binding and uptake occurring at a UDCA concentration of 0.8–1 mM. In addition, the receptor population uncovered by UDCA seems to be functional, as demonstrated by a parallel increase in binding, internalization and degradation of LDL induced by UDCA. These results support the hypothesis that the increase in LDL binding is responsible for the increased LDL uptake. The UDCA-induced stimulation of cell-associated LDL was found to occur within 10 min, reaching a plateau in approx. 60 min. The process thus took place within a time period during which LDL receptor cycling may occur [13], suggesting a possible stimulation of receptor cycling by UDCA. However, the internalized LDL represented 66 % of the bound LDL in both control and UDCA-treated cells, suggesting that an increase in cycling may not be involved in this effect. However, the effect of UDCA on LDL receptor cycling requires further study.

The fact that CDCA, the 7α-hydroxy epimer of UDCA, was not able to modify the binding of LDL implied that the bile acid

must possess a 7β-hydroxy group in order to be effective. However, UCA and NUDCA, which are 7β-hydroxy analogues of UDCA, did not affect LDL binding. The main differences in these bile acids from UDCA are the addition of a 12α-hydroxy group in UCA and the lack of the C-24 atom in NUDCA. These results indicate that modifications in the structure of the bile acid may result in perturbations of certain actions, such as the interaction of bile acids with their protein carrier or with the cell membrane. Moreover, it is of interest to note that the conjugation of UDCA with glycine or taurine did not affect the concentration-dependent stimulation of LDL binding. However, the slower stimulatory action of GUDCA underlines the narrow confines of the structural requirements for this effect on the LDL receptor.

Heuman *et al.* [70] have demonstrated that the regulation of 7α-hydroxylase and 3-hydroxy-3-methylglutaryl coA reductase, the rate-limiting enzymes for bile acid and cholesterol synthesis [71,72] respectively, was negatively correlated with the degree of hydrophobicity of the bile acid. CDCA, a hydrophobic bile acid [70,73], significantly inhibits the two enzymes, whereas UDCA, CA and UCA, which are more hydrophilic [73], have no suppressive effect on these enzymes [70]. However, in our model CA and UCA did not mimic the effect of UDCA. On the contrary, both bile acids significantly decreased LDL binding, with a maximum effect of 15–20% at concentrations of 0.1–0.2 mM. These results thus suggest that, although the degree of hydrophilicity may be important for a bile acid to increase LDL binding, this property of the molecule alone is not sufficient to elicit this effect.

In a previous study in dogs *in vivo*, Angelin *et al.* [74] observed a rapid decrease in the number of hepatic LDL receptors following intravenous infusion of taurine-conjugated CA. The proposed mechanism was an inhibition of bile acid synthesis with a consequent decrease in the demand for cholesterol substrate. Our findings suggest that a direct decrease in LDL binding may, in addition, be involved in the rapid down-regulation of the LDL receptor by CA. It could be hypothesized that modulation of the LDL receptor number at the surface of the cell by bile acids such as CA, UCA and UDCA is an initial event. This may be followed by an adjusted response of 3-hydroxy-3-methylglutaryl coA reductase and 7α-hydroxylase as well as of the synthesis of both the LDL receptor mRNA and the LDL receptor itself. Such hypotheses need to be tested by further investigation.

In summary, the present study shows that UDCA directly and specifically increases receptor-dependent uptake and degradation of LDL in isolated hamster hepatocytes. This enhanced uptake and degradation of LDL is mainly attributable to an increase in the number of LDL-binding sites. A specific structural conformation is required for a bile acid to elicit this effect. However, certain agents other than bile acids, such as ketoconazole, also have the ability to stimulate LDL binding, probably through a mechanism similar to that of UDCA.

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