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# Effects of Individual Fatty Acids on Receptor-Mediated Binding, Internalization and Degradation of [$^{125}$I]LDL

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**Abstract:** The ability of Hep-G2 cells to process [$^{125}$I]LDL under basal conditions was investigated. The receptor-binding and internalization of [$^{125}$I]LDL increased with the time of incubation in a saturable manner. After 4 h of incubation, 31.4 ng of [$^{125}$I]LDL was cell bound. The cells rapidly internalized [$^{125}$I]LDL *via* specific, receptor-mediated endocytosis. The amount of internalized [$^{125}$I]LDL reached a maximun of 96.7 ng at 2 h of incubation and remained constant for the next 2 h. The rate of degradation of internalized [$^{125}$I]LDL proceeded in a linear manner over the entire 4 h of incubation after an initial lag period. The effects of individial fatty acids (C18:0, C18:1, C18:2, and C18:3), differing in their degree of unsaturation, on the receptor-binding, internalization and degradation of [$^{125}$I]LDL were also investigated. Inclusion of 1.0 mM of each fatty acid into the culture medium significantly increased [$^{125}$I]LDL metabolism in Hep-G2 cells. Among the fatty acids tested, stearic acid had the least effect on the receptor-binding activity. There were no significant differences among the unsaturated fatty acids in LDL-receptor binding. The effect of individual fatty acids on the [$^{125}$I]LDL uptake was similar to that of the receptor-binding, showing a significantly lower effect with stearic acid. The amount of degraded material of internalized [$^{125}$I]LDL was the lowest with stearic acid when it was compared with unsaturated fatty acids.
**Key words:** fatty acids, Hep-G2 cells, low density lipoprotein, receptor-mediated uptake.

In humans, serum low density lipoprotein (LDL) is the major transport vehicle for cholesterol, and the elevation of LDL is one of the major risk factors for the development of atherosclerotic vascular disease (Rhoads *et al*., 1976; Grundy, 1986). The activity of hepatic LDL-receptor comprises an important aspect of LDL and cholesterol metabolism. The major determinants of hepatic cholesterol concentration are the intracellular cholesterol synthesis and the rate of cholesterol uptake via LDL receptors in relation to the rate of cholesterol secretion (Edwards *et al*., 1976; Packard *et al*., 1983). LDL uptake, in turn, depends upon the interaction between the hepatocyte membrane and cholesterol carrier, mainly LDL (Attie *et al*., 1981; Pangburn, 1981).

Plasma cholesterol concentrations are strongly influenced by the quantity and composition of fats in the diet (Mattson *et al*., 1985; Connor, 1986; Kritchevsky *et al*., 1988). The substitution of polyunsaturated fatty acids for saturated fatty acids in the diet results in a reduction of plasma LDL cholesterol level. Dietary saturated fatty acids amplify the action of intracellular cholesterol in suppressing the synthesis of LDL receptors, thereby increasing the concentration of plasma LDL cholesterol (Illingworth *et al*., 1984; Spady *et al*., 1990). However, at present it is not clear whether the different kinds of fatty acids equally affect the LDL receptor activity.

The purposes of this study are to examine the uptake and degradation of [$^{125}$I]LDL under basal conditions and to study the comparative effects of individual fatty acids on LDL receptor activity, using the human hepatoma cell line.

## Materials and Methods

### Cell culture

The established Hep-G2 cell line, derived from human liver tumor cells, was obtained from the American Type Culture Collection (Rockvill, USA). Hep-G2 cells were cultured in 24-well plates ($1\times10^6$ cells per plate) and grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal bovine

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serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). Stock cultures were maintained at 37°C under a humidified atmosphere of 95% air and 5% $CO_2$. The assays were performed on the cell in the indicated assay medium after washing the monolayer with phosphate-buffered saline (PBS). Cell viability was measured by their ability to exclude trypan blue (0.04%). Greater than 95% of the cells excluded the dye during a 5 min incubation.

### Preparation of plasma LDL

Pooled human blood (normolipidemic subjects) was obtained from the local blood bank. Plasma was separated by a low speed centrifugation (1,000×*g*) for 30 min at 4°C. LDL was isolated by sequential floatation in a preparative ultracentrifuge according to the method of Havel *et al.* (1955). Density to obtain LDL (d=1.019~1.063 g/ml) was adjusted with sodium bromide and centrifugation were carried out in a Beckman Model L-50 preparative ultracentrifuge (Palo Alto, USA) using a 60 Ti rotor

### Radioiodination of LDL

Plasma LDL was labeled with [$^{125}$I] (carrier-free form, 10 mCi in 0.1 N NaOH, Boston, USA) based on Bilheimer's modification of the iodine monochloride method of MacFarlane as described by Goldstein *et al.* (1983). The extent of radiolabeling of lipids in the [$^{125}$I] LDL solution was measured by extracting a small aliquot with chloroform-methanol (2:1, v/v), and protein-bound radioactivity was measured by precipitating an aliquot of [$^{125}$I]LDL with 10% trichloroacetic acid. The [$^{125}$I]LDL was not used for studies unless it met the following criteria: 1) protein concentration of 2.5~4.0 mg/ml with a specific radioactivity of 200~600 cpm/ng, 2) greater than 98% of the [$^{125}$I] radioactivity was precipitable by TCA, and 3) less than 5% of the [$^{125}$I] radioactivity was extractable into chloroform-methanol.

The efficiency of iodination was 29.5%. Characterization of [$^{125}$I] labeled LDL showed that 98.2% of radioactivity was precipitated by 10% TCA and 3.2% was extracted with chloroform-methanol. Protein concentration was 2.8 mg/ml with a specific radioactivity of 337 cpm/ng of LDL

### Assay for surface binding of [$^{125}$I]LDL

LDL uptake at 37°C consists of the sum of cellular membrane binding and internalization of LDL. Because receptor mediated endocytosis, rather than binding of LDL ceases at 4°C, LDL binding distinct from internalization was determined at this temperature as described by Goldstein *et al.* (1983).

The cells were grown in monolayer in 6-well clustered dishes containing 1.0 ml of complete medium. Since the expression of maximal receptor activity is achieved while the cells are actively growing, cells were used before confluence was reached. For the final 24 h of cell growth, the complete medium was replaced with medium containing 10% lipoprotein-deficient serum. After a 24 h incubation, the medium was replaced with serum-free DMEM containing 1.0 mM of individual fatty acid/albumin complexes or 0.25 mM of albumin for a control. The cells were then incubated for 4 h at 37°C.

Prior to binding experiments, the cells were placed for 30 min in a 4°C cold room. The various concentrations of [$^{125}$I]LDL in the presence or the absence of a 50-fold excess of unlabeled LDL were added to the medium, and the cells were incubated for 2 h at 4°C. In experiments performed at 4°C, 80~90% of the total cell-associated [$^{125}$I] radioactivity was found in the heparin-releasable fraction. Nonspecific binding was calculated by subtracting the value obtained when unlabeled LDL was included from that obtained in its absence.

### LDL-uptake assay

For measurements of the uptake of [$^{125}$I]LDL at 37°C, the cells were grown as indicated above. At the start of the experiment, the medium was removed and each dish received 1.0 ml of warm (37°C) serum-free DMEM containing 1.0 mM of individual fatty acid/albumin complexes (C18:0, C18:1, C18:2, and C18:3) and albumin alone for control. After 4 h incubation, 10 μg of [$^{125}$I]LDL in the presence or the absence of excess unlabeled LDL was added to the medium. The cells were then placed in a $CO_2$ incubator and kept at 37°C for 4 h.

### Assay for degradation of [$^{125}$I]LDL

Cells were incubated at 37°C with [$^{125}$I]LDL as described in the preceeding section. Afterward the medium from each cell monolayer was collected and added to a glass tube containing 50% TCA to precipitate undegraded [$^{125}$I]LDL. After incubation at 4°C for at least 30 min, the precipitable material was removed by low-speed centrifugation. An aliquot of the TCA soluble supernatant was mixed with 10 μl of 40% potassium iodide as a carrier, followed by the addition of 35% hydrogen peroxide. The [$^{125}$I]-iodine ions, which were converted from [$^{125}$I]-iodide by the hydrogen peroxide, were extracted into chloroform.

After standing for 15 min, an aliquot was removed from the upper aqueous layer and its content of [$^{125}$I]-radioactivity was determined. This aqueous material consists almost exclusively of [$^{125}$I]-monoiodotyrosine deriv-

ed from the degradation of [$^{125}$I]LDL in cellular lysosomes (Goldstein *et al.*, 1983). All incubations were conducted in the absence or presence of excess unlabeled LDL. The degradation of [$^{125}$I]LDL was calculated by subtracting the value obtained when unlabeled LDL was included from that obtained in its absence.

## Results

The ability of Hep-G2 cells to process [$^{125}$I]LDL under basal conditions was investigated. Fig. 1 shows the receptor-binding, internalization and degradation of [$^{125}$I] LDL by Hep-G2 cells. The receptor-binding and internalization of [$^{125}$I]LDL at 37°C increased with the time of incubation in a saturable manner. However, the degradation of internalized [$^{125}$I]LDL continued to increase with the time of incubation. The cellular binding of [$^{125}$I]LDL almost reached equilibrium within 2 h. At 4 h of incubation, 31.4 ng of [$^{125}$I]LDL was cell bound. The cells rapidly internalized [$^{125}$I]LDL *via* specific receptor-mediated endocytosis. The amount of internalized [$^{125}$I]LDL reached the maximum at 2 h and remained constant for the next 2 h. After 2 and 4 h of incubation, 96.7 and 100.8 ng of [$^{125}$I]LDL was internalized by high affinity receptor-mediated uptake, respectively.

When Hep-G2 cells were incubated with [$^{125}$I]LDL, the internalized LDL was degraded in the cellular lysosomes to a material that was soluble in 10% trichloroacetic acid. During the first one hour of incubation, the relatively slow rate of degradation corresponded to the length of time required to establish maximal uptake. The rate of formation of acid-soluble material proceeded in a linear manner over the entire 4 h incubation after an initial lag period. The amont of LDL degraded by the Hep-G2 cells exceeded the amount of LDL bound after 2 h of incubation.

Table 1 shows the time course of internalization of [$^{125}$I]LDL at 37°C. The cells internalized LDL by two pathways, a saturable, high-affinity pathway (specific binding) and an unsaturable, low-affinity pathway (nonspecific binding). During the 4 h incubation, 100.8 ng of radiolabeled LDL was internalized by the high-af-

**Table 1.** Internalization of [$^{125}$I]LDL by Hep-G2 as a function of time. Cells were incubated in medium containing 10% LPDS with constant [$^{125}$I]LDL (10 μg/ml) in the absence or presence of 500 μg/ml of unlabeled LDL. After 1, 2, and 4 h incubation at 37°C, the amount of internalized [$^{125}$I]LDL was measured. The receptor-mediated uptake was calculated by subtracting the nonspecific uptake from the total uptake

| Pathways | Hours | | |
|---|---|---|---|
| | 1 | 2 | 4 |
| | (ng/mg protein) | | |
| Specific | 72.4±6.3 | 96.7±4.3 | 100.8±4.6 |
| Non-specific | 3.8±0.2 | 6.9±0.4 | 13.1±0.7 |

Values are the mean of triplicate incubations.

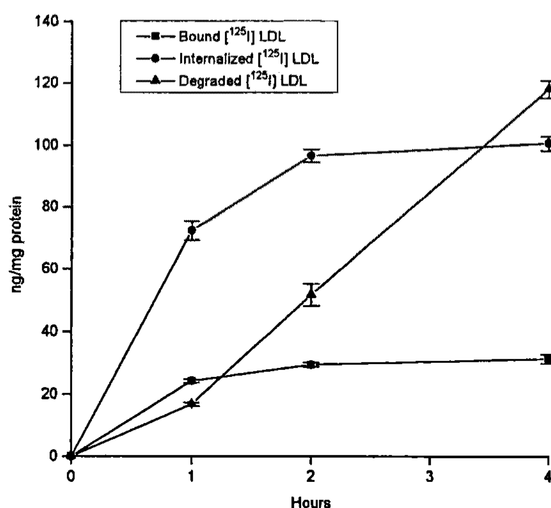


**Fig. 1.** Binding, internalization, and degradation of human LDL by cultures of Hep-G2. Cells were preincubated for 24 h in the medium supplemented with 10% LPDS. Binding, internalization, and degradation of LDL were measured after 1, 2, and 4 h of incubation at 37°C with [$^{125}$I]LDL (10 μg/ml) as described in the Materials and Methods sections. Values are the mean of triplicate incubations.

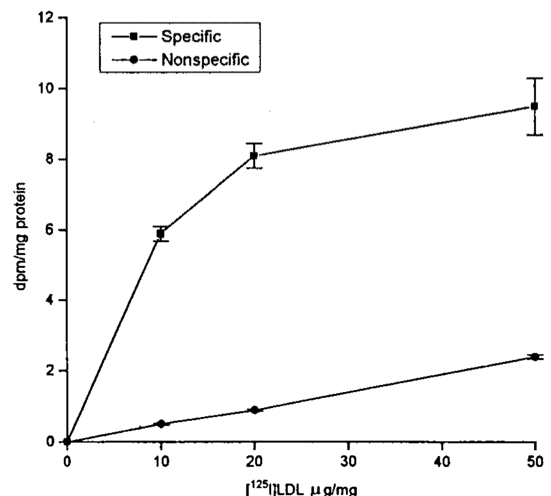


**Fig. 2.** Binding of [$^{125}$I]LDL by Hep-G2 at 4°C as a function of LDL concentration in absence or presence of 500 μg/ml of unlabeled LDL. After 2 h incubation at 4°C, the radioactivity associated with the cells were determined. The receptor-mediated uptake was calculated by subtracting the non-specific binding from the total binding. Values are the mean of triplicate incubations.

**Table 2.** Effect of fatty acids on the receptor-binding, internalization and degradation of [$^{125}$I]LDL. Four days after seeding, medium was replaced with DMEM containing 10% LPDS and cells were incubated for 24 h. After 24 h incubation, 1.0 mM of individual fatty acids were added to the medium with 10 μg/ml of [$^{125}$I]LDL in the presence or absence of 500 μg/ml of unlabeled LDL. Then the cells were incubated at 37°C for 4 h

| Incubation conditions | Binding | Internalization | Degradation | |
|---|---|---|---|---|
| | | | Cellular | Medium |
| | (dpm×10$^3$/mg protein/4 h) | | | |
| Control | 12.8±1.1$^a$ | 37.9±2.0$^a$ | 7.5±0.5 | 44.3±1.2$^a$ |
| 18:0 | 17.7±0.8$^b$ | 41.9±1.3$^b$ | 7.9±0.6 | 53.5±5.1$^b$ |
| 18:1 | 20.8±2.0$^c$ | 51.8±2.6$^c$ | 8.1±1.0 | 61.3±3.7$^c$ |
| 18:2 | 20.5±1.4$^c$ | 52.0±3.0$^c$ | 8.1±0.5 | 58.5±2.8$^c$ |
| 18:3 | 21.1±2.2$^c$ | 53.6±4.1$^c$ | 7.8±0.5 | 60.4±5.3$^c$ |

Values are the mean of triplicate incubations.
Values bearing superscripts in the same column indicate significant differences among the experimental groups.

finity LDL binding pathway. The uptake of LDL by this pathway reached equilibrium after 2 h. Non-specific uptake, which increases linearly, comprised approximately 13% of the total LDL uptake for a 4 h incubation with 10 μg/ml of [$^{125}$I]-labeled LDL and a 50-fold excess of unlabeled LDL.

The [$^{125}$I]LDL-binding at 4°C as a function of LDL concentration in the medium is shown in Fig. 2. Because the internalization of LDL by receptor- mediated endocytosis is inhibited at 4°C, the LDL-binding, independent of internalization, was examined at this temperature. Total LDL-binding increased rapidly as the LDL concentration increased from 0 to 10 μg/ml and continued to increase, although more slowly, through to the highest concentration range (50 μg/ml). The LDL-binding experiments were also carried out in the presence of excess unlabeled LDL where only non-saturable binding was observed. Non-specific binding did not reach a plateau but continued to increase up to the highest concentration of 50 μg/ml tested. At the higher concentration of [$^{125}$I]-labeled LDL, the contribution of non-saturable binding to the total binding increased. At an LDL concentration of 10 μg/ml, non-specific binding accounted for approximately 9.5% of the specific LDL binding whereas at an LDL concentration of 50 μg/ml, non-specific binding increased to approximately 22% of the specific binding.

Table 2 shows the effects of fatty acids (C18:0, C18:1, C18:2, and C18:3) on receptor-binding, internalization and degradation of [$^{125}$I]LDL at 37°C. The inclusion of 1.0 mM of each fatty acid in the medium significantly increased LDL uptake and degradation of [$^{125}$I]LDL compared to the control. Among the different fatty acids tested, stearic acid had the least effect on the receptor binding activity. There were no significant difference among the unsaturated fatty acids in LDL-receptor binding. The effect of individual fatty acids on the internalization of [$^{125}$I]LDL was similar to that of the receptor-binding, showing a significantly less effect with stearic acid. Among the unsaturated fatty acids tested, no significant difference was noted. The amount of degraded material in the medium was higher with the unsaturated fatty acids than those of stearic acid. However, the total cellular degradation of internalized [$^{125}$I]LDL was not significantly affected by the inclusion of fatty acids in the medium.

## Discussion

The present study demonstrates that the human-derived hepatoma cell line, Hep-G2, retains the function of receptor-mediated binding, internalization, and degradation of LDL. The affinity of binding of [$^{125}$I]LDL with these cells corresponds to the properties of LDL binding originally described in cultured human fibroblasts (Attie *et al.*, 1981; Havekes *et al.*, 1983) and subsequently observed in hepatocytes from other animal species (Pangburn *et al.*, 1981).

The model for receptor-mediated catabolism of LDL consists of three stages including the initial binding of LDL to high affinity receptors on the cell surface, internalization through endocytosis, and finally, degradation in lysosomes (Mahley *et al.*, 1977). The binding of LDL by Hep-G2 cells proceeds by two pathways: a high-affinity process and a low affinity process. The high-affinity binding of LDL is saturable and can be demonstrated at 4°C although its extent is greater at 37°C. It exhibits a maximal amount of binding of about 31.4 ng of LDL/mg of cell protein when the cells are incubated with 10 μg of [$^{125}$I]LDL for 4 h. This value is of the same magnitude as found by other researchers (Leichtner *et al.*, 1984; Wu *et al.*, 1984).

In contrast to the high affinity binding process, the low process is characterized by an apparent lack of saturability. This process represents nonspecific endocytosis of the LDL from the culture medium. The low-affinity component accounted for approximately 22% of binding at an [$^{125}$I]LDL concentration of 50 μg/ml, which is the concentration that was used for routine assays of LDL binding. This result is consistent with the findings of Havekes *et al.* (1983) who described LDL-receptor activity in Hep-G2 cells, as well as with other studies (Attie *et al.*, 1981; Pangburn *et al.*, 1981; Soltys *et al.*, 1982).

Several mechanisms have been proposed to account for the changes in LDL-receptor activity resulting from the intake of different fatty acids. First, they could influence the number of LDL-receptors synthesized. Second, they might affect the acitivity of the receptors in the cell membrane, and third, they could influence the affinity of the lipoproteins for the receptors (Brown *et al.*, 1976). The latter two mechanisms may be mediated by the fatty acid composition of phospholipid in either the membrane harboring LDL-receptors or the surface coat of lipoproteins containing apo B.

The present study also investigates the comparative effects of C18:0, C18:1, C18:2, and C18:3 on the LDL-receptor activity by measuring the receptor-mediated binding, internalization and degradation. Inclusion of individual fatty acids in the medium significantly increased the receptor-binding and internalization of LDL compared to the control. Receptor-mediated LDL uptake was significantly lower in the presence of stearic acid than those of unsaturated fatty acids. These results are supported by the clinical and animal studies (Shepherd *et al.*, 1980; Bonanome *et al.*, 1988) in which dietary saturated fatty acids decrease receptor-mediated clearance. Furthermore, Woollett *et al.* (1992) reported that decreased LDL-receptor activity by saturated fatty acids was accompanied by decreased LDL-apo B receptor mRNA levels.

The degradation of the cholesteryl ester moiety of LDL releases free cholesterol, and this lipoprotein-derived cholesterol consequently regulates cellular cholesterol metabolism by suppressing HMG-Co A reductase, activating microsomal acyl-Co A:cholesterol acyltransferase (ACAT) and down regulating the number of LDL receptors in the cell membrane (Goldstein *et al.*, 1983; Attie *et al.*, 1981). Therefore, it can be speculated that the supression of LDL-receptor synthesis by saturated fatty acids may occur by the redistribution of cholesterol among various cellular compartments to favor its inhibitory action on receptor synthesis by a mechanism of the well known cholesterol feed back inhibition (Grundy *et al.*, 1970; Spady *et al.*, 1988).

An alternate possibility for the LDL-receptor interfering action of saturated fatty acid has been postulated by Loscalzo *et al.* (1987) on the basis of an in vitro study. This study suggests that enrichment of cell membrane phospholipid with saturated fatty acids interferes with the normal function of LDL receptors within the cell membrane, possibly by reducing binding or internalization of circulating LDL. Choue *et al.* (1994) showed that the amounts of saturated fatty acid in the membrane phospholipids markedly increased by the inclusion of this fatty acid in the culture medium. However, the proof that the saturated fatty acid content of the cell membrane and lipoprotein can be increased *in vivo* by the feeding of diets high in saturated fatty acid is lacking. Alteration in the ability of apo B to bind to the receptor due to changes in the LDL-phospholipid fatty acid composition are also possible. This effect is due to the influence of membrane phospholipid on the activity of apo B in a manner similar to that described for cellular receptor protein by Loscalzo *et al.* (1987). However there is currently little evidence to support such an effect on the apo B.

The possible mechanism for the elevation in plasma LDL-cholesterol level by saturated fatty acids might be increased plasma levels of LDL resulting from an increased secretion of VLDL in the circulation. However, this might not be supported by the findings that the secretion of VLDL in the presence of saturated fatty acid did not increase (Choue *et al.*, 1994). With this in mind, it is reasonable to conclude that the mechanism for the elevation of LDL-cholesterol level by saturated fatty acids may be attributed to a reduced LDL-receptor activity. This, in turn, is most likely due to the reduced rate of removal of the smaller, more dense LDL (Heimberg *et al.*, 1972; Wilcox *et al.*, 1975). The production of smaller, more dense particles might be a direct consequence of the alteration in the amount of triglyceride, phospholipid, cholesterol and apo B in lipoprotein secretion in the presence of saturated fatty acids.

The available evidence suggests that substitution of unsaturated fatty acid for saturated fatty acid results in an increase in LDL-receptor activity (Spady *et al.*, 1989). This is in agreement with the present study which found that unsaturated fatty acids result in a greater uptake and degradation of [$^{125}$I]LDL when they were compared with the saturated fatty acid. Studies in laboratory animals further indicate that exchange of linoleic acid for saturated fatty acids increases the receptor- mediated uptake of LDL (Spady *et al.*, 1990). These observations, however, might not prove that linoleic acid actively stimulates the synthesis of LDL-receptors. In fact, when linoleic acid is fed in large quantities to monkeys, there is no evidence for an increase in mRNA abundance for LDL-receptors (Kuo *et al.*, 1989). The effects of linoleic acid, therefore, could be entirely passive, which is to say, saturated fatty acids may actively suppress activity or function of LDL receptors by mechanisms yet to be determined, while linoleic acid is neutral. Further studies will be necessary to determine the differential effects of dietary saturated, monounsaturated and polyunsaturated fatty acids on LDL-receptor activity.

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