Lack of a lipoprotein-induced insulin resistance in hepatoma cells in culture

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Summary. A lipoprotein-induced resistance to the action of insulin has been postulated. To test this hypothesis, cultured rat-derived hepatoma cells, designated FAO, and human-derived hepatoma cells, designated HEP-G2, were incubated for 20 h in the presence or absence of lipoproteins; specific ¹²⁵I-insulin receptor binding and labeled glucose incorporation into glycogen were then measured. Very low density lipoproteins ($d < 1.006$ g/ml) in physiologic (0.5 mg/ml) or pathophysiologic (5 mg/ml) concentrations did not modify insulin receptor binding of FAO or HEP-G2 cells. This was true for very low density lipoproteins derived from normal human, diabetic human, and streptozotocin-diabetic rat plasma. Low density

lipoproteins ($d = 1.019 - 1.063$ g/ml) isolated from normal human plasma similarly failed to modify insulin receptor binding. Concerning insulin action, the different very low density lipoprotein preparations did not modulate either basal or insulin-stimulated glucose incorporation into glycogen of the cells. Thus, very low density lipoproteins and low density lipoproteins did not induce insulin resistance in cultured hepatoma cells either at the insulin receptor level or at the post-receptor level.

Key words: Insulin resistance, lipoproteins, liver, insulin binding, insulin action, hepatoma cells.

Hyperlipoproteinaemias are frequent metabolic disturbances in patients with diabetes mellitus [1]. Several in vitro [2, 3] and in vivo [4–6] studies have led to the hypothesis that lipoproteins per se can modify insulin action at the cellular level, i. e. induce resistance to the action of the hormone.

Endogenous hypertriglyceridaemia (type IV) is frequently associated with insulin resistance [4–6]. Recently, decreased insulin receptor binding of monocytes and erythrocytes was demonstrated in such nonobese patients [4]. It was suggested that this might be directly due to elevated levels of very low density lipoproteins (VLDL) [4]. In fact, VLDL reduced insulin receptor binding of adipocytes and IM-9 lymphocytes in vitro [3]. In cultured aortic endothelial cells, malondialdehyde-altered low density lipoproteins (MDA-LDL) and beta-migrating VLDL (beta-VLDL) induced a post-receptor insulin resistance, but in this case there was no modification of insulin receptor binding induced by lipoproteins [2].

In view of these controversial results with extrahepatic cells, we asked if lipoproteins can induce insulin resistance in cells of hepatic origin. We used highly differentiated human- and rat-derived hepatoma cells in culture, designated HEP-G2 and FAO, which respre-

sent well characterized models for metabolic studies [7–12]. Hyperlipoproteinaemia was simulated by the addition to the culture medium of VLDL and LDL derived from normal human, diabetic human and streptozotocin-diabetic rat plasma. Effects of these lipoproteins on insulin receptor binding and on the incorporation of glucose into glycogen were examined.

Materials and methods

D-[U-¹⁴C]-glucose, specific activity 275 mCi/mmol and D-[1-³H]-glucose, specific activity 5.7 Ci/mmol were from Amersham International, Amersham, UK. ¹²⁵I-TYR-A14 monoiodoinsulin was a gift from Drs. Ronald Chance and Bruce Frank, Eli Lilly Corporation, Indianapolis, Ind, USA. Porcine monocomponent insulin, glycogen (from bovine liver, type IX), Hepes (N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid) and streptozotocin were from Sigma, St. Louis, Mo, USA. Dulbecco's modified eagle medium (DME), "Medium 199", fetal calf serum, trypsin/EDTA (trypsin 0.5 g/l, Na₂EDTA 0.2 g/l), penicillin/streptomycin were from Gibco, Grand Island, NY, USA. Bovine serum albumin (fraction V) and enzymatic triglyceride kit were from Boehringer, Mannheim, FRG. Gentamicin was from Irvine Scientific, Santa Ana, Calif, USA. Male Sprague-Dawley rats were from Ivanovas, Kisslegg, FRG. Plastic culture dishes and flasks were from Falcon Plastics, Los Angeles, Calif, USA. All other chemicals and reagents were of analytical grade.

Cell culture

HEP-G2 hepatoma cells, derived from a minimal deviation human hepatoma [9], were grown in Dulbecco's modified eagle medium (DME) supplemented with fetal bovine serum (10% v/v) and gentamicin (50 mg/l). FAO hepatoma cells, derived from a highly differentiated rat hepatoma [8], generously supplied by Dr. C. R. Kahn, Boston, Mass, USA, were cultured in "Medium 199" containing fetal bovine serum (5% v/v), penicillin (100 U/ml) and streptomycin (100 mg/l). Both cell lines were routinely cultured in a humidified incubator at 37 °C in an atmosphere of CO₂ (5% v/v) and air (95% v/v). The cells were split 1:5 every 7 days. For insulin binding and insulin action assays, cells were plated in 35-mm diameter plastic culture dishes (400,000–500,000 cells/dish) and incubated in their respective media. After 3 days in culture, the medium was removed and fresh culture medium was added. In the case of the HEP-G2 cells, this medium contained human lipoprotein-deficient serum (LDS, 10% v/v, d > 1.21 g/ml) in place of fetal bovine serum; medium for the FAO cells contained 5% (v/v) fetal bovine serum. To these media was added either the indicated lipoproteins or the corresponding volume of appropriate buffer (about 4% v/v). The cells were then incubated for 20 h with or without lipoproteins. After culture, the cells were washed with phosphate-buffered saline (PBS, pH 7.4, 4 °C).

Lipoproteins

Very low density lipoproteins (VLDL, d < 1.006 g/ml) and low density lipoproteins (LDL, d = 1.019–1.063 g/ml) were isolated by sequential preparative ultracentrifugation (2 × 20 h, 50,000 rpm) in the presence of EDTA (1 mg/ml) [13]. Both fractions were washed by refloatation at the higher density limit to eliminate albumin contamination. Lipoprotein-deficient serum (LDS, d > 1.21 g/ml) was derived from human plasma after adjusting the density to 1.21 g/ml. Densities were adjusted by the addition of NaBr. Prior to their use, the lipoproteins and the lipoprotein-deficient serum were exhaustively dialyzed against phosphate-buffered saline (pH 7.4, 4 °C) containing EDTA (1 mg/ml). In some cases, the lipoproteins were dialyzed against saline (NaCl 154 mmol/l, pH 7.4, 4 °C) containing glutathione (0.2 mg/ml) instead of EDTA. Before use, the lipoproteins were sterile filtered (0.44 µm).

Plasma for lipoprotein preparation was obtained from three sources: pooled human blood after an overnight fast; fasting, Type 2 (non-insulin-dependent) diabetic patients with moderate secondary hyperlipoproteinaemia (serum triglyceride 2–3 mg/ml); and streptozotocin-diabetic rats. Diabetes was induced in these rats according to the procedure described in detail by Bar-On et al. [14]. Male Sprague-

Dawley rats were injected via the tail vein with streptozotocin (45 mg/kg body weight); all animals were killed 7 days after this injection by exsanguination from the abdominal aorta. Prior to killing, the animals were fasted for 6 h. Mean blood glucose was 13.44 mmol/l at the time of killing, and mean serum triglyceride was 2.76 mg/ml. It was shown by electrophoresis that the diabetic rat VLDL fraction was free of chylomicrons.

The triglyceride concentrations of the VLDL preparations and media were measured using a commercial enzymatic kit and are indicated in terms of mg triglyceride/ml. LDL protein content was determined after delipidation of lipoproteins with a mixture of 3:1 (v/v) ethanol:diethylether. Protein was then determined by the Lowry method [15]. LDL concentrations are indicated as mg LDL protein/ml.

Electrophoretic mobilities of the lipoproteins were determined on agarose gels.

Insulin binding

For the determination of insulin receptor binding, cells were incubated in fresh Hanks Hepes buffer (Hepes 20 mmol/l, pH 7.4, 1.5 ml/dish) containing albumin (1 mg/ml). This buffer additionally contained ¹²⁵I-insulin (mono-¹²⁵I-[TYR-A14]-insulin, 0.1 µCi/dish, specific activity 200–300 µCi/µg) alone or together with the indicated concentrations of unlabeled insulin. Binding equilibrium was reached in both cell lines within 2 h at 20 °C. Nonspecific ¹²⁵I-insulin binding was measured by incubating cells with labeled insulin (0.1 µCi/dish) and an excess of unlabeled insulin (1000 U/l). At the end of the incubation period, the medium was aspirated and the cell layer washed with phosphate-buffered saline (pH 7.4, 4 °C, 4 ×). Cells were then solubilized by the addition of NaOH solution (0.2 mol/l, 2 × 0.5 ml) and scraped off the plates with a rubber policeman. The radioactivity associated with the cells was measured in a gamma counter. An aliquot of the solubilized cells was used for protein determination according to the method described by Lowry et al. [15]. All binding data were corrected for non-specific insulin binding.

Labeled glucose incorporation into glycogen

The assay for insulin-stimulated incorporation of labeled glucose into glycogen was carried out as previously described [16] according to the method of Hofmann et al. [17]. FAO cells were incubated with culture medium (37 °C, pH 7.4, 1 mg/ml albumin) containing 0.2 µCi D-[U-¹⁴C]-glucose/dish (specific activity 275 mCi/mmol) at a final glucose concentration of 2.5 mmol/l. HEP-G2 cells were incubated in culture

Table 1. Effect of very low density lipoproteins on specific insulin binding of FAO hepatoma cells

Lipoprotein in the culture medium during 20 h	Insulin specific bound (µU/mg cell protein)			
	¹²⁵ I-insulin alone in the assay medium	¹²⁵ I-insulin + 100 mU/l unlabeled insulin in the assay medium	¹²⁵ I-insulin + 1000 mU/l unlabeled insulin in the assay medium	n
Human VLDL				
Control	0.112 ± 0.033	1.848 ± 0.707	10.881 ± 4.804	4
VLDL-TG (5 mg/ml)	0.108 ± 0.040	1.848 ± 0.814	12.420 ± 4.779	4
Human Type 2 diabetic VLDL				
Control	0.253 ± 0.113	3.935 ± 1.663	22.537 ± 10.681	4
VLDL-TG (5 mg/ml)	0.248 ± 0.089	3.723 ± 1.450	19.894 ± 7.269	4
Rat diabetic VLDL				
Control	0.219 ± 0.040	3.111 ± 0.621	18.837 ± 4.080	3
VLDL-TG (5 mg/ml)	0.233 ± 0.061	3.335 ± 0.650	20.646 ± 1.367	3

FAO hepatoma cells were incubated during the last 20 h in culture in the absence (control) or the presence of the indicated level of VLDL. ¹²⁵I-insulin binding alone or in the presence of the indicated concentrations of unlabeled insulin was measured as described under "Materials and Methods". ¹²⁵I-insulin concentration was 4.44 mU/l for the experiments with human VLDL, 5.88 mU/l for those with human Type 2 diabetic VLDL and 6.08 mU/l for those with rat diabetic VLDL. Values represent mean ± SEM. All determinations were performed in duplicate. n = number of individual experiments. p > 0.05 for all insulin concentrations in each experimental group

medium (37°C, pH 7.4, 1 mg/ml albumin) containing 4 μ Ci D-[1-³H]-glucose/dish (specific activity 5.7 Ci/mmol), at a final glucose concentration of 5.5 mmol/l. Basal glycogen synthesis was measured in the absence of insulin and stimulated synthesis in the presence of the indicated insulin concentrations. After 2 h incubation at 37°C, the incubation medium was removed and the cells were washed with phosphate-buffered saline (pH 7.4, 4°C, 4 $\times$). Glycogen was extracted as previously described [16]. Blank values were obtained by parallel incubation of cells at a temperature of 2°C for 2 h with medium containing labeled glucose. The results were corrected for blank values and protein content.

Statistical analysis

The Student's *t*-test for paired observations was used for the evaluation of statistical differences. A value of $p < 0.05$ was considered significant.

Table 2. Effect of very low density lipoproteins on basal and insulin stimulated glycogen synthesis in FAO hepatoma cells

Lipoprotein in the culture medium	Glucose incorporated into glycogen (nmol glucose/mg cell protein per 2 h)		
	Basal	Insulin (1000 mU/l)	<i>n</i>
Human VLDL			
Control	1.820 $\pm$ 0.452	3.971 $\pm$ 0.382	5
VLDL-TG (5 mg/ml)	1.609 $\pm$ 0.396	4.032 $\pm$ 0.645	5
Human Type 2 diabetic VLDL			
Control	1.904 $\pm$ 0.707	3.493 $\pm$ 1.491	4
VLDL-TG (5 mg/ml)	1.920 $\pm$ 0.611	3.510 $\pm$ 1.348	4
Rat diabetic VLDL			
Control	2.182 $\pm$ 0.182	3.923 $\pm$ 0.483	3
VLDL-TG (5 mg/ml)	2.117 $\pm$ 0.207	4.176 $\pm$ 0.081	3

FAO hepatoma cells were incubated during the last 20 h in culture in the absence (control) or presence of the indicated VLDL. Basal and insulin-stimulated glucose incorporation into glycogen were then measured as described under "Materials and Methods". Values represent mean $\pm$ SEM. All determinations were performed in triplicate. n = number of separate experiments. $p > 0.05$ for all values

Results

Rat-derived FAO hepatoma cells were cultured in the absence or presence of pathophysiologic VLDL concentrations (5 mg/ml) for 20 h prior to measurement of insulin binding and insulin action. In preliminary experiments (data not shown), the effect of insulin on labeled glucose incorporation into glycogen was very low when insulin was added to the preincubation media, suggesting the accumulation of an inhibiting factor. Therefore, the media were changed for the assays of insulin binding or action. VLDL was not included during these short term assays. VLDL derived from normal human plasma had no effect on ¹²⁵I-insulin receptor binding compared to insulin binding to cells cultured in the absence of VLDL (Table 1). As parameter of insulin action in FAO hepatoma cells, insulin stimulation of labeled glucose incorporation into glycogen was measured. Insulin (1000 mU/l) stimulated glucose incorporation into glycogen to 218% above the basal control (Table 2). Basal glucose incorporation into glycogen was not different in VLDL treated cells, compared to cells incubated without lipoproteins. Insulin stimulation of glycogen synthesis was not significantly different ($p > 0.05$) in FAO cells incubated in the presence of VLDL compared to cells incubated in the absence of VLDL (Table 2).

The lack of a lipoprotein effect on insulin binding and action might have been attributed to compositional differences between lipoproteins from normal and diabetic men and animals [14, 18, 19]. To address this possibility, the FAO cells were also cultured in the presence of VLDL (5 mg/ml) isolated from the plasma of Type 2 diabetic patients and streptozotocin-induced diabetic rats (Tables 1 and 2). However, these VLDL preparations again had no effect on insulin receptor binding

Fig. 1. Effect of very low-density lipoproteins (left) and low-density lipoproteins (right) on specific ¹²⁵I-insulin receptor binding of HEP-G2 cells. Left: Cells were incubated for 20 h in the absence of VLDL (●—●) or in the presence of normal human VLDL at a concentration of 0.5 mg/ml (■—■) or 5 mg/ml (▲—▲). ¹²⁵I-insulin binding alone or in the presence of the indicated concentrations of unlabeled insulin was measured as described in "Materials and methods". Values are mean $\pm$ SEM of four individual experiments. All determinations were performed in duplicate. Bars illustrate SEM values at each point. Differences were not significant ($p > 0.05$) at any insulin concentration. Right: Cells were incubated for 20 h in the absence (●—●) or presence of normal human LDL (■—■) in a concentration of 0.5 mg/ml LDL protein. ¹²⁵I-insulin binding was measured as described above. Values are mean $\pm$ SEM of three individual experiments, with all determinations performed in duplicate. Differences were not statistically different ($p > 0.05$) at all insulin concentrations tested

Fig. 2. Influence of very low-density lipoproteins on glucose incorporation into glycogen of HEP-G2 cells. Cells were incubated for 20 h in the absence of lipoproteins (●----●) or in the presence of normal human VLDL in a concentration of 0.5 mg/ml (■----■) or 5 mg/ml (▲----▲). Basal and insulin-stimulated rates of labeled glucose incorporation into glycogen (insulin concentrations as indicated) were measured as described in "Materials and methods". Values are mean of 3 individual experiments. In 2 experiments the determinations were performed in triplicate, and in 1 experiment the determinations were performed in duplicate. SEM are shown for the control group and the cells incubated in the presence of VLDL (5 mg/ml). Differences were not significant ($p > 0.05$) for basal and all insulin-stimulated levels of glucose incorporation into glycogen. Basal glucose incorporation into glycogen (= 100%) was for cells incubated for 20 h in the absence of lipoproteins 4.72 ± 0.41 nmol glucose $\times$ mg protein⁻¹ $\times$ 2 h⁻¹ incorporated into glycogen (mean $\pm$ SEM); for cells incubated for 20 h in the presence of 0.5 mg/ml VLDL, it was 5.21 ± 1.12 ; for cells incubated in the presence of 5 mg/ml VLDL, 4.81 ± 0.16

(Table 1). Similarly, basal and insulin-stimulated incorporation of labeled glucose into glycogen in FAO cells was not significantly modified by VLDL isolated from diabetic rat and diabetic human plasma (Table 2).

As a model for human hepatocytes, highly differentiated HEP-G2 hepatoma cells were used [9]. Figure 1 shows cellular ¹²⁵I-insulin binding and its displacement by unlabeled insulin. Insulin binding data were not different whether the cells were cultured in the absence of VLDL (control), in the presence of physiologic (0.5 mg/ml) or pathophysiologic (5 mg/ml) concentrations of normal human VLDL (Fig. 1, left). Under identical incubation conditions with VLDL, insulin action was measured as shown in Figure 2. Insulin (100–100,000 mU/l) stimulated ³H-glucose incorporation into glycogen in a dose-dependent manner. Basal glucose incorporation was not modified when the HEP-G2 cells were incubated with normal human VLDL. In agreement with data for the rat cell line, insulin stimulation of labeled glucose incorporation into glycogen was not significantly different in HEP-G2 cells incubated in the absence of VLDL or incubated in the presence of physiologic (0.5 mg/ml) or pathophysiologic (5 mg/ml) VLDL levels (Fig. 2).

LDL levels are frequently elevated in diabetic patients [20]. Therefore, their effect on insulin receptor binding in HEP-G2 cells was examined. The LDL con-

centration used (0.5 mg/ml protein, approximately 1 mg/ml cholesterol) was within the physiologic range, and sufficient to downregulate high affinity LDL-receptor activity [7]. As shown in Figure 1 (right), LDL did not change ¹²⁵I-insulin receptor binding compared to that in cells incubated in the presence of lipoprotein-deficient serum (LDS) alone.

The lack of a lipoprotein-induced insulin resistance might have been the consequence of lipoprotein catabolism by the cells during the 20 h of incubation with the lipoproteins. Therefore, VLDL-triglyceride and LDL-cholesterol concentrations and electrophoretic mobility of the lipoproteins on agarose gels were determined after the 20 h incubation. The measured parameters were compared to those of lipoproteins not incubated with cells (data not shown). VLDL-triglyceride concentrations (0.5 and 5 mg/ml) and LDL-cholesterol concentration were not measurably different from original levels after 20 h of incubation with cells. Similarly, electrophoretic mobility of VLDL (0.5 and 5 mg/ml) and LDL (0.5 mg/ml) on agarose gels was not significantly different after 20 h of incubation with cells when compared to the mobility of lipoproteins not incubated with hepatoma cells.

To examine the possibility of a toxic effect of the lipoproteins on the cells, cellular protein content and viability were determined. Cellular protein content of HEP-G2 cells and FAO cells after incubation for 20 h in the presence or absence of lipoproteins was similar, regardless of the presence of VLDL (0.5 or 5 mg/ml) or LDL (0.5 mg/ml) during the incubation (data not shown). Viability of the cells as determined by trypan blue exclusion under the phase contrast microscope was identical in the cells incubated for 20 h with or without lipoproteins.

Discussion

In this study with hepatoma cells, VLDL and LDL did not modify insulin receptor binding. Similarly, Berliner et al. [2] observed that neither beta-migrating very low density lipoproteins (beta-VLDL) nor malondialdehyde-altered LDL (MDA-LDL) had any effect on insulin receptor binding in aortic endothelial cells in culture. In contrast to these data, Steiner et al. [3] observed reduced insulin receptor binding in rat adipocytes in suspension and cultured IM-9 lymphocytes of human origin incubated with VLDL. Taken together, these results suggest that triglyceride-rich VLDL can modify insulin receptor binding in extrahepatic tissues [3] but not in cells of hepatic origin. However, in contrast to the triglyceride-rich VLDL, cholesterol-rich lipoproteins like LDL, beta-VLDL and MDA-LDL do not modulate insulin receptor-interaction in the cells studied [2]. The possibility that only primary cells are sensitive to lipoprotein-induced modulation of cellular insulin action is argued against by the reduced insulin receptor binding

in the lymphocyte cell line after culture in the presence of VLDL [3].

In this study, VLDL did not modify insulin action. In the investigation with adipocytes and lymphocytes, no biologic effects of insulin were measured [3]. Thus, the metabolic significance of reduced insulin receptor binding in that study [3] is not clear. In contrast, in aortic endothelial cells [2] beta-VLDL and MDA-LDL induced a post-receptor insulin resistance, as measured by amino acid transport; such an effect could not be induced by LDL in the same cells [2]. Beta-VLDL and MDA-LDL were taken up by the aortic cells and increased their cholesterol content [2, 21] while LDL and VLDL do not comparably alter the cellular cholesterol content [21, 22]. This suggests that an increased cellular cholesterol could possibly induce resistance to the action of insulin and explain our lack of a lipoprotein effect [23]. Alternative explanations for the differences may lie in a tissue specificity between hepatic and extrahepatic cells. Hepatic cells are able to process lipids in ways that other cells cannot, e.g. in bile production, and that might explain the apparent discrepancy. It is also possible that amino acid transport and glucose incorporation into glycogen have a different susceptibility to a lipoprotein modification, thus explaining the differences in results.

The present in vitro studies should not be considered to exclude the possibility that in patients with diabetes mellitus or endogenous hypertriglyceridaemia, lipoproteins could indeed induce insulin resistance. For instance "remnant" particles derived from VLDL and chylomicrons [19, 24] might be taken up avidly enough by the liver [25] to affect insulin action, even though VLDL did not have this effect in vitro.

In summary, our results do not support the hypothesis of a VLDL-induced resistance to the action of insulin either at the insulin receptor or at the post-receptor level.

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