Effect of long-term treatment of 3T3-L1 adipocytes with chlorate on the synthesis, glycosylation, intracellular transport and secretion of lipoprotein lipase

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Lipoprotein lipase (LPL) is synthesized and glycosylated in the endoplasmic reticulum (ER), transported through the Golgi to the cell surface, and finally secreted. To examine the role of heparan sulphate proteoglycans (HSPG) in the synthesis, activity, intracellular transport and secretion of LPL, 3T3-L1 adipocytes were cultured for 7 days in the presence of 20 mM chlorate, an inhibitor of sulphation of HSPG. Treatment of cells with 20 mM chlorate for 7 days caused a 55% decrease in LPL activity in the intracellular compartment and a 79% decrease in the cell-surface compartment. The synthetic rate of LPL in chlorate-treated cells was identical with that in control cells as determined by bio-synthetic labelling. The study with endoglycosidase H (endo H) showed that the treatment with chlorate increased the proportion of LPL subunits which were totally endo H-sensitive. The study with a heparin–Sephacel column showed that 3T3-L1 adipocytes contained three forms of LPL. The first form, accounting for 35% of the LPL, did not bind to the heparin–Sephacel column and had little or no activity; the second form, accounting for 32%, bound to the column and was eluted with 0.4–0.75 M NaCl but had no activity; the third form, accounting for 33%, bound to the column and was eluted with 0.8–1.2 M NaCl and had activity. In chlorate-treated cells, the first form accounted

for 66% of the LPL, the second form 15% and the third form 19%. When cells were incubated for 1 h with brefeldin A, which translocates Golgi proteins to the ER [J. Lippincott-Schwartz, L. C. Yuan, J. S. Banifacino and R. D. Klausner (1989) *Cell* **56**, 801–813; J. Lippincott-Schwartz, J. Glickman, J. E. Donaldson, J. Robbins, T. E. Kreis, K. B. Seamon, M. P. Sheetz and R. D. Klausner (1991) *J. Cell Biol.* **112**, 567–577], the chlorate-induced decrease in cellular LPL activity was restored. These findings indicate that LPL synthesized in chlorate-treated cells can be processed to be fully active, but chlorate-treated cells are unable to transport LPL to the Golgi and accumulate inactive LPL with a lower affinity for heparin in the ER. The treatment with chlorate decreased the proportion of LPL subunits that were endo H-resistant, indicating that the processing of oligosaccharide chains of LPL in the *trans*-Golgi was impaired in chlorate-treated cells. The amount of ³⁵S-labelled LPL secreted by chlorate-treated cells was identical with that secreted by control cells, whereas the level of LPL activity in the medium of chlorate-treated cells was 25% of that in the medium of control cells, indicating that most of the LPL secreted by chlorate-treated cells was inactive.

INTRODUCTION

Lipoprotein lipase (LPL) is synthesized by the parenchymal cells of extrahepatic tissues and transported to the luminal surfaces of the vascular endothelial cells of the tissues, where it hydrolyses triacylglycerols in chylomicrons and very-low-density lipoproteins to non-esterified fatty acids and monoacylglycerols. This enzyme is released into the bloodstream by injecting heparin. Defect or dysfunction of this enzyme causes severe hypertriglyceridaemia [1,2].

Studies in cultured adipocytes indicate that the production of active LPL involves the synthesis and N-linked glycosylation of the LPL subunit in the endoplasmic reticulum (ER), the trimming of glucose and certain mannose residues from high-mannose type oligosaccharide chains in the ER and Golgi, the processing of chains to complex type chains in the Golgi, and the dimerization of subunits [3–9]. Mouse LPL has two N-linked oligosaccharide chains per subunit [6,7]. Studies with endoglycosidase H (endo H) indicate that mouse adipocytes contain three groups of LPL subunits: one is an endo H-resistant subunit with two complex type oligosaccharide chains and is the mature form of the

enzyme; another is a partly endo H-sensitive subunit with one high-mannose type chain and one complex type chain; and the third is a totally endo H-sensitive subunit with two high-mannose type chains [6–10].

Heparan sulphate proteoglycans (HSPG) provide the high-affinity binding sites for LPL on the cell surface. The negatively charged sulphate residues of HSPG interact with the positively charged amino acid residues on the C-terminus of LPL via electrostatic interaction [11]. Chlorate, which inhibits sulphurylase [12], causing a subsequent decrease in the production of phosphoadenosine phosphosulphate, the active sulphate donor for sulphotransferases [13], has been used in studies of HSPG biosynthesis and structure–function relationships of HSPG [14–17]. Humphries and Silbert [14] demonstrated that treatment of cultured endothelial cells with chlorate causes a marked decrease in the sulphation of proteoglycans and that the removal of chlorate from the culture medium results in the rapid resumption of sulphation. Hoogewerf et al. [15] also demonstrated that chlorate inhibits the sulphation of HSPG of cultured avian adipocytes, thus decreasing the binding of LPL to the cell surface.

Abbreviations used: BFA, brefeldin A; DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; endo H, endoglycosidase H; HSPG, heparan sulphate proteoglycans; LPL, lipoprotein lipase.

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This paper describes the effect of long-term treatment of 3T3-L1 adipocytes with chlorate on the synthesis, activity, intracellular transport and secretion of LPL. Treatment of cells with chlorate resulted in the production of inactive LPL that was totally endo H-sensitive and had a lower affinity for heparin.

MATERIALS AND METHODS

Cell culture

3T3-L1 fibroblasts were grown to confluence in a standard medium in 60 mm plates. Confluent cells were stimulated to differentiate into adipocytes by supplementing the standard medium with 1 μ M dexamethasone, 10 μ g/ml insulin and 0.5 mM methylisobutylxanthine for 2 days [9]. This medium was then replaced with a complete medium containing 0–20 mM sodium chlorate and was replenished every 2 days. The cells on the bottom of a plate excluded Trypan Blue, indicating that they were alive.

The standard medium contained 10 % (v/v) fetal bovine serum, 100 i.u./ml penicillin, 100 μ g/ml streptomycin and 0.25 μ g/ml amphotericin B in Dulbecco's modified Eagle's medium (DMEM). The complete medium contained 5 μ g/ml insulin in the standard medium.

Assay of LPL activity

3T3-L1 adipocytes were cultured for 7 days in a complete medium containing 0–20 mM chlorate. This medium was replaced with a fresh complete medium containing the appropriate additive, and the cells were incubated for 30 min at 37 °C. The culture medium was then separated from the cells, filtered through a filter with a pore size of 0.2 μ m and used for prompt assay of LPL activity. Cellular LPL activity was measured in the extract of acetone/ether-dried powder of cells [7].

A stock emulsion containing 5 mCi of tri[9,10(n)-³H]oleoyl-glycerol, 1.13 mmol of trioleoylglycerol, 60 mg of phosphatidylcholine and 9 ml of glycerol was prepared [7]. A mixture of 1 vol. of the stock emulsion, 19 vol. of 3 % (w/v) BSA in 0.2 M Tris/HCl buffer, pH 8.2, and 5 vol. of heat-inactivated (56 °C for 10 min) serum from starved rats was incubated at 37 °C for 15–30 min. For assay, 100 μ l of this activated substrate mixture was added to 100 μ l of the diluted extract of the powder or medium, and the mixture was incubated for 1 h at 37 °C. One unit of lipolytic activity was defined as that releasing 1 nmol of fatty acids/min at 37 °C.

[³⁵S]Methionine incorporation studies

3T3-L1 adipocytes were cultured for 7 days in a complete medium containing either 0 or 20 mM chlorate. Cells were washed twice with PBS, then the plates were replenished with 1.5 ml of methionine-deficient DMEM containing the appropriate additive. After 30 min, 160 μ Ci of [³⁵S]methionine was added and the cells were incubated for 1 h at 37 °C. The cells were then harvested in 0.2 M Tris/HCl buffer, pH 7.5, containing 3 % (v/v) Triton X-100, 1 % (w/v) *N*-lauroylsarcosine, 0.15 M NaCl and 1 mM PMSF, sonicated briefly at 0 °C, then centrifuged for 20 min at 4 °C and 12000 *g*. An aliquot of the infranatant was taken to measure the amount of [³⁵S]methionine incorporated into the total protein [precipitated with 10 % (w/v) trichloroacetic acid] [7]. Another aliquot of the infranatant was used to immunoprecipitate ³⁵S-labelled LPL.

The medium of the cells incubated for 1 h with [³⁵S]methionine was filtered through a filter with a pore size of 0.2 μ m and mixed with an equal volume of 20 % (w/v) trichloroacetic acid in the presence of 50 μ g of BSA as a carrier. The precipitated ³⁵S-

labelled proteins were solubilized, neutralized and used to immunoprecipitate ³⁵S-labelled LPL [9].

³⁵S-labelled LPL was immunoprecipitated with a chicken antiserum against bovine LPL and was resolved by SDS/PAGE [9]. The gels were stained with Coomassie Blue, destained, impregnated with En³Hance (Du Pont–New England Nuclear, Wilmington, DE, U.S.A.) and dried on a sheet of cellophane under vacuum. Autoradiographs were obtained by exposing the gel to Kodak X-Omat film at –80 °C for 14 days. The radioactive zones corresponding to LPL were cut out from the gels and dissolved in 1 ml of 30 % (w/v) H₂O₂ at 65 °C overnight. The radioactivity was determined in a liquid-scintillation counter.

Enzymic deglycosylation of LPL

³⁵S-labelled LPL, immunoprecipitated with a chicken antiserum against bovine LPL, was digested with endo H as described previously [18]. The digestion products were resolved by SDS/PAGE. The relative amounts of the endo H-digestion products of LPL were estimated densitometrically with a dual-wavelength flying-spot scanner CS-90040 (Shimadzu, Kyoto, Japan) that subtracted background and integrated the image produced by an autoradiographic band.

Heparin–Sephacrose column chromatography of LPL

3T3-L1 adipocytes were cultured for 7 days in a complete medium containing either 0 or 20 mM chlorate, washed once with ice-cold PBS, harvested in 0.5 ml of a lysis buffer (pH 8.2) containing 25 mM ammonia, 6 mM EDTA, 0.8 % (v/v) Triton X-100, 0.04 % SDS, 1 μ g/ml pepstatin, 10 μ g/ml leupeptin and 3.5 μ g/ml aprotinin, sonicated briefly at 0 °C, and centrifuged for 20 min at 4 °C and 12000 *g*. A 0.95 ml sample of the combined infranatants from two plates was applied to a heparin–Sephacrose column (column size 1 ml) equilibrated with 0.3 M NaCl in solution A [20 mM Tris/20 % (v/v) glycerol/0.1 % (v/v) Triton X-100 (pH 7.4)]. The proteins were eluted with a linear gradient of NaCl (0.3–2.0 M) in solution A. The level of LPL activity in duplicate samples of each fraction was measured.

Western blotting

An aliquot of each fraction eluted from the heparin–Sephacrose column was mixed with an equal volume of 0.125 M Tris/HCl buffer, pH 6.8, containing 4 % (w/v) SDS, 10 % (v/v) 2-mercaptoethanol, 20 % (v/v) glycerol and 0.002 % Bromophenol Blue, and heated for 5 min at 95 °C. Proteins in the fraction were separated by SDS/PAGE and then transferred electrophoretically to a nitrocellulose membrane. LPL was blotted with a chicken antiserum against bovine LPL, a rabbit anti-chicken IgG and ¹²⁵I-protein A as described previously [2]. To determine the relative amount of LPL protein, the band corresponding to LPL was cut out from a membrane and its radioactivity was determined in an Aloka ARC-600 γ -counter. Results are expressed as the percentage of radioactivity in the LPL of each fraction to that in the LPL of all fractions. The net counts in LPL were obtained by subtracting the counts in background from the counts in the band corresponding to LPL.

Chemical analysis

DNA was measured fluorometrically by the method of Hinegardner [19] with calf thymus DNA as the standard. Triacylglycerol was measured with a kit for triacylglycerol from Wako Pure Chemicals Co. (Osaka, Japan).

RESULTS

Effect of chlorate on DNA and triacylglycerol content

The confluent 3T3-L1 fibroblasts were stimulated for 2 days to allow differentiation into adipocytes. The DNA content of the cultures doubled during this 2-day stimulation, and then further increased gradually over the next 7 days. The presence of chlorate in the medium caused a decrease in the DNA content of the cultures (Table 1). The DNA content in the cultures of chlorate-treated cells was 91 % of that of control cells on day 2 of the treatment, and 85 % on day 7. There was no difference in the triacylglycerol content of the cultures of control and chlorate-treated cells (Table 1).

Effect of chlorate on the development of LPL activity

3T3-L1 adipocytes were cultured in a medium containing either 0 or 20 mM chlorate, and LPL activity was measured in the extract of acetone/ether-dried powder of the cells and in the medium. The 2-day treatment of cells with chlorate caused a 29 % increase in cellular LPL activity and a 485 % increase in LPL activity in the medium, whereas the 7-day treatment caused a 55 % decrease in cellular LPL activity and a 66 % decrease in LPL activity in the medium (Table 2).

Next the cells were cultured for 7 days in the medium containing 0–20 mM chlorate, and LPL activity was measured in the extract of acetone/ether-dried powder of the cells and in the medium. Treatment of cells with chlorate caused a dose-dependent decrease in LPL activity (Figure 1). The decrease in LPL activity in the medium was greater than that in cellular LPL activity.

To examine whether the chlorate-induced decrease in LPL activity was reversible, the plates of the cells cultured for 7 days in the medium containing 20 mM chlorate were replenished with a chlorate-free medium, after which the cells were cultured for an

Figure 1 Dose dependence on chlorate of the decrease in LPL activity

3T3-L1 adipocytes were cultured for 7 days in medium containing 0–20 mM chlorate. The plates were replenished with a fresh medium containing the appropriate additive; the cells were then incubated for 30 min at 37 °C. Cells were harvested, sonicated briefly at 0 °C and processed into acetone/ether-dried powder. Cellular LPL activity was measured in the extract of the powder (○). The medium was filtered through a 0.2 µm filter and the LPL activity in an aliquot of the medium was measured (●). Cellular LPL activity in control cells and LPL activity in the medium of control cells were 3484 ± 108 and 183 ± 9 m-units/mg of DNA respectively. Values are means $\pm$ S.D. for three plates.

additional 4 days. The increase in cellular LPL activity of the cells pretreated with chlorate was greater than that of the control cells, and 4 days after removing the chlorate the cellular LPL activity reached the same level as that of the control cells (Figure 2A). The LPL activity in the medium of cells pretreated with chlorate also recovered similarly (Figure 2B). Thus the decreased cellular LPL activity and the decreased LPL activity in the medium were restored by removing the chlorate. Therefore we

Table 1 Effect of chlorate on the DNA and triacylglycerol content of the cultures

3T3-L1 fibroblasts were plated in 60 mm plates and cultured in a standard medium. When cells became confluent (–2 days) the medium was replaced with a standard medium supplemented with 1 µM dexamethasone, 10 µg/ml insulin and 0.5 mM methylisobutylxanthine. The medium was replaced with a complete medium containing either 0 or 20 mM chlorate 2 days later (0 days) and was replenished every 2 days. At the indicated intervals, cells were washed with PBS, harvested and sonicated briefly at 0 °C. The DNA and triacylglycerol contents of the homogenates were measured. Values are means $\pm$ S.D. for three plates. * $P < 0.05$; ** $P < 0.01$ (Student's t test, compared with control cells).

Cells	Time (days) ...	DNA content (µg per plate)					Triacylglycerol content (µg/µg of DNA)				
		–2	0	2	4	7	–2	0	2	4	7
Control		14.9 $\pm$ 1.3	30.0 $\pm$ 1.5	50.1 $\pm$ 0.6	60.6 $\pm$ 1.8	66.3 $\pm$ 2.9	1.08 $\pm$ 0.17	1.56 $\pm$ 0.10	5.41 $\pm$ 0.17	11.00 $\pm$ 0.36	16.71 $\pm$ 0.86
Chlorate-treated		–	–	45.4 $\pm$ 0.6**	49.2 $\pm$ 4.3*	56.5 $\pm$ 3.3*	–	–	5.34 $\pm$ 0.06	11.66 $\pm$ 0.42	15.88 $\pm$ 2.04

Table 2 Effect of chlorate on the development of LPL activity in cells and medium

3T3-L1 fibroblasts were plated in 60 mm plates and cultured in a standard medium. When cells became confluent (–2 days) the medium was replaced with a standard medium supplemented with 1 µM dexamethasone, 10 µg/ml insulin and 0.5 mM methylisobutylxanthine. The medium was replaced with a complete medium containing either 0 or 20 mM chlorate 2 days later (0 days) and was replenished every 2 days. At the indicated intervals, the plates were replenished with a fresh complete medium containing the appropriate additive, and cells were incubated for 30 min at 37 °C. The medium was filtered through a filter with a pore size of 0.2 µm and used for the prompt assay of LPL. Cells were harvested and sonicated briefly at 0 °C; an aliquot of the homogenate was used to make an acetone/ether-dried powder. Cellular LPL activity was measured in the extract of the powder. Values are means $\pm$ S.D. for three plates. * $P < 0.05$; ** $P < 0.01$ (Student's t test, compared with control cells).

Cells	Time (days) ...	LPL activity in cells (m-units/mg of DNA)					LPL activity in medium (m-units/mg of DNA)				
		–2	0	2	4	7	–2	0	2	4	7
Control		270 $\pm$ 98	247 $\pm$ 69	1845 $\pm$ 36	1789 $\pm$ 157	2426 $\pm$ 156	5 $\pm$ 4	6 $\pm$ 3	52 $\pm$ 1	61 $\pm$ 3	59 $\pm$ 6
Chlorate-treated		–	–	2379 $\pm$ 154*	1890 $\pm$ 70	1087 $\pm$ 146**	–	–	304 $\pm$ 7**	143 $\pm$ 31*	20 $\pm$ 2**

Figure 2 Recovery of LPL activity after removal of chlorate

3T3-L1 adipocytes were cultured for 7 days in a medium containing either 0 (○) or 20 (●) mM chlorate. On day 0 the medium was replaced with a chlorate-free complete medium and then changed every 2 days. At the indicated intervals the culture medium was replaced with fresh chlorate-free complete medium and the cells were incubated for 30 min at 37 °C. The medium was then separated from cells, filtered through a 0.2 µm filter and used for the prompt assay of LPL activity. Cells were harvested, sonicated briefly at 0 °C and processed into acetone/ether-dried powder. LPL activity was measured in the extract of acetone/ether-dried powder of cells (A) and in the medium (B). Values are means ± S.D. for three plates. * $P < 0.01$ (Student's t test, compared with control cells).

used cells cultured for 7 days in the presence of 20 mM chlorate as chlorate-treated cells in the following experiments.

Effect of chlorate on LPL activity in the intracellular and cell-surface compartments and in the medium

Cellular LPL activity corresponds to the sum of LPL activity in the intracellular and cell-surface compartments. The LPL activity of the cell-surface compartment is released by heparin; the level of activity remaining in the cells after heparin treatment corresponds to that of the intracellular compartment. Treatment of cells with chlorate decreased the levels of LPL activity in the intracellular compartment by 55%. In the presence of heparin, the release of LPL activity into the media of control and chlorate-treated cells increased 4.0-fold and 3.5-fold respectively but the level of the heparin-releasable LPL activity of chlorate-treated cells was 21% of that of control cells (Table 3).

Both control and chlorate-treated cells spontaneously secreted LPL into the medium. The level of LPL activity in the medium of chlorate-treated cells was 25% of that in the medium of control cells (Table 3).

Effect of chlorate on the half-life of LPL activity in the cells and the medium

To examine the effect of chlorate on the half-life of cellular LPL activity, 3T3-L1 adipocytes were incubated with 15 µM cycloheximide and 3 µg/ml heparin for 0–90 min and LPL activity in the extract of acetone/ether-dried powder of cells was measured. Addition of cycloheximide/heparin to the medium of control cells caused a rapid decrease in cellular LPL activity, with a half-

Table 3 Effect of chlorate on LPL activity in the intracellular and cell-surface compartments and the medium

3T3-L1 adipocytes were cultured for 7 days in a medium containing either 0 or 20 mM chlorate. The medium was removed and replaced with 1.5 ml of ice-cold medium containing either 0 and 20 µg/ml heparin. Cells were then incubated for 15 min on crushed ice. An aliquot of the medium was taken for assay of LPL activity. Cells were harvested, sonicated at 0 °C and processed into acetone/ether-dried powders. Values are means ± S.D. for three or four plates, and are representative of two independent experiments. * $P < 0.05$; ** $P < 0.01$ (Student's t test, compared with LPL activity measured in the absence of heparin). Heparin-releasable LPL activity is defined as (LPL activity in the medium with heparin) – (LPL activity in the medium without heparin).

Cells	Heparin (µg/ml)	LPL activity (m-units/mg of DNA)		
		In cells	In medium	Heparin-releasable
Control	0	3030 ± 93	138 ± 2	
	20	2834 ± 44*	553 ± 24**	415
Chlorate-treated	0	1359 ± 187	34 ± 5	
	20	1279 ± 74	120 ± 13**	86

life of 38 min (Figure 3A). In chlorate-treated cells, cellular LPL activity did not change during the first 15 min of the cycloheximide/heparin treatment but then decreased following a first-order kinetic process. The half-life of the LPL activity was 51 min (Figure 3A).

To determine whether the decrease in the LPL activity observed in the medium of chlorate-treated cells resulted from the decreased stability of LPL in the medium, the medium was separated from cells and filtered, then incubated again at 37 °C. Aliquots of the media were taken at the indicated intervals for assay of LPL activity. The inactivation of LPL followed a first-order kinetic process. The half-life (17 min) of the LPL activity in the medium of chlorate-treated cells was identical with that in the medium of control cells (Figure 3B).

Effect of chlorate on the synthesis and secretion of ³⁵S-labelled LPL

3T3-L1 adipocytes cultured for 7 days in a medium containing either 0 or 20 mM chlorate were incubated for 1 h with [³⁵S]methionine, after which the amount of radioactivity incorporated into the total protein was measured. The chlorate-treated cells incorporated more radioactivity into the total protein than the control cells [control cells ($n = 3$), $(3.39 \pm 0.08) \times 10^8$ c.p.m./mg of DNA; chlorate-treated cells ($n = 3$), $(4.50 \pm 0.09) \times 10^8$ c.p.m./mg of DNA; $P < 0.01$]. This finding indicated that chlorate-treated cells synthesize larger amounts of proteins than control cells.

³⁵S-labelled LPL was immunoprecipitated with a chicken anti-serum against LPL from the extract of cells incubated for 1 h with [³⁵S]methionine and was resolved by SDS/PAGE. Both control and chlorate-treated cells contained two groups of LPL subunits with molecular masses of 53–55 and 55–58 kDa (Figure 4). There was no difference in total radioactivity in both subunits between the control and chlorate-treated cells [control cells ($n = 3$), $(2.60 \pm 0.71) \times 10^4$ c.p.m./mg of DNA; chlorate-treated cells ($n = 3$), $(2.88 \pm 0.21) \times 10^4$ c.p.m./mg of DNA].

A component with a molecular mass of 36–37 kDa was also immunoprecipitated from the cell extract with a chicken anti-serum against LPL (Figure 4), suggesting that LPL was cleaved proteolytically. The production of the degraded component was greater in chlorate-treated cells than in control cells.

To analyse the types of oligosaccharide chain of the LPL subunits, ³⁵S-labelled LPL was digested with endo H and resolved

Figure 3 Effect of chlorate on the half-life of LPL activity in cells (A) and medium (B)

(A) The plates of 3T3-L1 adipocytes cultured for 7 days in a medium containing either 0 or 20 mM chlorate were washed once with PBS and then replenished with 1.5 ml of a medium containing the appropriate additive. After the addition of 15 μ M cycloheximide and 3 μ g/ml heparin to each plate, cells were incubated at 37 °C, harvested at the indicated intervals, sonicated briefly at 0 °C and processed into acetone/ether-dried powder. LPL activity was measured in the extract of acetone/ether-dried powder of cells. The LPL activity is shown as a percentage of the initial LPL activity. The initial LPL activity was 1827 $\pm$ 37 m-units/mg of DNA in control cells and 605 $\pm$ 30 m-units/mg of DNA in chlorate-treated cells. Values are means $\pm$ S.D. for four plates. (B) The plates of 3T3-L1 adipocytes cultured for 7 days in a medium containing either 0 or 20 mM chlorate were washed once with PBS and then replenished with 1.5 ml of a medium containing the appropriate additive. Cells were incubated for 30 min at 37 °C and the medium was then separated from cells and filtered through a 0.2 μ m filter. The filtered medium was incubated again at 37 °C. LPL activity in an aliquot of the medium was measured at the indicated intervals. The LPL activity is shown as a percentage of the initial LPL activity. The initial LPL activity was 107 $\pm$ 20 m-units/mg of DNA in the medium of control cells and 24 $\pm$ 5 m-units/mg of DNA in the medium of chlorate-treated cells. Values are means $\pm$ S.D. for four plates.

by SDS/PAGE. As did the control cells, chlorate-treated cells contained three types of LPL subunit (Figure 5). One type was endo H-resistant with a molecular mass of 55–58 kDa; the second was partly endo H-sensitive, yielding a 52 kDa product; the third was totally endo H-sensitive, yielding a 50 kDa product. The relative amounts of the digestion products were estimated densitometrically. In control cells, 53 % of the LPL was totally endo H-sensitive and 25 % was endo H-resistant. In chlorate-treated cells, 76 % of the LPL was totally endo H-sensitive and 11 % was endo H-resistant (Table 4). There was no significant difference in the proportion of LPL subunits that were partly endo H-sensitive between control and chlorate-treated cells.

As did the control cells, chlorate-treated cells secreted LPL subunits with a molecular mass of 55–58 kDa (Figure 4). The amount of 35 S-labelled LPL secreted by the chlorate-treated cells was identical with that secreted by the control cells [control cells ($n = 3$), $(10.2 \pm 1.7) \times 10^2$ c.p.m./mg of DNA; chlorate-treated cells ($n = 3$), $(9.9 \pm 0.4) \times 10^2$ c.p.m./mg of DNA]. LPL subunits in the medium of both control and chlorate-treated cells were resistant to endo H digestion (results not shown).

Figure 4 Effect of chlorate on synthesis and secretion of LPL

(A) The plates of 3T3-L1 adipocytes cultured for 7 days in a medium containing either 0 or 20 mM chlorate were washed twice with PBS and then replenished with 1.5 ml of methionine-deficient DMEM containing the appropriate additive. After 30 min, 160 μ Ci of [35 S]methionine was added; the cells were then incubated for 1 h at 37 °C. Cells were harvested, sonicated and centrifuged. 35 S-labelled LPL was immunoprecipitated from the cell extract with a chicken antiserum against bovine LPL and was resolved by SDS/PAGE. The results are representative of three plates of each group. (B) 35 S-labelled LPL was immunoprecipitated from the medium of cells incubated for 1 h with [35 S]methionine with a chicken antiserum against bovine LPL and was resolved by SDS/PAGE. The results are representative of three plates of each group. In both panels the molecular masses of LPL subunits (in kDa) are indicated.

Chromatography of LPL on a heparin–Sephacrose column

LPL in the extracts of control and chlorate-treated cells was chromatographed on the heparin–Sephacrose column (Figure 6). Proteins in each fraction eluted from a heparin–Sephacrose column were separated by SDS/PAGE, transferred to a nitrocellulose membrane and blotted with a chicken antiserum against bovine LPL, a rabbit anti-chicken IgG and 125 I-protein A (Figure 7). 3T3-L1 adipocytes contained three forms of LPL. The first form, accounting for 35 % of the LPL, did not bind to the column and had little or no activity. The second form, accounting for 32 %, bound to the column and was eluted with 0.4–0.75 M NaCl but had no activity. The third form, accounting for 33 %, bound to the column, was eluted with 0.8–1.2 M NaCl and had activity (Figure 6A). In chlorate-treated cells the first form accounted for 66 % of the LPL, the second form 15 % and the third form 19 % (Figure 6B).

Effect of brefeldin A (BFA) on LPL activity in control and chlorate-treated cells

To determine whether Golgi enzymes are normal in chlorate-treated cells, cells were incubated with BFA, which is known to translocate Golgi proteins to the ER [20,21], and LPL activity in the extract of acetone/ether-dried powder of cells was measured. Chlorate-treated cells contained 1162 $\pm$ 92 m-units of LPL activity/mg of DNA, 47 % of that in control cells. BFA increased in 1 h the LPL activity in chlorate-treated cells to 2293 $\pm$ 187 m-units/mg of DNA, 92 % of that in untreated control cells (Table 5). Thus the chlorate-induced decrease in cellular LPL activity

(A) Control cells

(B) Chlorate-treated cells

Figure 5 Digestion with endo H of ^{35}S -labelled LPL extracted from control and chlorate-treated cells

^{35}S -labelled LPL immunoprecipitated with a chicken antiserum against bovine LPL, from extracts of control (A) and chlorate-treated (B) cells that had been incubated for 1 h with [^{35}S]methionine, was digested with endo H; the digestion products were resolved by SDS/PAGE. The relative amounts of LPL subunits (molecular masses indicated in kDa) were estimated densitometrically with a dual-wavelength flying-spot scanner. The results are representative of three plates of each group.

Table 4 Effect of chlorate on glycosylation of LPL

3T3-L1 adipocytes were cultured for 7 days in a complete medium containing either 0 or 20 mM chlorate. The plates were washed twice with PBS and replenished with 1.5 ml of methionine-deficient DMEM containing the appropriate additive. After 30 min, 160 μCi of [^{35}S]methionine was added and the plates were incubated for 1 h at 37 °C. ^{35}S -labelled LPL was immunoprecipitated with a chicken antiserum against bovine LPL from the cell extracts and digested with endo H; the digestion products were resolved by SDS/PAGE. The relative amounts of three subunits were estimated densitometrically. The typical densitometric pattern of the endo H digestion products of LPL are shown in Figure 4. Values are means $\pm$ S.D. for three plates. * $P < 0.05$ (Student's t test, compared with control cells).

Cells	Relative amounts of subunits (%)		
	50 kDa	53–55 kDa	55–58 kDa
Control	53 $\pm$ 2	21 $\pm$ 3	25 $\pm$ 5
Chlorate-treated	76 $\pm$ 7*	10 $\pm$ 8	11 $\pm$ 5*

was restored by adding BFA to the medium. BFA also increased cellular LPL activity of control cells by 53%.

DISCUSSION

We have studied the effect of long-term treatment of 3T3-L1 adipocytes with chlorate, an inhibitor of sulphation of HSPG [13–17], on the synthesis, glycosylation, activity, intracellular transport and secretion of LPL. LPL is located in three compartments of the culture: the intracellular and cell-surface compartments and the medium. In the cultures of 3T3-L1

Figure 6 Heparin-Sepharose column chromatography of LPL in control and chlorate-treated cells

3T3-L1 adipocytes cultured for 7 days in a medium containing either 0 (A) or 20 (B) mM chlorate were harvested, sonicated and centrifuged. A sample of 0.95 ml of the combined infranantants from two plates was applied to a heparin-Sepharose column equilibrated with 0.3 M NaCl in solution A. Proteins were eluted with a linear gradient of NaCl (0.3–2.0 M) in solution A. The level of LPL activity ($\circ$) in 20 μl of each fraction was measured. Proteins in another aliquot of each fraction were separated by SDS/PAGE, transferred to a nitrocellulose membrane and blotted as described in the Materials and methods section. The bands corresponding to LPL were cut out and their radioactivity was measured. Results are the radioactivity in the LPL of each fraction expressed as a percentage of that in the LPL of all fractions ($\bullet$). The conductivity of each fraction was measured and translated into NaCl molarity by comparison with NaCl standards in solution A ($\blacklozenge$).

adipocytes on day 9 of confluence, 84% of LPL activity was in the intracellular compartment, 12% was in the cell-surface compartment and 4% was in the medium. The 7-day treatment of cells with chlorate caused a 55% decrease in LPL activity in the intracellular compartment, a 79% decrease in the cell-surface compartment and a 75% decrease in the medium.

The study with a heparin-Sepharose column showed that 3T3-L1 adipocytes contained three forms of LPL: one that did not bind to the column and was inactive, a second that bound to the column and was eluted with 0.4–0.75 M NaCl but was inactive, and a third that bound to the column was eluted with 0.8–1.2 M NaCl and was active. Inactive LPL, which had a lower affinity for heparin, accounted for 67% of cellular enzyme protein in control cells and 81% of that in chlorate-treated cells. There was no difference in the synthetic rate of LPL between control and chlorate-treated cells as determined by biosynthetic labelling. Thus the chlorate-induced decrease in cellular LPL activity resulted from an increased production of inactive LPL with a lower affinity for heparin rather than a decreased synthetic rate

Control cells

Chlorate-treated cells

Figure 7 Western blots of LPL in fractions eluted from heparin-Sepharose

Proteins in each fraction eluted from heparin-Sepharose in Figure 6 were separated by SDS/PAGE, transferred to a nitrocellulose membrane and blotted with a chicken antiserum against bovine LPL, a rabbit anti-chicken IgG and 125 I-protein A.

Table 5 Effect of BFA on LPL activity in control and chlorate-treated cells

3T3-L1 adipocytes were cultured for 7 days in a medium containing either 0 or 20 mM chlorate. The plates were replenished with 2.5 ml of a medium containing the appropriate additive; the cells were then incubated for 1 h at 37 °C in the absence or presence of 1 μ g/ml BFA. Cells were harvested, sonicated briefly at 0 °C and processed into acetone/ether-dried powder. LPL activity was measured in the extract of acetone/ether-dried powder of cells. Values are means $\pm$ S.D. for four plates. * $P < 0.01$ (Student's t test, compared with LPL activity in control cells incubated in the absence of BFA).

Cells	BFA (μ g/ml)	LPL activity (m-units/mg of DNA)
Control	0	2495 $\pm$ 181
	1	3815 $\pm$ 122*
Chlorate-treated	0	1162 $\pm$ 92*
	1	2293 $\pm$ 187

of enzyme protein. There are many reports that active LPL is a non-covalently bound homodimer with a high affinity for heparin and that dissociation of the dimeric form of LPL to the monomeric form results in loss of activity and low affinity for heparin [2,22–26]. These results and our present findings indicate that chlorate might impair the formation of dimers of LPL.

The expression of activity and intracellular transport and the acquisition of a high affinity for heparin of LPL involve post-translational modification [3–10,27,28], as well as LPL gene transcription and mRNA processing and translation. LPL in brown adipocytes of combined lipase-deficient *cld/cld* mice is inactive and totally endo H-sensitive, has a lower affinity for heparin and is retained in the ER [7]. Recently, Park et al. [29] demonstrated, using a zonal centrifugation technique, that approx. 60% of the LPL in brown adipocytes of this mutant mice is aggregates of LPL monomers, perhaps higher oligomers. Vannier et al. [3] reported that carbonyl cyanide *m*-chlorophenylhydrazone, which blocks the budding process involved in the formation of vesicles that carry secretory proteins from the ER to the Golgi [30–32], caused the accumulation of inactive LPL in the ER. Studies with tunicamycin, an inhibitor of N-linked glycosylation in the ER [33], and castanospermine, an inhibitor of glucosidase I in the ER [34], also showed that LPL

retained in the ER is inactive and has a lower affinity for heparin [8,9,35]. That most of LPL in chlorate-treated cells was inactive and totally endo H-sensitive and had a lower affinity for heparin suggests that chlorate might block the transport of LPL from the ER to the Golgi and cause the accumulation of inactive LPL in the ER.

BFA, which is known to block the transport of proteins from the ER to the Golgi, to cause disassembly of the Golgi complex, and to translocate Golgi glycosidases to the ER [20,21,36–38], increased LPL activity in brown adipocytes from *cld/cld* mice or castanospermine-treated brown adipocytes from normal mice [29]. Similarly the chlorate-induced decrease in cellular LPL activity was restored by adding BFA to the medium. The level of cellular LPL activity of chlorate-treated cells incubated with BFA was identical with that of control cells incubated without BFA. BFA completely blocks the secretion of LPL by adipocytes [10,27,29]. This was confirmed by the present study (results not shown). This result raises the possibility that an increase in LPL activity in chlorate-treated cells treated with BFA resulted from a blockade of secretion of active LPL. However, the observation that LPL activity secreted for 1 h by chlorate-treated cells was very low [LPL activity secreted by control cells ($n = 3$), 229 $\pm$ 23 m-units/mg of DNA; LPL activity secreted by chlorate-treated cells ($n = 3$), 43 $\pm$ 5 m-units/mg of DNA] indicates that the BFA-induced increase in LPL activity in chlorate-treated cells did not result solely from an accumulation of active LPL. These findings indicate that LPL synthesized in chlorate-treated cells can be processed to be fully active, but chlorate-treated cells are unable to transport LPL to the Golgi.

Mouse LPL is a glycoprotein with two complex type oligosaccharide chains that is resistant to endo H digestion [6–10,35]. Glycosylation is initiated by transfer of the common precursor oligosaccharide, Glc₃Man₉GlcNAc₂, to newly synthesized protein in the ER, and after the removal of three glucose residues and six of nine mannose residues in the ER and the *cis*- and medial Golgi, the endo H-sensitive oligosaccharide chains are processed to the endo H-resistant type by the addition of sugars, such as galactose and sialic acid, in the *trans*-Golgi. That chlorate decreased the proportion of LPL subunits that were endo H-resistant indicates that transport of LPL to the *trans*-Golgi was impaired in chlorate-treated cells. The possibility that chlorate inhibits the activity of glycosidases in the *cis*- and medial Golgi

can be excluded because LPL in chlorate-treated cells incubated with BFA became fully active. However, the effect of chlorate on the activity of glycosidases in the *trans*-Golgi remains to be resolved. To our knowledge there is no report that HSPG is necessary for the localization of glycosidases in the Golgi or for the stability of their activity.

Olivecrona et al. [4] reported that much of the LPL secreted spontaneously by 3T3-L1 adipocytes was inactive. However, the site of inactivation of LPL has been unknown. Vannier et al. [3] reported, but did not prove, that the degradation of LPL takes place after discharge of enzyme molecules from the *trans*-Golgi into transport vesicles. In the present study we found that chlorate caused a marked decrease in LPL activity in the medium without a decreased secretion rate of LPL, but there was no difference in the half-life of LPL activity present in the medium between control and chlorate-treated cells. Thus the chlorate-induced decrease in LPL activity in the medium did not result from the decreased stability of LPL in the medium. That LPL subunits in the media of both control and chlorate-treated cells were endo H-resistant indicates that their oligosaccharide chains had been normally processed in the Golgi. Together the results suggest that LPL might be inactivated near or at the cell surface.

Chlorate has been used to inhibit the sulphation of HSPG [14,15]. The present study showed that the effect of chlorate on cellular LPL activity and LPL activity in the medium depends on the duration of treatment. The short-term (48 h) treatment of 3T3-L1 adipocytes with chlorate increased LPL activity in the medium but decreased heparin-releasable LPL activity on the cell surface (results not shown). These results agree well with those of Hoogewerf et al. [15]. They demonstrated that the chlorate-induced increase in LPL secretion resulted from a blockade of sulphation of HSPG on the cell surface. However, the long-term (7 days) treatment of 3T3-L1 adipocytes with chlorate decreased LPL activity in all compartments of the culture: the intracellular and cell-surface compartments and the medium. The discrepancies between the results obtained from the short-term and long-term treatments with chlorate remain to be resolved. One possibility is that longer periods of chlorate treatment are needed to influence the intracellular component other than HSPG, such as a component that contributes to the activation of LPL. Park et al. [29] reported that activation of LPL might require some modification in the Golgi other than the processing of oligosaccharide chains, such as phosphorylation, proteolysis and acylation.

LPL is synthesized and glycosylated in the ER and then sequentially transported through the *cis*-, medial and *trans*-Golgi to the cell surface, where it binds to HSPG by electrostatic interaction. However, the intracellular transport system of LPL has been unknown. The ability of LPL to bind to HSPG is acquired in the ER because the LPL transported from the ER has a high affinity for heparin [35], whereas the ability of HSPG to bind to LPL is acquired in the Golgi because sulphation of proteoglycans occurs in the *cis*- and *trans*-Golgi [39]. On the basis of these findings we supposed that LPL bound to HSPG in the Golgi and a complex of LPL with HSPG was transported to the cell surface. Thus the binding of LPL to HSPG via electrostatic interaction might be necessary for the intracellular transport of LPL.

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