The effects of chemically modifying serum apolipoproteins on their ability to activate lipoprotein lipase

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1. Lipoprotein lipase activity was measured in an acetone-dried-powder preparation from rat epididymal adipose tissue using pig serum or pig serum lipoprotein, which had been chemically modified, as activator. 2. Modification of acidic amino acids of lipoproteins with *NN*-dimethyl-1,3-diamine resulted in a complete loss of ability to activate lipoprotein lipase. 3. Modification of 34% of lipoprotein arginine groups with cyclohexanedione resulted in the loss of 75% of the activation of lipoprotein lipase; approx. 42% of the original activation was recovered after reversal of the modification. This effect was dependent on the cyclohexanedione concentration. 4. Modification of 48% of lipoprotein lysine groups with malonaldehyde decreased the maximum activation by 20%, but three times as much lipoprotein was required to achieve this. 5. Non-enzymic glycosylation of lipoprotein with glucose, under a variety of conditions resulting in up to 28 nmol of glucose/mg of protein, had no effect upon the ability to activate lipoprotein lipase. In contrast non-enzymic sialylation resulted in a time-dependent loss of up to 60% of ability to activate lipoprotein lipase. 6. Reductive methylation and acetoacetylation of serum did not affect the ability to activate lipoprotein lipase. 7. The results are compared to the effects of similar modifications to low density lipoproteins on receptor-mediated endocytosis.

INTRODUCTION

The lipoprotein apoproteins play an important role in several metabolic reactions. Apolipoprotein-CII (apo-CII) is required to activate lipoprotein lipase (triacylglycerol-protein acylhydrolase, EC 3.1.1.34), an enzyme involved in the removal of triacylglycerol-rich lipoproteins from blood (Havel *et al.*, 1980). By contrast, apo-CIII appears to inhibit lipoprotein lipase (Brown & Baginsky, 1972). Apo-B (Shireman *et al.*, 1977) and apo-E (Pitas *et al.*, 1979) are necessary for the interaction of low-density lipoproteins (LDL) with cell-surface receptors that mediate endocytosis of these cholesterol-rich lipoproteins. Apo-AI is required for the activity of lecithin:cholesterol acyltransferase (phosphatidylcholine:sterol *O*-acyltransferase; EC 2.3.1.43) (Chung *et al.*, 1979).

Several chemical modifications of lipoprotein apoproteins have resulted in the inhibition of receptor-mediated endocytosis. These have included modifications of lysine residues by reductive methylation, acetoacetylation, carbamylation (Weisgraber *et al.*, 1978) or acetylation (Basu *et al.*, 1976), modification of arginine residues with cyclohexanedione (Mahley *et al.*, 1977), modification of carboxy groups by attaching a cationic group (Basu *et al.*, 1976) and cross-linking lysine residues with malonaldehyde (Haberland *et al.*, 1982). Lipoprotein apoproteins may also be modified by non-enzymic glycosylation *in vivo* or *in vitro* (Schleicher *et al.*, 1981; Sasaki & Cottam, 1982; Kim & Korup, 1982). Schleicher *et al.* (1981) found higher concentrations of glycosylated lipoprotein in diabetic than in non-diabetic patients.

In view of the derangements in lipid metabolism, particularly the occurrence of increased serum lipoprotein concentrations, associated with diabetes mellitus (Nikkila & Kekki, 1973; Howard *et al.*, 1978; Taskinen & Nikkila, 1979), and in view of the role of lipoprotein lipase in clearing triacylglycerols from the blood, the effects of a number of chemical modifications of serum lipoproteins, including non-enzymic glycosylation, upon their ability to activate lipoprotein lipase were investigated.

MATERIALS AND METHODS

Materials

Benzylpenicillin, streptomycin sulphate, *N*-acetylneuraminic acid (AcNeu), NaBH₄, sodium cyanoborohydride, casein (purified powder), trioleoylglycerol, phosphatidylcholine, guanidinium chloride and mercaptoacetic acid were obtained from Sigma Chemical Co., Poole, Dorset, U.K. Malonaldehyde dimethylacetal, cyclohexanedione, 1-ethyl-3-(3-dimethylaminopropyl)-carbodi-imide hydrochloride, *NN*-dimethylpropane-1,3-diamine, diketene and 2,4-dinitrofluorobenzene were obtained from Aldrich Chemical Co. Ltd., Gillingham, Dorset, U.K. Hydroxylamine, mannitol, L-fructose, D-glucose, thiobarbituric acid, oxalic acid, SDS and Cocktail T [a liquid-scintillation cocktail containing 2,5-diphenyloxazole and 1,4-bis-(5-phenyloxazol-2-yl)-benzene in Triton X-100/toluene (1:2, v/v)] were obtained from BDH Chemicals, Poole, Dorset, U.K. Tri[9,10(n)-³H]oleoylglycerol was obtained from Amer-

Abbreviations used: apo-, apolipoprotein-; LDL, low-density lipoprotein; AcNeu, *N*-acetylneuraminic acid; HDL, high-density lipoprotein.

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sham International, Amersham, Bucks., U.K. and further purified by t.l.c. on silica gel before use.

Separation of lipoproteins

Venous blood was obtained from pigs (*Sus scrofa*, Large White $\times$ Landrace cross) which had been fasted overnight. The blood was allowed to clot for 3 h at room temperature and serum prepared by centrifuging the blood at 850 *g* for 30 min at 4 °C. The supernatant serum was used either directly as an activator in lipoprotein lipase assays or for the preparation of the lipoprotein fraction as follows. The density of the serum was adjusted to 1.21 g/ml by the addition of solid KBr and 16 ml aliquots were overlaid with 3.2 ml of KBr solution [density (ρ) = 1.21 g/ml] and centrifuged at 150 000 *g* at 12 °C for 45 h. The top 3 ml, containing the total lipoprotein, were removed from each tube and dialysed against 250–300 vol. of 0.15 M-NaCl/0.01% EDTA, pH 7.4, for two 24 h periods. Aliquots of the lipoprotein (1.1 ml) were transferred to plastic vials and stored at –15 °C. The concentration of lipoprotein in the solution was approx. 4.35 times greater than in serum.

Modifications of lipoproteins and serum

The modification of carboxylic acid groups on lipoproteins to cationic groups was performed as follows (Basu *et al.*, 1976). To 1-ethyl-3-(3-dimethylamino-propyl)carbodi-imide hydrochloride (100 mg) was added 2 M-*NN*-dimethylpropane-1,3-diamine, pH 6.5 (1 ml) and lipoprotein solution (1 ml). There was no appreciable change in pH, and after 2–3 h at room temperature the reaction mixture was stored at 4 °C for 16 h. The same procedure was followed for the control, except that the carbodi-imide was omitted. At the end of the incubation the solutions were dialysed against at least 100 vol. of 0.15 M-NaCl/0.01% EDTA, pH 7.0, at 4 °C with three changes of dialysis medium over 24 h. Aliquots of the samples were then stored at –15 °C.

Modification of arginine groups on lipoproteins with cyclohexanedione was carried out by the method of Mahley *et al.* (1977). Lipoprotein solution (containing 6.1 mg of protein/ml) (1 vol.) and 2 vol. of the chosen concentration of cyclohexanedione (up to 0.9 M; controls contained no cyclohexanedione) in 0.2 M-sodium borate/HCl buffer, pH 8.1, were mixed and incubated at 35 °C for 2 h then dialysed as described for the 'cationized' preparations. A portion of the dialysis residue was stored as described above and the remainder used to regenerate the free arginine groups, by incubating with an equal volume of 1 M-hydroxylamine/0.3 M-mannitol, pH 7.0, at 36 °C for 16 h. These solutions were also dialysed and stored as described above.

Malonaldehyde-cross-linked lipoproteins were prepared by the method of Haberland *et al.* (1982). Malonaldehyde was generated from malonaldehyde dimethylacetal (0.165 ml) by treating it with 12 M-HCl (0.2 ml) for 5 min at room temperature, after which 4.5 ml of 0.1 M-sodium phosphate buffer, pH 6.4, were added. The pH was adjusted to 6.4 with 10 M-NaOH and 1 ml of this malonaldehyde solution was added to 1 ml of lipoprotein solution in a stoppered tube. As a control, the phosphate buffer was substituted for the malonaldehyde solution. The mixtures were incubated for 3 h at 37 °C then dialysed and stored as described above.

Reductive methylation of serum proteins was by the

method of Weisgraber *et al.* (1978). To 15 mg of NaBH₄ in a capped tube, cooled in an ice/salt mixture to 0 °C, was added 2.25 ml of diluted serum (2 vol. of serum to 1 vol. of 0.3 M-sodium borate/HCl buffer, pH 9.0). Aq. 37% (v/v) formaldehyde (15 μ l) was added, followed by five further 15 μ l additions at 6 min intervals. After each addition the tube was centrifuged at 2000 rev./min at 4 °C to reduce foaming. In a control reaction NaBH₄ was omitted. The solution was then dialysed as before and stored at 4 °C before use.

Lysine groups of serum proteins were acetoacetylated by an adaptation of the method of Weisgraber *et al.* (1978). Serum (6 vol.) was mixed with 3 vol. of 0.3 M-sodium borate/HCl buffer, pH 8.5, and 1 vol. of diketene reagent (which had been made by diluting 0.5 ml of diketene to 10 ml with 0.1 M-sodium borate/HCl, pH 8.5). After 5 min at room temperature, the mixture was dialysed against 0.2 M-Na₂CO₃/NaHCO₃ buffer, pH 9.5, for 4 h at room temperature. The dialysis was repeated overnight in fresh buffer at 4 °C before changing to dialysis against 0.15 M-NaCl/0.01% EDTA, pH 7.0, overnight at 4 °C. The dialysis was repeated and the serum stored at 4 °C. Although this modification may be reversed (Weisgraber *et al.*, 1978), the procedure is not presented here in view of the absence of any effect of the original modification (see the Results section).

Glycosylation of lipoprotein preparations was carried out by a procedure modified from that described by Day *et al.* (1979). The reaction mixtures consisted of phosphate-buffered saline (made from 100 vol. of 0.154 M-NaCl and 21 vol. of 0.1 M-sodium phosphate buffer, pH 7.3) containing benzylpenicillin (100 units/ml), streptomycin sulphate (100 μ g/ml), glucose (200 mM) and lipoprotein (2.4 mg of protein/ml). For the preparation of sialylated lipoprotein, 140 mM-AcNeu replaced glucose. The incubations were maintained at 36–38 °C for up to 120 h. Control mixtures in which the glucose or AcNeu was replaced by water were incubated for the same period. In some incubations, as indicated in the Results section, lipoproteins were incubated with 400 mM-glucose and 207 mM-sodium cyanoborohydride for 72 h. In this case parallel incubations contained either no sodium cyanoborohydride, or no glucose and no sodium cyanoborohydride. The solutions were dialysed and stored as described above.

Preparation of acetone-dried powder of rat epididymal fat-pad

Epididymal fat-pads weighing 23 g in total were removed from four rats and homogenized, using a Teflon/glass homogenizer, at 4 °C in 80 ml of 50 mM-NH₃/HCl, pH 8.1, containing 2.5% (w/v) casein to act as a protein carrier. An acetone/diethyl-ether-dried powder, hereafter referred to as 'acetone powder' was prepared by the method of Salaman & Robinson (1966). The dried powder was divided into 100 mg portions and stored at –20 °C. Before each assay, 30–50 mg of acetone powder was rehomogenized in 10 ml of 50 mM-NH₃/HCl buffer, pH 8.1.

Activation of lipoprotein lipase by lipoproteins

The method was based on that used by Nilsson-Ehle & Schotz (1976). An emulsion of [³H]trioleoylglycerol was prepared in phosphatidylcholine and glycerol, giving a concentration of 60 mM-trioleoylglycerol (0.68 mCi/mmol). Before each assay a 'working solu-

tion' of [^3H]trioleoylglycerol was prepared by mixing 100 μl of 60 mM-trioleoylglycerol, 600 μl of 500 mM-Tris/HCl, pH 8.0, and 200 μl of bovine serum albumin (185 mg/ml). The standard assay mixture contained, in a volume of 200 μl , 45 μl of [^3H]trioleoylglycerol (working solution), 30 μl of bovine serum albumin (40 mg/ml), up to 60 μl of lipoprotein, diluted as necessary with 0.15 M-NaCl, and 50 μl of rehomogenized acetone powder, the balance being water. The final concentrations in the assay were, therefore, 1.5 mM-[^3H]trioleoylglycerol (0.68 mCi/mmol), 15.25 mg of bovine serum albumin/ml and 75 mM-Tris/HCl, pH 8.0. The reaction was started by the addition of lipoprotein, followed 5 s later by the acetone powder. This procedure prevented preincubation of the lipoprotein with the substrate, which can cause an inhibition of enzyme activity (Baginsky, 1981). The assay tubes were incubated at 37 °C for 30 min and stopped by the addition of 3.25 ml of chloroform/methanol/heptane (125:141:100, by vol.), followed by 1.05 ml of 0.1 M- K_2CO_3 /borate buffer, pH 10.5 (Belfrage & Vaughan, 1969). The mixture was agitated on a vortex mixer, the phases separated by centrifugation (3000 rev./min for 10 min), and 1 ml of the upper phase transferred to a counting vial to which was also added 100 μl of 2.5 M-HCl, 500 μl of water and 10 ml of Cocktail T. The radioactivity of the sample was determined by liquid-scintillation counting and corrected for quench by the sample-channels-ratio method. The result was also corrected for recovery of oleic acid into the top phase of the extraction, which had been determined to be 82% in a separate experiment.

When serum was to be used as the source of the lipoprotein lipase activator it was heated at 62 °C for 10 min to destroy any lipolytic activity (this had been shown to be unnecessary when lipoprotein was used). Serum was used in place of lipoprotein in the standard assay. The albumin content of the serum was assumed to be equivalent to 40 mg of bovine serum albumin/ml, so the bovine serum albumin content of the assay mixture was decreased to take account of this.

Protein estimation

The acetone powder and serum were assayed by a modified biuret method (Hübscher *et al.*, 1965). The protein content of the modified lipoprotein fraction was determined by the method of Lowry *et al.* (1951); the unmodified lipoprotein, whose protein content had been determined by the biuret method, was used as the standard. In both cases it was necessary to extract the lipid from the developed colour by using water-saturated diethyl ether.

Estimation of the extent of glycosylation

The extent of glucosylation of lipoproteins was measured by a modification of the method of Murtiashaw *et al.* (1983) in which 5-hydroxymethylfurfural liberated from glucosylated protein by reaction with oxalic acid was determined after reaction with thiobarbituric acid. In the modification used, the lipoprotein fraction contained 0.1% SDS and 6 M-guanidinium chloride. Complete solubilization of the mixture was achieved by heating at 100 °C for 60 s. The mixture was left at 37 °C for 12 h before being allowed to react with oxalic acid. The standard curve was constructed with fructose solutions (up to 40 nmol/assay), which also contained 0.1% SDS and 6 M-guanidinium chloride. Results were quoted as nmol of fructose equivalent/mg of protein.

Amino acid analysis

Approx. 0.5 mg samples of lipoprotein or serum were freeze-dried, then extracted with chloroform/methanol (2:1, v/v) to remove the lipid. Residual solvent was removed *in vacuo* and the protein residue stored under N_2 at -15 °C. The samples were hydrolysed with 1 ml of 6 M-HCl at 110 °C for 22 h. Amino acid analysis was carried out on a Biotronik LC5000 amino acid analyser (Biotronik Wissenschaftliche Geräte G.m.b.H., Munich, Germany) with a sodium citrate buffer programme.

The hydrolysis of cyclohexanedione-modified lipoproteins was carried out in the presence of 20 μl of mercaptoacetic acid to avoid regeneration of arginine (Mahley *et al.*, 1977).

Glycosylated lipoproteins were reduced with NaBH_4 before lipid extraction and hydrolysis in order to decrease their susceptibility to acid hydrolysis (Hurrell & Carpenter, 1974). To about 0.2 mg of solid NaBH_4 was added 0.2 ml of lipoprotein solution, containing about 0.5 mg of protein, and 0.2 ml of 0.4 M-sodium phosphate buffer, pH 7.4. The mixture was left at room temperature for 3 h, then dialysed as described above, under 'Modifications of lipoproteins and serum' before being freeze-dried and extracted as normal.

The amino acid composition of the lipoproteins or serum which had been modified by malonaldehyde or acetoacetate was determined by an indirect method, since lysine was wholly regenerated, in the case of acetoacetate, or partly regenerated, in the case of malonaldehyde, by the procedure to hydrolyse the proteins. This method estimated the unmodified lysine groups as dinitrophenyl derivatives after reaction with dinitrofluorobenzene. Any free lysine in the amino acid analysis should then be due to lysine groups blocked with either acetoacetate or malonaldehyde that are subsequently liberated on hydrolysis (Weisgraber *et al.*, 1978).

To about 0.6 mg of delipidated protein residue was added 200 mg of NaHCO_3 and 1 ml of 100% ethanol. The protein precipitate was dispersed and 0.1 ml of dinitrofluorobenzene was added. The mixture was left in the dark for 28 h (malonaldehyde) or 96 h (acetoacetate). The precipitate was collected by sedimenting at 1000 g for 10 min in a bench centrifuge and washed three times each with ethanol, water and acetone. Excess acetone was removed under N_2 , and the sample dried *in vacuo* and stored under N_2 at -15 °C before hydrolysis as described above.

Treatment of unmodified lipoprotein with dinitrofluorobenzene showed that 93% of the lysine groups reacted to form dinitrophenyl derivatives; the corresponding value for serum was 87%. Hydrolysis regenerated 100% of lysine groups after acetoacetate modification, but 20% of the lysine groups remained blocked by malonaldehyde after hydrolysis. Both the proportion of free lysine after dinitrofluorobenzene treatment and the proportion of lysine groups remaining modified after acid hydrolysis were corrected for in calculating the percentage of lysine groups that had been modified.

RESULTS AND DISCUSSION

Preliminary experiments showed that the release of oleate from trioyleoylglycerol was in proportion to the time of incubation for more than 40 min and to the concentration of acetone-powder protein used up to at

Fig. 1. Activation of lipoprotein lipase by serum or lipoprotein

The standard assay was performed (see the Materials and methods section) in which the indicated volume of serum (●) or an amount of lipoprotein equivalent to the indicated volume of serum from which it was derived (○) was included. The serum contained 96 mg of protein/ml and the lipoprotein 6.1 mg of protein/ml equivalent of serum. Error bars show $\pm$ S.D. for three determinations.

least 0.4 mg of protein per assay. No significant change in lipoprotein lipase activity was observed at concentrations of trioleoylglycerol between 1 and 4 mM. Fig. 1 shows the stimulation of lipoprotein lipase activity brought about by pig serum and by pig lipoprotein. The maximum stimulation achieved with serum used here is greater than that achieved with lipoprotein: 5.75 ± 1.00 -fold (mean $\pm$ S.D. from 12 determinations) with serum, compared with 2.59 ± 0.47 -fold from 11 determinations with lipoprotein. The serum used as an activator and that used to prepare lipoproteins were obtained from different pigs, but the stimulation produced by lipoprotein was almost identical with that produced by serum from the latter pig. The discrepancy was due, therefore, to an inherent difference between the two samples of serum and was not a result of the manipulations required to isolate lipoproteins. Serum-activated lipase activity was inhibited by 93% by the inclusion of 1 M-NaCl in the incubation mixture. Inhibition by 1 M-NaCl and activation by serum are characteristics of genuine lipoprotein lipase activity (Korn, 1959).

Modification of acidic amino acids

The modification of LDL with *NN*-dimethyl-1,3-diamine, giving them a net positive charge, resulted in an increase in LDL degradation by fibroblasts with an impaired LDL-receptor mechanism (Basu *et al.*, 1976). In our hands, triacylglycerol-rich lipoproteins modified in this manner completely failed to stimulate lipoprotein lipase activity (Fig. 2). It has been reported that the 29 amino acids at the C-terminus of human apo-CII (i.e. residues 50–78) constitute the active portion of the molecule (Musliner *et al.*, 1979). This region contains two glutamic acid residues (77 and 78) and two aspartic acid residues (50 and 68) (Jackson *et al.*, 1977). 'Cationization' of an acidic amino acid in this region might indicate that one of these residues plays a direct role in the binding or activation of lipoprotein lipase by apo-CII.

Kinnunen *et al.* (1977) have shown that removal of the

Fig. 2. Effect of 'cationization' of lipoprotein on lipoprotein lipase activation

The standard assay was used in which the indicated amount of 'cationized' (○) or control (●) lipoprotein was included as activator. The results are scaled to show the extent of activation as follows: the unactivated case (no lipoprotein included) is given the value 0; the maximum activation achieved is given the value 1; all other points are scaled with reference to these two points. In this case $0 = 1.91 \pm 0.17$ (mean $\pm$ S.D. from three determinations) nmol of oleate released/min per mg of acetone-powder protein and $1 = 5.10 \pm 1.58$ (3) nmol/min per mg of protein. Results are means $\pm$ S.D. from three determinations.

tripeptide from the C-terminus of apo-CII, which is Gly-Glu-Glu, removed 95% of the ability to activate lipoprotein lipase. It seems likely that 'cationization' of the terminal two glutamic acids might have had a similar effect. This portion of the apo-CII molecule forms part of the 'lipoprotein lipase-binding region' (Smith *et al.*, 1980).

Alternatively the loss of apo-CII activity may have been due to a loss of conformation due to reversal of the charge of some of the eight acidic amino acids in the remainder of apo-CII molecule. The combination of properties exhibited by 'cationized' lipoproteins (Basu *et al.*, 1976; Fig. 2) make them potentially useful in distinguishing various routes of lipoprotein metabolism.

Modification of arginine residues

Incubation of lipoproteins with cyclohexanedione resulted in a loss of up to 34% of the arginine residues detected by amino acid analysis; these were all regained after incubation with hydroxylamine and mannitol. Fig. 3 shows that treatment of lipoproteins with 0.9 M-cyclohexanedione decreased the stimulatory capacity by approx. 75%, whereas hydroxylamine/mannitol alone decreased this activity by about 15%. Reversal of the modification of the arginine residues restored the activity to about 42% of its original value; this value may well have been greater had it not been for the inhibitory effect

Fig. 3. Effects of cyclohexanedione treatment and reversal on lipoprotein lipase activation

The experiment was carried out as described in Fig. 2, except that the activator was either a control (●), treated with hydroxylamine/mannitol (▲), treated with cyclohexanedione (○) or treated with cyclohexanedione followed by hydroxylamine/mannitol (△). $0 = 2.09 \pm 0.35$ (12) nmol/min per mg of protein; $1 = 5.33 \pm 0.88$ (3) nmol/min per mg of protein. Results are means $\pm$ S.D. for three determinations.

of the reversal reagent on its own. It would appear that approx. 30% of the inhibitory effect of cyclohexanedione was due to reversible covalent modification of the arginine residues, the remainder being due perhaps to a conformational effect on the protein. Incubation of lipoproteins with lower concentrations of cyclohexanedione produced lesser extents of inhibition. Mahley *et al.* (1977) showed that specific modification of the arginine of apo-B and apo-E of LDL resulted in the loss of their ability to interact with high-affinity receptors on fibroblasts. Our results indicate that unmodified arginine groups are necessary for normal functioning of apo-CII and that the structure of apo-CII is more easily lost than the structure of apo-B or apo-E, where regeneration of the arginine groups resulted in 80% recovery of activity. It is noteworthy that human apo-CII contains only one arginine residue at position 49 (Jackson *et al.*, 1977) and that it lies just outside the active section of the molecule (Musliner *et al.*, 1979). It is therefore unlikely that the arginine interacts directly with lipoprotein lipase. Its role may be in maintaining the tertiary structure, perhaps through a charge interaction with an acidic amino acid.

Malonaldehyde modification

Malonaldehyde, which can be generated *in vivo*, is a well-known cross-linking reagent that reacts with the ϵ -NH₂ group of lysine. Modification of more than 15% of the lysine in LDL leads to decreased uptake by LDL receptors and increased uptake by scavenger receptors (Haberland *et al.*, 1982). Under the conditions used, 48% of the lysine was modified, as revealed by amino acid analysis. Fig. 4 shows that this extent of modification

Fig. 4. Effects of malonaldehyde modification on lipoprotein lipase activation

The procedure was as described in Fig. 2, except that the indicated amount of malonaldehyde-modified (○) or control (●) lipoprotein was included as activator. $0 = 2.16 \pm 0.37$ (6) nmol/min per mg of protein; $1 = 4.61 \pm 0.94$ (3) nmol/min per mg of protein. Results are means $\pm$ S.D. for three determinations.

decreased the maximum amount of activation of lipoprotein lipase by only 20%, but that a 3-fold greater concentration of lipoprotein was necessary to achieve maximum activation. This suggests that, unlike the other modifications, the malonaldehyde-modified apo-CII forms an inactive reversible complex with lipoprotein lipase, for which it competes with the unmodified apo-CII.

Methylation and acetoacetylation of lysine ϵ -NH₂ groups

Modification of lipoprotein apoproteins by reductive methylation or acetoacetylation of ϵ -NH₂ groups of lysine has been shown to inhibit receptor binding of lipoproteins by Weisgraber *et al.* (1978). In our hands, similar treatments of pig serum, which resulted in 28% of lysine groups being blocked by methylation or 5% by acetoacetylation, both produced no change in the activation curves for lipoprotein lipase (Figs. 5a and 5b). This contrasts somewhat with the findings of Musliner *et al.* (1979), who showed that maleyl, citraconyl and especially succinyl derivatives of the 29-residue peptide from the C-terminus of human apo-CII had decreased ability to activate lipoprotein lipase. There are two possible reasons for this discrepancy: the acylation employed by Musliner *et al.* (1979) modified -OH groups not -NH₂ groups; and the groups would have been more accessible to the reagents in the case of a purified 29-amino acid peptide than in an intact lipoprotein. On this latter point, however, reductive methylation of a partially delipidized lipoprotein preparation (P. F. Dodds & M. I. Gurr, unpublished work), also failed to modify the lipoprotein lipase activation curve.

Fig. 5. Effects of methylation and acetoacetylation of serum on lipoprotein lipase activation

The procedure was as described in Fig. 2, except that in (a) methylated (○) or control (●) serum was included as activator, and in (b) acetoacetylated (○) or control (●) serum was included as activator. In (a), $0 = 1.28$ nmol/min per mg of protein and $1 = 7.26$ nmol/min per mg of protein. In (b), $0 = 1.75$ nmol/min per mg of protein and $1 = 10.23$ nmol/min per mg of protein.

Non-enzymic glycosylation of lipoproteins

Glucose. Incubation of lipoproteins with 300 mM-glucose for up to 120 h appeared to have no effect on their ability to activate lipoprotein lipase (Fig. 6). Amino acid analysis of the lipoproteins revealed that 5.8% of the total lysine was recovered as fructosyl-lysine. This experiment was repeated, but with 1 M-glucose and incubation was for 72 h. The extent of glycosylation was measured by a thiobarbituric acid method and was shown to increase from 8.0 nmol/mg of protein in the control to 27.8 nmol/mg of protein after incubation. In this case also there was no noticeable difference in the lipoprotein lipase activation curves, which were similar to that in Fig. 6. Kesaniemi *et al.* (1983) reported that it was necessary to reduce the ketoamine link between the lysine group and the glucose molecule with sodium cyanoborohydride in order to obtain a good yield of glucosylated LDL. Accordingly the experiment was repeated with lipoproteins incubated with glucose in the presence or absence of sodium cyanoborohydride. That the sodium cyanoborohydride was an effective reducing agent was shown

Fig. 6. Effects of non-enzymic glycosylation on lipoprotein lipase activation

The procedure was as described in Fig. 2, except that the activator was either a control (●), or had been incubated for 120 h with glucose (○) or with AcNeu (Δ). $0 = 1.92 \pm 0.17$ (6) nmol/min per mg of protein; $1 = 4.66 \pm 0.34$ (3) nmol/min per mg of protein. Results are means $\pm$ S.D. for three determinations.

by its action in stabilizing the glucose-lysine bond, which would no longer give a positive thiobarbituric acid assay result. In spite of this, it remained impossible to distinguish the lipoprotein lipase activation curves of the lipoprotein preparations from each other or from the control.

These results with glucose are similar to those obtained by methylation or acetoacetylation. The results with malonaldehyde (Fig. 4) and AcNeu (Fig. 6) suggest that apo-CII lysine molecules are available for modification, so it appears that glucose is benign with respect to lipoprotein lipase activation. It would appear from these results that non-enzymic glucosylation of apo-CII, as part of an intact lipoprotein in the bloodstream, does not play a role in the disruptions of lipoprotein metabolism seen in diabetics. Non-enzymic glycosylation of LDL or HDL apoproteins, however, appears to produce important effects on receptor binding of lipoproteins (Sasaki & Cottam, 1982; Sasaki *et al.*, 1982; Witzum *et al.*, 1982a,b).

AcNeu. Incubation of lipoproteins with 140 mM-AcNeu for 120 h resulted in a loss of 60% of the maximum stimulation of lipoprotein lipase using up to 50 μg of lipoprotein protein per assay. At higher concentrations the modified lipoproteins inhibited strongly (Fig. 6). This loss of activity may have been due to an irreversible loss of conformation of the apo-CII, but the time course of the inactivation (Fig. 7) is more typical of relatively slow non-enzymic modification of free $-NH_2$ groups by monosaccharides (e.g. Day *et al.*, 1979; McKinney *et al.*, 1982; A. Lopez-Johnston, V. A. Welch & P. F. Dodds, unpublished work). We were not able to demonstrate, by amino acid analysis, the presence of a sialylated amino acid or a decrease in the unmodified lysine content of the lipoproteins, but this is not surprising, since McKinney *et al.* (1982) showed that the covalent link between AcNeu and human serum albumin was labile under hydrolysing

Fig. 7. Effect of time of incubation with AcNeu on lipoprotein activation of lipoprotein lipase

The experiment was carried out as described previously, except that 12.5 (●), 4 (▲) or 2 μl (■) of lipoprotein (equivalent volumes to serum for which it was derived) that had been incubated with 140 mM-AcNeu for the indicated times was included as activator. The scale is the same as that in Fig. 6. Results are means $\pm$ S.D. for three determinations.

conditions. Human apo-CII contains six lysine residues (Jackson *et al.*, 1977), and modification at Lys-75 could disrupt the binding of apo-CII to lipoprotein lipase. The sialylation of lysine would result in a charge reversal at these points and so could exert its effect via a loss of conformation of apo-CII.

General discussion

From the results presented here it would seem that the major disruptions to the ability of serum lipoproteins to activate lipoprotein lipase were due to those modifications which reversed a charge (cationization with dimethylpropanediamine or glycosylation with AcNeu) as well as modification of the arginine residue with cyclohexanedione. More subtle effects were observed with malonaldehyde, whereas reductive methylation, acetoacetylation and non-enzymic glucosylation appeared to have no effect. These findings provide information about the structural requirements of the apolipoproteins which modify the activity of lipoprotein lipase. It seems likely that the activating apoprotein, apo-CII, mediated most of the effects observed, but the participation of other apoproteins, notably apo-CIII, cannot be ruled out in experiments which used intact lipoproteins as the

activator. The resolution of the effects of modifying individual apoproteins must await experiments in which purified apoproteins are used as activators.

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