Effects of C Apoproteins on the Activity of Endothelium-Bound Lipoprotein Lipase

By THOMAS W. LUKENS and JAYME BORENSZTAJN*

Department of Pathology, University of Chicago, Chicago, IL 60637, U.S.A.

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Rat apoprotein C-II activated the hydrolysis of triacylglycerol in apoprotein-depleted chylomicrons by lipoprotein lipase *in vitro* and in the perfused rat heart. Apoproteins C-I and C-III-3 inhibited the hydrolysis of the triacylglycerol moiety in intact and apoprotein C-II-re-activated chylomicrons *in vitro*, but had no effect on the hydrolysis *in situ*.

One of the initial events in the metabolism of plasma chylomicrons and VLD lipoproteins is the hydrolysis of their triacylglycerol moiety. This hydrolysis is catalysed by lipoprotein lipase on the surface of the endothelial cells of the capillaries of various tissues, most notably adipose tissue and muscle (Robinson, 1970). Suggestions have been made that the catalytic action of the endothelium-bound lipoprotein lipase may be modulated by the apoproteins normally present on the surface of the chylomicrons and VLD lipoproteins, in particular apoproteins C-I, C-II and C-III (Krauss *et al.*, 1973; Bar-On *et al.*, 1976; Schonfeld *et al.*, 1976; Carlson & Ballantyne, 1976; Rogers *et al.*, 1976). No direct evidence is available, however, to support this hypothesis. Studies on the effects of various apoproteins on lipoprotein lipase activity have only been carried out *in vitro* with solubilized enzyme preparations and unphysiological substrates (see Lukens & Borensztajn, 1978).

We have shown (Lukens & Borensztajn, 1978) that the physiological role of apoproteins on the catalytic action of endothelium-bound lipoprotein lipase can be investigated by using apoprotein-depleted chylomicrons perfused through the isolated perfused rat heart preparation. In the present paper, we examined the effects of rat apoproteins C-I, C-II and C-III-3, singly and in combination, on the hydrolysis of the triacylglycerols of intact and apoprotein-depleted chylomicrons by lipoprotein lipase *in vitro* and *in situ*.

Methods

Apoprotein fractionation

C-apoproteins were isolated from unlabelled rat lymph chylomicrons. As a source of these apoproteins, lymph chylomicrons were chosen, rather

than plasma lipoproteins, because they can be obtained in large quantities with a minimum of preparation and expense. Chylomicrons were washed as described by Lukens & Borensztajn (1978), freeze-dried and delipidated by the method of Windmueller *et al.* (1973). The apoproteins were fractionated by the method of Herbert *et al.* (1974) for rat HD lipoproteins, except that Sephacryl S-200 (Pharmacia Fine Chemicals, Uppsala, Sweden) was substituted for Sephadex G-200. The isolated apoproteins C-I, C-II and C-III-3 were judged to be pure by electrophoresis on polyacrylamide gels containing 8M-urea (Herbert *et al.*, 1974). When isolated in parallel, C apoproteins from lymph chylomicrons and rat plasma HD lipoproteins showed identical elution patterns and electrophoretic mobility (T. W. Lukens & J. Borensztajn, unpublished observations).

Abbreviations used: VLD lipoprotein, very-low-density lipoprotein; HD lipoprotein, high-density lipoprotein.
* To whom correspondence should be addressed.
Present address: Department of Pathology, Northwestern University Medical School, Chicago, IL 60611, U.S.A.

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Lipoprotein lipase assay

The lipoprotein lipase assay medium (0.15ml) contained ³H-labelled chylomicrons (1.15mg of triacylglycerol), 3% (w/v) bovine serum albumin (Sigma Chemical Co., St Louis, MO, U.S.A.) and 0.1M-Tris/HCl buffer, pH8.1. Where indicated, chylomicrons were incubated with apoproteins for 20min at 37°C before being mixed with the other assay components. The enzyme reaction was started by adding 0.1ml of rat heart lipoprotein lipase and incubation was carried out at 37°C for 20min in a shaking water bath. The ³H-labelled non-esterified fatty acids released into the assay medium were extracted by the method of Belfrage & Vaughan (1969), and their radioactivity was measured in an Isocap 300 liquid-scintillation spectrophotometer (Searle Analytic, Elk Grove Village, IL, U.S.A.). In all experiments, the amount of substrate was not limiting during the period of incubation.

Other procedures

Chylomicrons were obtained by cannulating the mesenteric lymph duct of fed rats given corn oil by

stomach tube. Chylomicrons with ^3H -labelled triacylglycerol fatty acids were obtained by feeding rats with $500\mu\text{Ci}$ of $[9,10(n)-^3\text{H}]$ palmitic acid ($500\mu\text{Ci/mol}$) (Amersham/Searle, Arlington Heights, IL, U.S.A.). ^{14}C -labelled chylomicrons used for the perfused-heart studies were obtained as described by Lukens & Borensztajn (1978). The methods for washing the labelled chylomicrons, their treatment with soluble trypsin or a mixture of trypsin plus trypsin inhibitor (control chylomicrons), collection of rat heart perfusate as a source of lipoprotein lipase, perfusion of the hearts and measurement of their $^{14}\text{CO}_2$ production, and protein and triacylglycerol determinations were as described by Lukens & Borensztajn (1978).

Statistical significance of the difference between means was analysed by Student's *t* test.

Results

Fig. 1 shows the results of an experiment in which the effects of apoproteins C-I, C-II and C-III-3 on the hydrolysis *in vitro* of trypsin-treated chylomicron triacylglycerols were examined. The trypsin-treated chylomicrons were incubated with increasing amounts of each apoprotein before being added to the lipoprotein lipase assay system. It is apparent that apoprotein C-II was the most effective in promoting the re-activation of the substrate. In the absence of apoproteins, the hydrolysis of the trypsin-treated chylomicrons was only 6% of that of the control

Fig. 1. Effect of apoproteins on the hydrolysis of triacylglycerol of trypsin-treated chylomicrons

Chylomicrons that had been treated with soluble trypsin ($20\mu\text{g/mg}$ of triacylglycerol) were incubated with increasing amounts of apoproteins before being used as substrate for lipoprotein lipase, as described in the Methods section. Results are expressed as percentage hydrolysis of control chylomicrons (100%), and each point represents the average of assays carried out in duplicate. $\circ$, Apoprotein C-I; $\bullet$, apoprotein C-II; $\square$, apoprotein C-III.

chylomicrons (100%). With increasing amounts of apoprotein C-II the rate of hydrolysis was rapidly increased and at a concentration of $4.8\mu\text{g}$ of apoprotein C-II/assay it reached levels similar to that of the control chylomicrons (100%). Maximal stimulation of the rate of hydrolysis (about 30% higher than control chylomicrons) was observed at an apoprotein concentration of $8\text{--}14\mu\text{g/assay}$. This corresponds to a ratio (w/w) of apoprotein to triacylglycerol of 1:100. At concentrations greater than $14\mu\text{g}$ of apoprotein per assay the rate of hydrolysis of the substrate was progressively less (Fig. 1). Apoproteins C-I and C-III-3 at low concentrations also stimulated the hydrolysis of chylomicron triacylglycerol, although this effect was only a fraction of that observed with apoprotein C-II.

Table 1 shows the results of experiments in which the effects of apoproteins C-I and C-III-3 on the hydrolysis of intact lymph chylomicrons or trypsin-treated chylomicrons re-activated with apoprotein C-II were examined. When intact chylomicrons were incubated with apoprotein C-I or C-III-3 at a concentration of $13\mu\text{g/mg}$ of triacylglycerol and then used as substrate for lipoprotein lipase, the triacylglycerol hydrolysis was inhibited by 40%. Doubling the concentration of the apoproteins resulted in a corresponding increase in the inhibitory effect. Inhibition of similar magnitude was also observed when trypsin-treated chylomicrons that had been re-activated with $13\mu\text{g}$ of apoprotein C-II/mg of triacylglycerol were incubated with 13 and $26\mu\text{g}$ of apoprotein C-I (Table 1). With the same concentrations of apoprotein C-III-3, the inhibitory effect was even more pronounced (Table 1).

To determine whether the inhibitory effects of apoproteins C-I, C-II and C-III-3 observed *in vitro* (Table 1) could be reproduced in a system in which lipoprotein lipase is present at its physiological site of action, intact and trypsin-treated ^{14}C -labelled chylomicrons were incubated with the various apoproteins and then perfused through isolated rat hearts. The $^{14}\text{CO}_2$ formed as a result of the uptake and oxidation of the ^{14}C -labelled chylomicron triacylglycerol fatty acids was used as an indicator of the capacity of the heart lipoprotein lipase to hydrolyse the triacylglycerols (Lukens & Borensztajn, 1978). The results in Table 2 show that, in agreement with our previous observations (Lukens & Borensztajn, 1978), the $^{14}\text{CO}_2$ formed by hearts perfused with trypsin-treated chylomicrons was decreased by about 70% when compared with hearts perfused with untreated chylomicrons ($P < 0.001$). Preincubation of trypsin-treated chylomicrons with apoprotein C-II completely restored the ability of this substrate to be hydrolysed by the endothelium-bound lipoprotein lipase. Incubation of apoproteins C-I and C-III-3 ($13\mu\text{g/mg}$ of triacylglycerol) with intact chylomicrons or trypsin-treated chylomicrons

re-activated with apoprotein C-II (13 $\mu\text{g}/\text{mg}$ of triacylglycerol) had no effect on the hydrolysis of the lipoprotein triacylglycerols in this physiological system (Table 2). Even when the amount of apoprotein C-I added to the re-activated chylomicrons was doubled (26 μg), no inhibition could be detected (Table 2).

Table 1. *Effects in vitro of C apoproteins on the hydrolysis of triacylglycerol of intact and re-activated chylomicrons by lipoprotein lipase*

Chylomicrons that had been treated with soluble trypsin (20 $\mu\text{g}/\text{mg}$ of triacylglycerol) were pre-incubated with apoprotein C-II (13 $\mu\text{g}/\text{mg}$ of triacylglycerol). These chylomicrons, as well as control chylomicrons, were then incubated with 13 or 26 μg (*) of apoproteins C-I or C-III-3 before being used in the lipoprotein lipase assay system *in vitro* as described in the Methods section. The results are expressed as percentages of the maximal rates of hydrolysis with each substrate and represent the averages of assays carried out in duplicate.

Apoproteins added	Triacylglycerol hydrolysis (%)	
	Control chylomicrons	Trypsin-treated chylomicrons
—	100	6
C-I	58	—
C-I*	30	—
C-III-3	62	—
C-III-3*	34	—
C-II	—	100
C-II+C-I	—	62
C-II+C-I*	—	48
C-II+C-III-3	—	43
C-II+C-III-3*	—	25

Discussion

The present investigation with a physiological system offers direct evidence that apoprotein C-II promotes the hydrolysis of chylomicron triacylglycerols by endothelium-bound lipoprotein lipase. Such a role for apoprotein C-II had previously only been inferred from the results of experiments carried out *in vitro* and from the observation that in one individual the absence of detectable circulating apoprotein C-II was associated with high concentrations of plasma triacylglycerols (Breckenridge *et al.*, 1978). The re-activation by apoprotein C-II of the hydrolysis of trypsin-treated chylomicrons *in vitro* (Fig. 1) and *in situ* (Table 2) resembles that observed when HD lipoproteins were incubated with this substrate (Lukens & Borensztajn, 1978). It is noteworthy, however, that in the presence of excess apoprotein C-II hydrolysis was inhibited (Fig. 1), whereas no such inhibitory effect was detected when trypsin-treated chylomicrons were incubated with high concentrations of HD lipoprotein (Lukens & Borensztajn, 1978). Inhibition of triacylglycerol hydrolysis by lipoprotein lipase in the presence of apoprotein C-II has also been observed in systems with artificial substrates (Havel *et al.*, 1973; Ekman & Nilsson-Ehle, 1975). In the experiment using the isolated rat heart (Table 2), the single concentration of apoprotein C-II used (13 $\mu\text{g}/\text{mg}$ of triacylglycerol) was sufficient to restore the capacity of trypsin-treated chylomicron triacylglycerols to be hydrolysed at a rate similar to that of intact chylomicrons. It remains to be investigated whether increasing concentrations of the apoprotein would have an effect on the rate of triacylglycerol hydrolysis *in situ*.

Table 2. *Effects of C apoproteins on the oxidation of ^{14}C -labelled chylomicron triacylglycerol fatty acids by the perfused rat heart*

^{14}C -labelled chylomicrons that had been treated with soluble trypsin (20 $\mu\text{g}/\text{mg}$ of triacylglycerol) were preincubated with apoprotein C-II (13 $\mu\text{g}/\text{mg}$ of triacylglycerol). These chylomicrons as well as untreated chylomicrons were then incubated with 13 μg or 26 μg (*) of apoproteins C-I or C-III-3 before being diluted in the perfusion medium to a concentration of 0.22 $\mu\text{equiv.}$ of triacylglycerol fatty acids/ml. Groups of four hearts from rats starved overnight were then perfused for 10 min with the various ^{14}C -labelled chylomicron preparations, and the $^{14}\text{CO}_2$ formed was measured as previously described (Lukens & Borensztajn, 1978). The results are expressed as means $\pm$ s.d. and have been corrected for $^{14}\text{CO}_2$ that could have been derived from the oxidation of the ^{14}C -labelled free fatty acids present in the chylomicron preparations (Lukens & Borensztajn, 1978).

^{14}C -labelled chylomicrons	Apoproteins added	Perfusion time (min)	...	$^{14}\text{CO}_2$ produced ($\mu\text{equiv.}$ of triacylglycerol fatty acid/g of heart)		
				0-5	5-10	0-10
Trypsin-treated	—			0.05 $\pm$ 0.006	0.30 $\pm$ 0.05	0.35 $\pm$ 0.05
Trypsin-treated	C-II			0.22 $\pm$ 0.05	0.90 $\pm$ 0.08	1.12 $\pm$ 0.17
Trypsin-treated	C-II+C-I			0.23 $\pm$ 0.06	0.98 $\pm$ 0.25	1.21 $\pm$ 0.28
Trypsin-treated	C-II+C-I*			0.26 $\pm$ 0.02	1.05 $\pm$ 0.28	1.31 $\pm$ 0.28
Trypsin-treated	C-II+C-III-3			0.19 $\pm$ 0.03	0.83 $\pm$ 0.05	1.02 $\pm$ 0.08
Control	—			0.20 $\pm$ 0.04	0.95 $\pm$ 0.16	1.16 $\pm$ 0.18
Control	C-I			0.21 $\pm$ 0.04	0.98 $\pm$ 0.15	1.19 $\pm$ 0.16
Control	C-III-3			0.18 $\pm$ 0.04	0.76 $\pm$ 0.09	0.94 $\pm$ 0.13

similar to that *in vitro*, i.e. an increase and then a decline at sufficiently high apoprotein concentrations. It should be pointed out that the optimal ratio of apoprotein C-II to triacylglycerol required for the maximal rate of hydrolysis *in vitro* varied with different batches of trypsin-treated chylomicrons (T. W. Lukens & J. Borensztajn, unpublished observations). Although we have not investigated further the reasons for these variations, it is possible that differences in the average particle size of the various preparations may have contributed to these results (Kashyap *et al.*, 1977).

Whereas apoprotein C-II promoted an increase in lipoprotein lipase activity both *in vitro* (Fig. 1) and *in situ* (Table 2), the inhibitory effects of apoproteins C-I and C-III-3 on the hydrolytic action of the enzyme *in vitro* (Table 1) were not apparent in the isolated heart system (Table 2). The inhibition *in vitro* is in agreement with the observations by other authors made with the use of artificial substrates and human apoproteins (Brown & Baginsky, 1972; Havel *et al.*, 1973; Bensadoun *et al.*, 1974; Ekman & Nilsson-Ehle, 1975). The mechanism whereby apoproteins C-I and C-III-3 inhibit the hydrolysis of chylomicrons or artificial substrates *in vitro* is not known. One possible explanation is that these apoproteins become attached to the surface of the substrate by specific binding or non-specific adsorption and in this way interfere with the interaction of the soluble lipoprotein lipase with apoprotein C-II. If this explanation is correct, it remains to be explained why apoproteins C-I and C-III-3 inhibited lipoprotein lipase activity *in vitro* (Table 1), but not the endothelium-bound enzyme (Table 2). Another possible explanation of this inhibitory effect *in vitro* is that much of the apoprotein added to the chylomicrons may have remained in solution and complexed with the solubilized lipoprotein lipase, thus preventing its interaction with the substrate. Evidence in favour of this possibility has been provided by Quarfordt *et al.* (1977), who incubated an artificial emulsion of triacylglycerol with arginine-rich apoprotein and found that the unbound apoprotein inhibited soluble lipoprotein lipase activity, whereas no such inhibition was observed with the substrate-bound fraction. In the present study, the amount of added apoprotein bound to the chylomicrons was not measured. It is possible that, *in vitro*, excess apoproteins C-I and C-III-3 were available in solution to complex with and inhibit the action of the soluble enzyme. In this regard, it has been shown that human apoproteins C-I and C-III can complex with lipoprotein lipase in the absence of substrate (Miller & Smith, 1973). Such an interpretation is not inconsistent with the lack of inhibition of the hydrolysis of chylomicron triacylglycerol by lipoprotein lipase observed *in situ*. In order to be perfused through the isolated rat heart, all chylomicron preparations were diluted in a large

volume of perfusion medium. Thus any unbound apoproteins C-I and C-III-3 would also be diluted and may not have been of sufficient concentration to inhibit the hydrolytic action of the endothelium-bound enzyme. Regardless of the possible mechanism of the inhibition of lipoprotein lipase activity by apoproteins *in vitro*, the results of the present investigation show the failure of rat apoproteins C-I and C-III-3 to inhibit the hydrolysis of chylomicron triacylglycerols in a physiological system (Table 2). This indicates that suggestions made on the basis of experiments *in vitro* (Krauss *et al.*, 1973; Bar-On *et al.*, 1976; Schonfeld *et al.*, 1976; Carlson & Ballantyne, 1976; Rogers *et al.*, 1976) with regard to the role of apoproteins C-I and C-III in the catabolism of chylomicrons and VLD lipoproteins must now be reassessed.

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