Mice lacking acylation stimulating protein (ASP) have delayed postprandial triglyceride clearance

I. Murray, A. D. Sniderman, and K. Cianflone[1]

Mike Rosenbloom Laboratory for Cardiovascular Research, McGill University Health Centre, Montreal, Quebec, Canada H3A 1A1

Abstract **Acylation stimulating protein (ASP) is a 76 amino acid fragment of the third component of complement (C3) which is generated by the interaction of adipsin and factor B with C3. In vitro studies have shown that ASP can markedly increase triglyceride synthesis in adipocytes. To test the ASP pathway in vivo, C3-deficient mice, and therefore ASP-deficient mice, were generated and oral fat loads were conducted in wild-type (C3+/+) and mutant (C3−/−) animals. The principal results were: *1*) postprandial triglyceride clearance was significantly delayed in mutant compared to wild-type mice; *2*) this difference was more pronounced in males compared to females; *3*) in both males and females, the differences were more pronounced in the second half of the postprandial period; *4*) fasting and postprandial free fatty acid (FFA) were higher in C3(−/−) than in C3(+/+) males; and *5*) intraperitoneal administration of ASP accelerated triglyceride clearance in C3(−/−) males. The data are consistent therefore, with the hypothesis that the ASP pathway is an important physiologic determinant of normal postprandial triglyceride clearance.**—Murray, I., A. D. Sniderman, and K. Cianflone. **Mice lacking acylation stimulating protein (ASP) have delayed postprandial triglyceride clearance.** ***J. Lipid Res.*** **1999.** 40: **1671–1676.**

Supplementary key words complement C3a • transgenic • alimentary lipemia

Storage of energy when it is in excess and release of energy when it is needed are the critical biologic functions of adipose tissue. Lipoprotein lipase (LPL) and insulin are both widely recognized to play important roles in the regulation of lipogenesis. Recently, in vitro studies have demonstrated that the acylation stimulating protein (ASP) pathway may also be a determinant of the rate at which adipocytes can store energy (1). Accordingly, the present study was designed to test this hypothesis in vivo using a mouse model in which synthesis of the obligate precursor of ASP, complement C3, was disrupted by targeted deletion.

ASP is generated by the interaction of factor B and adipsin with the third component of complement (C3), all three of which are synthesized and secreted by both murine (2) and human adipocytes (3–5). The product, C3a, is a non-glycosylated 77 amino acid N-terminal fragment of the α chain of C3. The terminal arginine is then rapidly removed by carboxypeptidase N to produce ASP. ASP is identical to C3adesArg as established by amino terminal sequence analysis, ion spray ionization mass spectroscopy, and amino acid analysis (6). Subsequently, a recombinant ASP was produced and shown to be identical in bioactivity to native ASP (7). Both native (plasma) and recombinant ASP were bioactive in specific target tissues such as adipose tissue but not in other cells such as macrophages (7). ASP, therefore, differs in function as well as composition from C3a.

In vitro studies have shown that ASP increases triglyceride (TG) synthesis by increasing the activity of the last enzyme involved in the synthesis of a TG molecule, diacylglycerol acyltransferase (8) and by increasing specific membrane transport of glucose through translocation of GLUT 1 and GLUT 4 from intracellular vesicles to the plasma membrane (9, 10). Of importance, ASP and insulin have independent and additive effects on TG metabolism (9, 11). Interaction with a cell membrane receptor (7, 12) and subsequent activation of a protein kinase C signal transduction pathway (13) appear to be critical to the production of these coordinated effects on TG synthesis. The structure of ASP is broadly conserved phylogenetically (14) consistent with the demonstration in vitro of the bioactivity of human ASP in a variety of species including murine cells (7).

In vivo studies in humans have shown that ASP is produced in adipose tissue and that the production of ASP is markedly accelerated in the second half (i.e., 3–6 h) of the postprandial period (15). Furthermore, the increase in ASP production during this period correlates with maximal TG clearance and fatty acid uptake by adipocytes. Of interest also, obese subjects have higher plasma ASP than normal, but their plasma ASP diminishes with prolonged

Abbreviations: ASP, acylation stimulating protein; LPL, lipoprotein lipase; C3, complement C3; TG, triglyceride; FFA, free fatty acid; AUC, area under the curve.

[1] To whom correspondence should be addressed.

fasting and weight loss (16). Thus plasma ASP correlates with expansion and contraction of adipose tissue mass.

There is, therefore, considerable circumstantial in vivo and in vitro evidence in humans indicating that the ASP pathway plays a physiologically important role in postprandial TG clearance. However, a direct test of this hypothesis has not yet been made. To do so, C3 knockout (−/−) mice were engineered. In the absence of C3, the precursor to ASP, these mice are unable to produce ASP. Accordingly, TG clearance after an oral fat load was determined in C3(−/−) mice and compared to that observed in wild-type C3(+/+) mice.

METHODS

Targeted disruption of the C3 locus

C3(−/−) and (+/+) mice were kindly provided by Dr. H. Colton and Dr. R. Wetsel. Development of the C3 knockout mice has been described in detail elsewhere (17, 18). The murine C3 locus was disrupted by replacing 2.4 kb of the 5′ flanking region and the first 105 bp of exon 1 of the C3 gene with the neomycin-resistant gene oriented in the opposite direction from C3 transcription (1.14 kb Neocassette from pMC1 NEO vector (Stratagene, LaJolla, CA)). RW4 and D3 ES (from 129 SVJ mice) were transfected with the targeting vector. Homologous recombinant RW4 ES cells were micro-injected into C57Bl/6 blastocysts. Chimeric founders were established (F_0) and germ line transmission into C57Bl/6 background was accomplished via mating to C57BI/6 females (F_1). C3 heterozygous (129SVJ × C57Bl/6 strain) F_1 were interbred with each other (brother/sister). Because these animals are not interbred strains, there is some genetic variation, and to control for this heterozygotes were intercrossed to produce heterozygotes (+/−), homozygotes (−/−) and wild type (+/+) (F_3 and F_4). Thus in all studies paired littermates (−/− and +/+) were used to control for and randomize genetic variation.

Genotyping of mice

Animal care was in accordance with Royal Victoria Hospital Animal Care Committee institutional guidelines. For genotyping analysis to identify hetrozygotes (+/−), homozygotes (−/−), and wild-type (+/+) mice, tail DNA was extracted. PCR was performed using 800 nm each of the following primers: C3 sense: CTT AAC TGT CCC ACT GCC AAG AAA CCG TCC CAG ATC; C3 antisense: CTC TGG TCC CTC CCT GTT CCT GCA CCA GGG ACT GCC CAA AAT TTC GCA AC. Neomycin sense: ATC GCA TCG AGC GAG CAC GTA CTC GGA; neomycin antisense: AGC TCT TCA GCA ATA TCA CGG CTA GCC. PCR conditions were: 30 cycles, 94°C, 1 min; 67°C 2 min; and 72°C 3 min. Products were separated by electrophoresis on a 7% polyacrylamide gel and visualized with ethidium bromide staining.

Baseline characterization of mice

At 8 weeks of age, having been on a Purina chow 5075 diet from the time of weaning (3 weeks), the mice (littermates) were weighed and fasting lipid levels, glucose, and insulin determined. Blood was obtained by tail bleeding after an overnight fast. Plasma FFA and TG were measured using colorimetric enzymatic kits (Boehringer Mannheim, Laval, Quebec, Canada). Fasting insulin was measured using a rat insulin radioimmunoassay kit that fully crossreacts with mouse insulin (Linco Research Inc., St. Charles, MO). Fasting plasma glucose was measured using a Trinder glucose kit (Sigma, St. Louis, MO). HDL lipids were measured after precipitation of apoB lipoproteins with heparin Mn^{2+}, and non-HDL (VLDL + LDL) cholesterol and triglyceride were calculated from the difference (total HDL).

Postprandial fatload on mice

An oral fat load was administered by intragastric feeding to C3(−/−) and wild-type mice (+/+) (10–12 weeks old). The mice were hand held and were not anesthetized. After an overnight fast, 400 μL of olive oil (followed by 100 μL air) was given as described (19–21). Blood samples (40 μL) were collected from each mouse at 0, 1, 2, 3, 4, and 6 h into EDTA-containing tubes and plasma was isolated to measure TG and FFA. Twenty-four C3(−/−) mice were studied (9 males, 15 females) and 13 C3(+/+) wild-type (7 males, 6 females). There was no evidence of fat malabsorption (loose stools, diarrhea). Results at each time point during the postprandial period are expressed as the mean ± SEM of the difference in TG from fasting levels. Individual time points were compared. As well, the area under the TG curve was determined using a linear trapezoidal equation (Sigma Stat, Jandel Scientific, San Rafael, CA) and the results in the two groups were compared.

The effect of ASP injection on postprandial lipemia was tested in separate studies (n = 8 male C3(−/−) mice, 10–12 weeks old). Two h after administration of the fatload (as described above), ASP (500 μg in 250 μl) was injected intraperitoneally with half of the animals receiving a sham injection (buffer only containing 1 mg/mL (bovine serum albumin in phosphate-buffered saline pH 7.2). Two weeks later, the same fatload was repeated with the ASP/sham injections reversed. ASP was purified and assessed for purity as previously described (13).

Statistical analysis

Data were analyzed by two-way ANOVA for genotype (−/− vs. +/+) and time effect (0–6 h) and significance of individual points was determined by Bonferroni *t*-test.

RESULTS

At 8 weeks of age, the mice (−/− and +/+ littermates) were weighed and fasting cholesterol, triglyceride (TG), glucose, and insulin levels were measured. These results are shown in **Table 1**. Both male C3(−/−) and wild-type mice C3(+/+) weighed more than the females ($P < 0.005$). The fasting TG and cholesterol values tended to be higher in the C3(−/−) males but these differences were not statistically significant. By contrast, fasting plasma FFA were significantly higher in the male C3(−/−) compared to the wild-type C3(+/+) mice ($P < 0.05$).

The results of the changes in plasma TG during the oral fat load in the ASP C3(−/−) and the wild-type C3(+/+) animals are illustrated in **Fig. 1** for both males and females separately. In both the male and female C3(+/+) mice, there was only a moderate increase in postprandial TG reaching a maximum of 54% increase over baseline (Fig. 1). None of the individual time points were significantly increased above fasting TG although the overall curve in the male C3(+/+) was significantly different from fasting ($P < 0.01$). By contrast, TG clearance from plasma after the oral fat load was significantly delayed in the C3(−/−) group with a significant postprandial increase in both male C3(−/−) ($P < 0.01$) and in female C3(−/−) ($P < 0.001$) mice. In the females, the 3- and 4-h

TABLE 1. Characteristics of C3(−/−) and wild type (+/+) mice

Variable	Male		Female	
	C3(−/−) (n = 9)	C3(+/+) (n = 7)	C3(−/−) (n = 15)	C3(+/+) (n = 6)
Wt (g)	28.3 ± 0.8	27.7 ± 1.1	23.0 ± 0.4	22.6 ± 0.9
TG (mg/dL)	100.6 ± 16.1	83.5 ± 12.9	70.1 ± 10.5	64.2 ± 3.1
Chol (mg/dL)	67.1 ± 4.3	63.0 ± 4.6	57.6 ± 4.0	56.6 ± 7.0
FFA (mm)	1.97 ± 0.52[a]	0.87 ± 0.18	1.54 ± 0.40	0.91 ± 0.23
Glu (mg/mL)	1.12 ± 0.08	1.12 ± 0.16	0.91 ± 0.03	1.06 ± 0.077
Ins (ng/mL)	0.295 ± 0.021	0.234 ± 0.033	0.200 ± 0.014	0.217 ± 0.016

Fasting blood was obtained in mice 8 weeks of age. Data are the means ± SEM; Wt, weight; TG, plasma triglycerides; Chol, total plasma cholesterol; FFA, plasma free fatty acids; Glu, plasma glucose; Ins, plasma insulin.

[a] $P < 0.05$ C3(−/−) vs. C3(+/+) mice by one-way ANOVA.

time points were significantly above fasting ($P < 0.05$). The differences, however, were most marked in the male C3(−/−) mice in which plasma TG increased 112% above basal. At the 2, 3, and 4-h time points, postprandial TG were significantly increased above basal TG (81–112%) and even at 6 h were still significantly increased above basal TG (50%, $P < 0.01$ by ANOVA for the postprandial curve).

The triglyceride curves from the C3(−/−) mice were also directly compared to the C3(+/+) mice. Both the 4- and 6-h time points were significantly higher in the C3(−/−) males compared to the C3(+/+) males (112 ± 28 mg/dL vs. 39 ± 12 mg/dL, $P < 0.025$ and 51 ± 37 mg/dL vs. −8 ± 8 mg/dL, $P < 0.05$, respectively). Similarly, the total TG area under the curve (AUC) was substantially greater in the male C3(−/−) mice compared to the male wild-type group (+/+) (AUC = 558 ± 120 mg/dL/h vs. 264 ± 45 mg/dL/h, $P < 0.05$). A similar although less marked trend was present in the C3(−/−) females (AUC = 357 ± 63 mg/dL/h vs. 247 ± 64 mg/dL/h) (Fig. 1, right panel). Importantly, because the fasting TG levels were not significantly different amongst the groups, the difference in postprandial TG clearance cannot be a simple consequence of disproportionately elevated fasting TG levels in the C3(−/−) animals.

Because in vivo studies in humans have shown that maximal activation of the ASP pathway and TG clearance by adipocytes does not occur until the second half of the postprandial period (15), the areas under the TG (AUC) curve in the first and second halves of the postprandial periods in the C3(−/−) and C3(+/+) mice were calculated separately and these early and late phases were compared. These data are given in **Table 2**. In all cases, there was no significant difference between C3(−/−) and wild-type C3(+/+) animals in the early portion of the TG curve. By contrast, the late TG (AUC) curve is significantly greater for the C3(−/−) mice vs. the C3(+/+). The differences were particularly pronounced in the male C3(−/−)

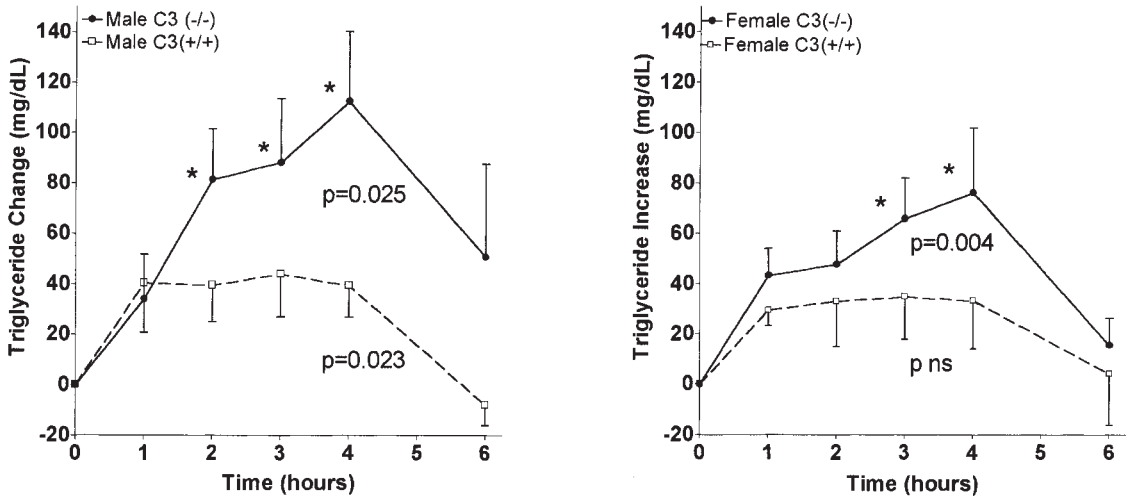

Fig. 1. Changes in plasma triglyceride during the postprandial period in male and female C3(−/−) and wild type (+/+) mice. The differences in plasma triglycerides (mean ± SEM) from fasting mice (shown in Table 1) were determined at each time point in each animal for males (left panel) and females (right panel) where * $P < 0.05$ vs. fasting TG (Bonferroni *t*-test). Triglyceride area under the curve was calculated in male C3(−/−) (n = 9) and male C3(+/+) (n = 7) (558 ± 120 mg/dL/h C3(−/−) vs. 264 ± 45 mg/dL/h, C3(+/+), $P < 0.05$). Female C3(−/−) (n = 15) and C3(+/+) (n = 6) mice were compared separately (357 ± 63 mg/dL/h C3(−/−) vs. 247 ± 64 mg/dL/h C3(+/+). For each curve, the ANOVA results vs. time are given in the figure where ns = not significant. By two-way ANOVA, there was a significant effect of genotype (−/− vs. +/+) for males ($P = 0.0052$) whereas the effect was not quite significant for females ($P = 0.07$).

TABLE 2. Area under the plasma triglyceride curves during the early and late postprandial periods

Mice	n	Early	Late
		mg/dL/h	
All			
C3 (−/−)	24	181 ± 30	252 ± 41
C3 (+/+)	13	132 ± 23	124 ± 21[a]
Male			
C3 (−/−)	9	226 ± 68	331 ± 63
C3 (+/+)	7	137 ± 34	127 ± 21[b]
Female			
C3 (−/−)	15	153 ± 23	204 ± 50
C3 (+/+)	6	128 ± 30	119 ± 38

Plasma triglyceride was determined at each time point shown and the difference from the fasting value determined (Figs. 1 and 2). The area under the plasma triglyceride curve was then determined for the first half (0–3 h) and the second half (3–6 h) of the postprandial period. Data are shown as mean ± SEM; n = number of animals.

[a] $P < 0.025$ for C3(−/−) vs. C3(+/+).

[b] $P < 0.01$ for C3(−/−) vs. C3 (+/+).

TABLE 3. Fasting lipoproteins and glucose/insulin ratio in male C3 (−/−) mice

	C3 (+/+) (n = 5)	C3 (−/−) (n = 8)	% Change
			%
HDL cholesterol mg/dL	53.4 ± 3.1	45.8 ± 3.9	↓ 14
VLDL + LDL cholesterol mg/dL (non-HDL cholesterol)	5.6 ± 0.5	16.9 ± 1.9	↑ 302 $P < 0.005$
VLDL + LDL triglyceride mg/dL (non-HDL triglyceride)	7.7 ± 2.3	45.2 ± 11.2	↑ 587 $P < 0.05$
Glucose/insulin mg/ng	5.00 ± 78	3.92 ± 33	↓ 22

vs. male wild-type C3(+/+) mice, with almost a 3-fold difference in the late postprandial area under the curve. While the difference between female C3(−/−) vs. female wild-type C3(+/+) mice was not statistically significant, the same trend is apparent in these animals as well.

As noted previously, the fasting plasma FFA concentration was significantly higher in the male C3(−/−) mice compared to the three other groups. As can be seen in **Fig. 2**, postprandial FFA in the C3(+/+) mice were only significantly increased postprandially at 3 and 4 h ($P < 0.05$), returning to basal levels by 6 h. By contrast, although fasting FFA were already significantly increased in the C3(−/−) mice (Table 1), they continued to increase postprandially and at 4 and 6 h were still significantly increased over basal ($P < 0.05$). At 6 h, when the C3 (+/+) mice had returned to fasting levels, C3(−/−) mice were reaching their highest point (67% above basal, $P < 0.05$) and were significantly increased vs. C3 (+/+) mice ($P < 0.05$).

These data prompted more detailed studies in C3(−/−) and C3(+/+) males. As shown in **Table 3**, there was a slight decrease (30%) in the glucose/insulin ratio. There was also a small, but not significant, decrease in fasting HDL cholesterol levels. By contrast there was a substantial (3- to 5-fold) increase in fasting non-HDL (VLDL + LDL) cholesterol and triglyceride (Table 3) ($P < 0.005$ and $P < 0.05$, respectively).

As an additional test of our hypothesis, because C3(−/−) mice have no functional ASP, we examined the effects of injecting human ASP intraperitoneally after administration of the fat load. As shown in **Fig. 3**, in the absence of

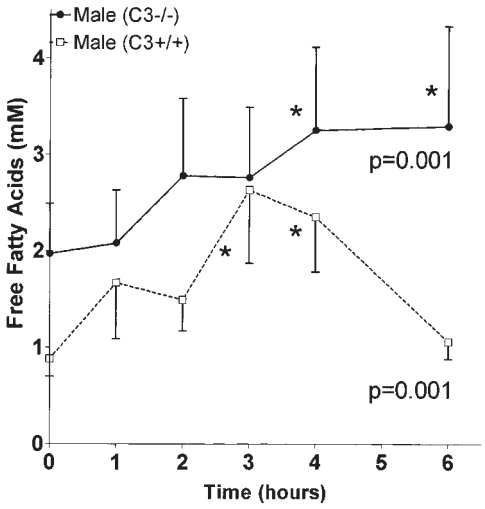

Fig. 2. Free fatty acid levels in male C3(−/−) and wild type mice during the postprandial period. Plasma free fatty acids were determined at each time point in each animal after the fat load as described in Fig. 1. Nine male C3(−/−) animals and 6 male wild-type (+/+) animals were studied, where * $P < 0.05$ vs. fasting FFA (Bonferroni *t*-test), and the results for one-way ANOVA for each curve are given in the figure. By two-way ANOVA, there was a significant effect of genotype ($P = 0.013$) for (−/−) vs. (+/+) mice.

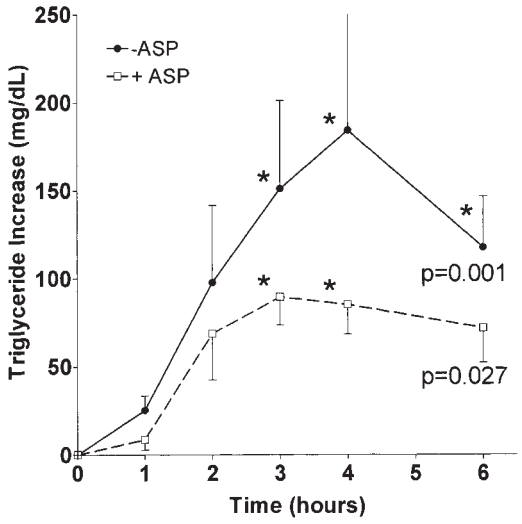

Fig. 3. Changes in plasma triglyceride during the postprandial period in male C3(−/−) mice with injected ASP. The differences in plasma triglycerides (mean ± SEM) from fasting mice were determined at each time point in each mouse where fasting TG = 56 ± 6.0 mg/dL without ASP (n = 8) and 59 ± 9.8 with ASP injection (n=8); * $P < 0.05$ vs. fasting plasma triglyceride (Bonferroni *t*-test). The results for one-way ANOVA on each curve are given in the figure. By two-way ANOVA, there was a significant effect of ASP ($P = 0.026$) for +ASP vs. −ASP.

ASP, plasma TG increased 324% above basal (4 h). Administration of ASP at 2 h caused a rapid decrease in plasma triglyceride towards basal and a substantial (42%) decrease in the area under the curve over 6 h vs. C3(−/−) mice that received a sham injection (669 ± 216 control vs. 391 ± 77 with ASP, $P < 0.05$).

DISCUSSION

These findings represent direct evidence that the ASP pathway plays an important role in postprandial TG clearance from plasma. They constitute, therefore, an important step towards proof of concept that the ASP pathway, by increasing the rate of adipocyte TG synthesis, increases the rate of chylomicron TG clearance from plasma.

In vitro studies have shown that fatty acids themselves do not cause increased ASP generation by adipocytes (5). Insulin does produce a modest increase in ASP whereas chylomicrons cause a profound increase in ASP generation by adipocytes (5). As noted previously, in vivo studies in humans have demonstrated that production and release of ASP by adipocytes markedly increases in the second half of the postprandial period (15), a delay that would appear to reflect the time required to maximally activate the pathway by chylomicrons and insulin. The correspondence between these in vivo findings in humans and the present in vivo data in the C3(−/−) mice are of considerable interest. In the C3(−/−) mice injected intraperitoneally with ASP, the major differences also occur in the second half of the postprandial period.

Through activation of the ASP pathway by chylomicrons, the efficiency of postprandial TG clearance can be increased. If the substantial amounts of fatty acids released from chylomicrons were not quickly removed from the capillary space, then LPL action would be inhibited, and TG hydrolysis reduced (22). Thus, enhanced (21) or decreased (20) lipoprotein lipase activity can profoundly affect plasma triglyceride clearance as shown in overexpressing or knockout mice models. Thus, a decreased efficiency of triglyceride clearance may be reflected in our C3(−/−) mice by increased circulating free fatty acids, not only postprandially but also in the fasting state, as well as by delayed postprandial triglyceride clearance.

The ASP pathway provides, therefore, a form of metabolic integration. Chylomicrons stimulate the generation of ASP by adipocytes and ASP, by increasing fatty acid uptake and esterification into adipocytes, allows continued action of LPL and therefore allows continued hydrolysis of chylomicron TG. There is independent evidence supporting this model of modulation of LPL activity by fatty acids. In vitro experiments indicate that LPL forms complexes with fatty acids resulting in decreased lipolysis and detachment of both lipase and lipoproteins from their endothelial sites (22–24). In vivo support of this hypothesis has also been obtained (25).

Based on the present in vivo study and our previous in vitro data, we propose that a major site of action of ASP in vivo is on adipose tissue. However, it is also possible that ASP may act at other tissues, such as muscle. In vitro data indicate that ASP stimulates glucose transport via translocation of Glut1, Glut3, and Glut4 to the plasma membrane in L6 myotubes (9–11). Studies are underway in the present model to determine the tissues to which the dietary fatty acids are targeted.

Just as normal function of the ASP pathway appears integral to the normal deposition of dietary fatty acids into adipocytes during the postprandial period, dysfunction of the ASP pathway may be associated with lipoprotein abnormalities that are linked to the pathogenesis of coronary artery disease. If fatty acid trapping and storage by adipocytes are reduced in the postprandial period, an excessive proportion of dietary fatty acids will be diverted to the liver and hepatic secretion of apoB-100 lipoproteins (VLDL and LDL) will increase (26, 27). In fact, in our C3(−/−) mice, there were indeed increases in [VLDL + LDL] cholesterol and triglycerides. The elevated plasma LDL particle number which results from the increased secretion of apoB-100 lipoprotein particles by the liver increases the risk of coronary disease in humans. Unquestionably, further study will be required to define the role of the ASP as a determinant of the rate of retention and release of fatty acids from adipose tissue. The present data, however, indicate that for normal TG clearance after an oral fat load, the ASP pathway must be intact and thus the ASP pathway may play an important role in energy storage.

This study was supported by grants from Medical Research Council of Canada (to KC, #MT-13716; to ADS, #MT-5480), and Servier Amérique (to ADS and KC). K. Cianflone is a Research Scholar of the Heart & Stroke Foundation of Canada. I. Murray is a recipient of the Research Institute Award of the Royal Victoria Hospital.

Manuscript received 5 February 1999 and in revised form 6 May 1999.

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