Regulation by retinoic acid of acylation-stimulating protein and complement C3 in human adipocytes

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Acylation-stimulating protein (ASP), a product of complement C3, stimulates triacylglycerol synthesis in adipocytes. Previous studies have identified transthyretin, associated with chylomicrons, as a stimulator of C3 and ASP production. Since both transthyretin and chylomicrons transport retinyl ester/retinol, our goal was to investigate whether retinoic acid (RA) could be a potential hormonal mediator of the effect. Inhibitors of protein synthesis and protein secretion eliminated the stimulatory effects of chylomicrons on both C3 and ASP production in human differentiated adipocytes, suggesting that *de novo* protein synthesis and secretion are both required. Incubation with chylomicrons increased C3 mRNA levels ($37 \pm 1.5\%$). RA alone or with chylomicrons had a stimulatory effect on C3 production

(29-fold at 16.6 nM RA) and ASP production. An RA receptor antagonist blocked stimulation of C3 mRNA and C3 secretion by both RA and chylomicrons. Finally, RA and chylomicrons activated a 1.8 kb C3-promoter-luciferase construct transfected into 3T3-F442 and 3T3-L1 cells (by $41 \pm 0.2\%$ and $69 \pm 0.3\%$ respectively), possibly via RA receptor half-sites identified by sequence analysis. This is the first evidence documenting stimulation by RA of the C3 gene. Thus we propose RA as a novel cellular trigger in chylomicrons that subsequently results in increased ASP production by adipocytes after a meal.

Key words: adipose tissue, C3adesArg, chylomicrons.

INTRODUCTION

Complement C3 is a plasma protein that plays a major role in the complement system [1]. C3 is synthesized not only in the liver, but also in extra-hepatic tissues: fibroblasts, mononuclear phagocytes, endothelium, myoblasts and adipocytes [2–5]. Furthermore, complement factor B and adipsin (factor D) are also produced by adipocytes [2–5]. These three proteins are synthesized and secreted in a differentiation-dependent manner in both murine and human adipocytes.

Complement C3 interacts with factor B and adipsin (factor D) [2,3,5,6] to generate the cleavage product C3a. The C-terminal arginine residue of C3a is removed by carboxypeptidase to produce C3adesArg, which is identical to acylation-stimulating protein (ASP) [6]. *In vitro*, ASP is a potent stimulator of triacylglycerol (TG) synthesis in human adipocytes [7], and *in vivo* it accelerates the clearance of postprandial TG in both lean and obese (*db/db*) mice when administered intraperitoneally [8,9]. C3 knockout male mice (which are thus ASP-deficient) have delayed TG clearance, which can be partially corrected by administration of ASP [9]. In contrast, Wetsel et al. [10] did not observe any change in TG clearance with C3 knockout mice. These differences may be due to the influence of the background strains of these mice (K. Cianflone, I. Murray, S. Phelis, H. Vu, L. Y. Chen and A. D. Sniderman, unpublished work). Further, we have also demonstrated a decrease in adipose tissue mass in ASP-deficient female mice, even when TG clearance is normal [11]. These opposing viewpoints have been discussed in two recent review articles [12,13]. In spite of the fact that adipsin and factor B are also involved in ASP production via the alternative complement pathway, to date there is no published information on lipid profiles and fat metabolism in factor B or adipsin knockout mice [14,15].

Although, in humans, ASP levels in the general circulation do not increase postprandially [16–19], ASP production from subcutaneous adipose tissue increases in the local environment, concurrent with increased TG lipolysis after a meal [16,17], and elevated circulating ASP levels are found in obese subjects [20,21]. These findings all re-inforce a connection between the alternative complement pathway and adipose tissue metabolism.

The regulation of C3 production has been examined in various tissues and cells. Cytokines such as interleukin-1 α and -1 β [22], interferon γ [22] and interleukin-6 have been shown to increase C3 production. Hormones such as insulin [23], glucocorticoids [24] and oestradiol [25] are also involved in augmenting C3 production. By contrast, tumour necrosis factor- α [26] has been shown to decrease C3 production. We have shown previously that chylomicrons had a profound stimulatory effect on the production of C3 (the precursor to ASP) and of ASP in cultured human differentiated adipocytes [23,27]. The effects of chylomicrons on ASP and C3 production are time- and concentration-dependent [23,27]. Recently, transthyretin (TTR), a protein loosely associated with chylomicrons, was identified as being involved in mediating the stimulatory effect of chylomicrons on C3 and ASP production [27].

TTR is a plasma protein that is found complexed with retinol-binding protein [28]. This complex binds both thyroxine and retinol, and transports the prohormones throughout the plasma compartment to specific tissue sites. It has been proposed that there is receptor-mediated uptake and internalization of TTR; however, no specific receptor has been identified. Intracellularly, thyroxine and retinol are activated to form 3,3',5-triiodothyronine and retinoic acid (RA) respectively, which bind to specific DNA-binding sites and regulate gene transcription. Since TTR alone was not sufficient to stimulate the production of C3 and ASP [27], we have proposed that it acts as a vehicle to shuttle

Abbreviations used: ASP, acylation-stimulating protein; C/EBP, CCAAT enhancer binding protein; DMEM/F12, Dulbecco's minimum essential medium/Ham's F12; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PPAR, peroxisome-proliferator-activated receptor; RA, retinoic acid; RAR, retinoic acid receptor; RT, reverse transcriptase; RXR, retinoid X receptor; TG, triacylglycerol; TTR, transthyretin.

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specific hormones to the adipocyte. In the present study, we have examined the potential role of RA as the hormonal mediator of the effect of chylomicrons on the regulation of C3 secretion and ASP production.

MATERIALS AND METHODS

Materials

BSA (essentially fatty acid free), collagenase type II, human TTR, all-*trans*-RA and 17 β -oestradiol were from Sigma (Oakville, Ontario, Canada). All tissue culture media, Dulbecco's PBS, fetal bovine serum (FBS) and tissue culture supplies were from Gibco (Burlington, Ontario, Canada). Antiserum to human TTR was from Cedarlane Laboratories (Hornby, Ontario, Canada). Trizol reagent was purchased from Gibco BRL. The RA antagonist Ro 41-5253 was a gift from Dr L. Forni (Hoffman-LaRoche Ltd., Mississauga, Ontario, Canada). The C3-Luciferase plasmid was a gift from Dr D. P. McDonnell (Duke University, Durham, NC, U.S.A.). All chemicals used for the transfection assay were purchased from Sigma. The 3T3-F442 cell line was a gift from Dr R. Germinario (Lady Davis Institute, McGill University, Montreal, Quebec, Canada), and the 3T3-L1 cell line was purchased from A. T. C. C. (Rockville, MD, U.S.A.). The 100 bp DNA ladder (N3231S) was purchased from New England BioLabs Ltd. (Mississauga, Ontario, Canada).

Culture of human differentiating adipocytes

Human adipose tissue was obtained, with informed consent, from patients undergoing reduction mammoplasty, and then processed as reported previously [23]. Briefly, adipose tissue was cleaned of connective tissue and small blood vessels, then minced and treated with 0.1% collagenase. The cell suspension was centrifuged (1500 *g* for 30 min) to pellet the stromal-vascular cells (containing the preadipocytes) and the resuspended pellet was subsequently treated with buffer for 10 min to lyse the red blood cells. After filtration through a 50 μ m filter and gentle centrifugation (1500 *g* for 10 min), the cell pellet was resuspended in minimum essential medium containing 10% (v/v) fetal bovine serum. Preadipocytes were plated out on 24-well culture plates (cells from 10 g of cleaned tissue per 24-well plate) at a concentration of 3×10^4 cells per cm². After 24 h, cells were changed to serum-free Dulbecco's minimum essential medium/Ham's F12 medium (DMEM/F12) supplemented with 7.5 mg/l insulin, 1 μ M dexamethasone, 33 μ M biotin, 17 μ M pantothenate and 0.2 nM tri-iodothyronine. Differentiating adipocytes were maintained in a 37 °C incubator in 5% CO₂, and the medium was changed twice a week for 2–3 weeks, after which time the cells exhibited adipocyte-like morphology with multiple fat droplets.

Experimental incubation for measurement of C3 and ASP secretion

On day 21 of culture, differentiated adipocytes were changed to serum-free, supplement-free DMEM/F12 overnight prior to initiation of experiments. The next day, the medium was changed to fresh medium supplemented with components as indicated. All additions were diluted with PBS to a final volume of 125 μ l and added to the cells with 375 μ l of medium (DMEM/F12). Lipoprotein lipase (Sigma) was added at a concentration of 0.25 unit/ml to all chylomicron-containing samples. Monensin, colchicine, cycloheximide, TTR, RA and the RA receptor antagonist (Ro 41-5253) were added to chylomicrons and incubated (1 h, 37 °C) prior to addition to cells. RA was solubilized in ethanol (8.32 mM stock), and Ro 41-5253 was solubilized in

DMSO (1 mM stock). Both were then diluted in the media to give a maximum of 0.1% (v/v) ethanol or DMSO respectively. This was performed according to the product information supplied by commercial supplier of RA (Sigma product no. R2625) and by Dr L. Forni, Hoffman-LaRoche Ltd. [29] respectively. Following incubation (6 h, unless otherwise indicated), the medium was removed and frozen immediately at –70 °C for later analysis of medium ASP and C3 levels. The cells were washed twice with ice-cold PBS, and then 0.5 ml of 0.1 M NaOH was added to dissolve cell proteins, which were measured by the method of Bradford [30] using a commercial kit (Bio-Rad, Mississauga, Ontario, Canada).

Medium ASP determination

ASP was measured using a sandwich ELISA, as described previously [16,23,27]. A murine (in-house) monoclonal antibody to the eight-amino-acid C-terminus of ASP was used as capture antibody. The monoclonal antibody was coated at 7 μ g/ml in PBS (100 μ l per well in a 96-well plate) overnight at 4 °C and blocked with 1.5% (w/v) BSA for 2 h at room temperature. The plate was washed three times with wash solution between each step (0.05% Tween 20 in 0.9% NaCl). Standard ASP solutions (0–2.13 ng/ml), samples (conditioned culture media diluted appropriately) and control plasma samples were added at 100 μ l per well. The plate was incubated for 1 h at 37 °C and then washed, followed by incubation for 1 h at 37 °C with 100 μ l of rabbit antiserum to human ASP (raised against the holoprotein), diluted appropriately (1:2000) in PBS/0.05% Tween 20. The plate was then incubated for 30 min at 37 °C with 100 μ l of goat anti-(rabbit IgG) conjugated to horseradish peroxidase (1:5000; Sigma) diluted in PBS/0.05% Tween 20. Following the final wash, the colour reaction was initiated with 100 μ l of *o*-phenylenediamine dihydrochloride (1 mg/ml) in 100 mM sodium citrate/0.05% Tween 20. After visual development, the reaction was stopped with 50 μ l of 2 M H₂SO₄ and absorbance was read at 490 nm. ASP concentration was plotted against absorbance, and linear regression analysis was performed.

Medium C3 determination

Medium C3 was also determined by a sandwich ELISA, as described previously [23,27]. A murine monoclonal antibody to the C3d fragment of C3 (Quidel, San Rafael, CA, U.S.A.) was coated at 1 μ g/ml in PBS (100 μ l per well) overnight at 4 °C and blocked with 1.5% (w/v) BSA for 2 h at room temperature. The plate was washed three times with wash solution (0.05% Tween 20 in 0.9% NaCl) between each step. A standard solution (0–10 ng/ml) of C3 (Calbiochem), as well as test samples (conditioned culture media diluted appropriately) and in-house control plasma samples (diluted 1:100000), were added at 100 μ l per well. Subsequent steps were identical to those in the ASP sandwich ELISA. Goat polyclonal anti-C3 (1:5000; Quidel) and anti-(goat IgG) conjugated to horseradish peroxidase (1:1250; Sigma) were used for the C3 sandwich ELISA. C3 concentration was plotted against absorbance, and sample C3 concentrations were calculated by linear regression.

Chylomicron preparation and fractionation

Blood was obtained from healthy subjects with normal lipoprotein profiles 3 h after a high-fat meal and collected on ice into Vacutainer tubes containing EDTA as anticoagulant. Plasma was isolated immediately by low-speed centrifugation at 4 °C, and the chylomicrons were isolated by discontinuous preparative ultracentrifugation according to the procedure of Havel et al.

[31]. Plasma was layered under a salt solution of density 1.006 g/ml, and chylomicrons were isolated after centrifugation for 30 min at 40000 *g* (11 °C). TG and cholesterol concentrations of the chylomicrons were measured using commercially available colorimetric enzyme assays (Boehringer Mannheim, Laval, Quebec, Canada).

mRNA analysis by reverse transcriptase-PCR (RT-PCR)

Cells were treated as described above for experimental incubations. Following incubations, the medium was removed and total RNA was isolated using Trizol reagent (Gibco BRL) as described by manufacturer. Reverse transcription was performed as described previously using a random hexa-oligomer primer (Gibco) to generate cDNA [2,5]. Total RNA (3 µg) was denatured in the presence of RNase inhibitor (Gibco) for 5 min at 65 °C. The RT reaction mixture [final concentrations per reaction: 1 × buffer, 10 units of RNA, 200 units of MMLV (Moloney murine leukaemia virus) RT, 0.01 mM dithiothreitol and 0.5 mM each of dATP, dCTP, dGTP and dTTP] was added to the denatured RNA and incubated for 2 h at 37 °C. The reaction was quenched at 95 °C (5 min) and diluted to 100 µl.

cDNA was amplified using in-house primers for C3 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (house-keeper gene), as described previously [2,5]. We have reported previously that the product signal for GAPDH was linear for up to 29 cycles, and that for C3 was linear for up to 37 cycles, in differentiated adipocytes. The cycle number utilized was 35 cycles for C3 and 25 cycles for GAPDH. The cDNA (4 µl of the RT reaction) was added to the PCR reaction mixture (final concentrations/tube: 0.5 unit of *Taq* polymerase, 1 × buffer for *Taq*, 2 mM MgCl₂, 10 µM tetramethylammonium chloride and 1 µM each of 5' and 3' primers) and amplified (1 min at 95 °C, 1 min at 60 °C and 1 min at 72 °C). The PCR product generated was linear with between 1 µl and 5 µl of cDNA from the RT reaction. The PCR products were separated on a 7% piperazine diacrylamide polyacrylamide gel alongside a 100 bp DNA mass ladder (New England Biolabs; N3231S). The gels were stained using silver staining (Bio-Rad) and scanned using a densitometer for quantification. The PCR product signal was linear over a 4-fold range (5–20 µl), and 10 µl was routinely applied to the gel. The DNA mass ladder was used to construct a standard curve which was linear from 18 ng to 97 ng ($r^2 = 0.90–0.98$). All samples quantified were within the range of the mass standards utilized. Values were expressed as a ratio of C3 to GAPDH.

3T3 cell culture and C3 promoter activity

3T3-F442 and 3T3-L1 cells were maintained in DMEM/F12 containing 10% (v/v) fetal calf serum. Cells were plated in 24-well plates 24 h prior to transfection. DNA was introduced into the cells using LipofectAMINE reagent (Gibco) as described by the manufacturer. The C3-Luciferase plasmid contained a 1.8 kb fragment of the C3 promoter (–1807 to +58 bp). Transfection of the DNA (189 ng/well) into 3T3-F442 and 3T3-L1 cells was allowed to proceed for 5 h, and then the cells were washed, fresh Phenol Red-free medium containing 10% (v/v) fetal bovine serum was added, and the cells were cultured for 36 h. Subsequently, the cells were lysed and assayed for luciferase activity. On each plate, triplicate wells were transfected with pCMV (cytomegalovirus) β-galactosidase (62 ng/well) to assess transfection efficiency, which ranged from 28% to 37% (average 32%).

Statistics

Values are reported as means ± S.E.M. (with all determinations for each point in each experiment performed at least in triplicate). Statistical significance was set at $P = 0.05$, and was determined using either Student's *t*-test or one-way ANOVA (with multiple comparisons by Bonferroni's method) as indicated.

RESULTS

Effects of inhibitors of protein synthesis and secretion

In previous work we demonstrated that chylomicrons stimulate the production of ASP, as well as that of its precursor C3, in cultured human adipocytes [23,27]. The specific mechanism might result from increased synthesis of C3 or increased secretion of a preformed pool of C3. To examine these issues, we first looked at the effects of inhibitors of protein synthesis or secretion on the production of C3 and ASP. Based on the fact that the effect of chylomicrons is both concentration- and time-dependent [23,27], an incubation time of 6 h in the presence of 50 µg/ml chylomicron TG, which gave linear stimulation previously, was utilized [23,27]. Cultured differentiated adipocytes were incubated with inhibitors of protein secretion (monensin and colchicine) in the presence and absence of chylomicrons (Table 1). Monensin and colchicine alone had little effect on the basal production of C3 or ASP, which was already very low. In the presence of chylomicrons, the production of C3 and ASP was increased markedly; however, ASP constituted only a small portion of total C3. The addition of monensin (with chylomicrons) decreased C3 secretion by $90 \pm 1\%$ ($P < 0.001$) as compared with the chylomicron positive control. Similarly, the addition of colchicine (with chylomicrons) decreased C3 secretion by $72 \pm 1\%$ ($P < 0.001$). For ASP, there was complete loss of production as a result of the addition of monensin or colchicine as compared with the respective chylomicron control [$-99 \pm 1\%$ ($P < 0.001$) and $-96 \pm 0.5\%$ ($P < 0.001$) respectively].

A protein synthesis inhibitor (cycloheximide) was also tested in the presence and absence of chylomicrons (Table 2). The addition of cycloheximide (with chylomicrons) decreased C3 production by $75 \pm 5\%$ ($P < 0.001$) compared with the chylomicron positive control. Cycloheximide almost completely inhibited ASP production ($-92 \pm 2\%$; $P < 0.001$). Overall, the inhibition of both *de novo* protein synthesis and secretion of C3 (precursor protein) resulted in diminished ASP production. Therefore the stimulatory effect of chylomicrons on C3 and ASP production involves both protein synthesis and secretion.

Table 1 Effects of inhibitors of protein secretion on the production of C3 and ASP in human differentiated adipocytes

Differentiated human adipocytes were exposed to monensin (10 µM) or colchicine (10 µg/ml) in the absence (–) or presence (+) of chylomicrons (50 µg of TG/ml of medium) for a period of 6 h. PBS was used for the basal incubations. Medium C3 and ASP levels were measured by sandwich ELISAs. The results are means ± S.E.M. ($n = 12$), where $P < 0.001$ for the differences between C3 and ASP (ANOVA), and $*P < 0.001$ compared with values in the presence of chylomicrons (multiple comparison analysis).

Conditions	Concentration (pmol/mg of cell protein)			
	C3		ASP	
	–	+	–	+
Basal	2.1 ± 0.9	111.2 ± 83	0.2 ± 0.5	24.1 ± 3.6
+ Monensin	2.3 ± 0.9	11.5 ± 0.9*	1.0 ± 0.5	1.0 ± 0.5*
+ Colchicine	4.2 ± 1.1	32.0 ± 0.9*	0.2 ± 0.5	1.7 ± 0.5*

Table 2 Effects of inhibitors of protein synthesis on the production of C3 and ASP by human differentiated adipocytes

Differentiated human adipocytes were exposed to cycloheximide (10 $\mu\text{g/ml}$) in the absence or presence of chylomicrons (50 μg of TG/ml of medium) for a period of 6 h. C3 and ASP levels were measured in cell media. Values are means $\pm$ S.E.M. ($n = 12$). Significance of differences: * $P < 0.001$ compared with chylomicrons alone (ANOVA).

Conditions	Concentration (pmol/mg of cell protein)	
	C3	ASP
Basal	2.07 $\pm$ 0.40	0.23 $\pm$ 0.09
Cycloheximide	2.03 $\pm$ 0.75	0.94 $\pm$ 0.79
Chylomicrons	111.23 $\pm$ 8.29	24.13 $\pm$ 3.62
Chylomicrons + cycloheximide	27.53 $\pm$ 12.30*	1.86 $\pm$ 0.67*

Effects of RA on C3 and ASP secretion

In plasma, TTR and retinol-binding protein form a transport complex for thyroxine and retinol. The thyroxine is bound to TTR and the retinol to retinol-binding protein. Although we have shown previously that TTR increased C3 and ASP production significantly, the addition of TTR alone was not sufficient to give stimulation equivalent to that due to chylomicrons [27]. Accordingly, we proposed that TTR acts as a vehicle to shuttle hormone(s) from chylomicrons to the adipocyte. It has been demonstrated previously that retinol is transported in the lipid core of chylomicrons as a fatty acid ester [33], and can be translocated into adipocytes [34] and converted into RA, the bioactive form of retinol. RA was therefore investigated as the possible active component of chylomicrons that is responsible for the stimulation of C3 and ASP production, and was used within a range of concentrations used previously in adipocytes [35–37].

At low concentrations of RA (Figure 1, left panel), there was a concentration-dependent effect on levels of C3 in the medium. An RA concentration of 15 μM had the most pronounced effect on C3 production as compared with basal secretion (70.8 $\pm$ 36.7 compared with 2.4 $\pm$ 0.8 pmol of C3/mg of cell protein). However, the effects of RA on ASP production were much smaller, and not statistically significant. When RA was added to chylomicrons (Figure 1, right panel), the patterns for C3 and ASP

were similar. There was no further stimulation over the effect of the chylomicrons alone, and an inhibitory effect was seen at higher RA doses. There was no change in cell protein measured under the different conditions when compared with the controls, suggesting that the RA is not toxic.

Effects of RA antagonist on secretion of C3 and ASP

The stimulatory effects of RA on C3 and ASP production were investigated further using an antagonist to the RA receptor (RAR). The antagonist (Ro 41-5253) is a synthetic retinoid that binds the RA receptors RAR α (high affinity), RAR β and RAR γ (low affinity), and subsequently blocks binding of RA [29]. Adipose tissue expresses mRNAs for RAR α , RAR β and RAR γ , and for retinoid X receptor α (RXR α), RXR β and RXR γ , with RAR α , RAR γ , RXR α and RXR β being the most abundant isoforms in mature adipocytes [38]. Addition of antagonist blocked the stimulation by RA of C3 production at RA concentrations of 7.5 μM ($-86 \pm 28\%$; $P = 0.01$) and 15 μM ($-99 \pm 1\%$; $P = 0.002$) (Figure 2, left panel). In addition, the antagonist effectively blocked the stimulation of C3 production by chylomicrons, even in the presence of RA or TTR: chylomicrons, $-88 \pm 15\%$ ($P = 0.039$); chylomicrons + 3.75 μM RA, $-97 \pm 1\%$ ($P < 0.001$); chylomicrons + TTR, $-69 \pm 8\%$ ($P < 0.001$). There was also a noticeable decrease in ASP production on addition of 7.5 μM antagonist of $-91 \pm 26\%$ ($P < 0.05$) (Figure 2, right panel). However, there was only partial inhibition by the antagonist of the stimulation by chylomicrons of ASP production. Thus the stimulatory effect of chylomicrons on C3 appears to be mediated through RA, while the subsequent changes seen in the ASP concentration are likely to represent an indirect effect of the change in concentration of the substrate (C3).

Effects on C3 mRNA expression in human adipocytes

From the above data, it can be seen that chylomicrons stimulate both *de novo* C3 synthesis and C3 secretion, and that RA appears to be involved in this effect. Since RA executes its actions via a family of nuclear receptors (RARs and RXRs) [34], thus regulating gene transcription, the chylomicron stimulatory effect was investigated at the level of gene transcription for C3. Cells were incubated with and without chylomicrons for 2, 6 or 7 h, and C3

Figure 1 Effects of RA on C3 production in differentiated human adipocytes

Differentiated human adipocytes were exposed to increasing amounts of RA in the absence of chylomicrons (left panel; $n = 15$) or in the presence of a constant concentration of chylomicrons (right panel; 50 μg of lipoprotein TG/ml of medium; $n = 9$) over a period of 6 h. C3 and ASP levels were measured in cell media, and the results (means $\pm$ S.E.M.) are expressed as pmol/mg of cell protein (c.p.). Significance of differences are as follows. Left panel: C3, $P = 0.006$ (ANOVA) where * $P < 0.001$ compared with no addition by *post hoc* Bonferroni test; ASP, not significant. Right panel: C3, $P = 0.008$ by ANOVA and * $P = 0.012$ compared with chylomicron alone; ASP, $P < 0.001$ by ANOVA and * $P < 0.001$ compared with chylomicron alone.

Figure 2 RA receptor antagonist blocks the stimulation by chylomicrons and RA of C3 and ASP production

Differentiated human adipocytes were exposed to RA (at the indicated concentration in μM), chylomicrons (Chylo; 50 μg of TG/ml) and/or TTR (1.25 μg of TTR/ml) for 6 h. CTL, control. The RA receptor antagonist Ro 41-5253 was added as indicated (+ RA Antag; 3.3 μM). C3 (left panel) and ASP (right panel) levels were measured in cell media, and the results (means $\pm$ S.E.M.; $n = 12$) are expressed as pmol/mg of cell protein (c.p.). Multiple-comparison analysis was performed (ANOVA) to compare results in the presence and the absence of RA receptor antagonist: * $P < 0.005$.

Figure 3 RA antagonist and anti-TTR antibody block the stimulation by chylomicrons of C3 mRNA

Differentiated human adipocytes were incubated with or without chylomicrons (50 μg of TG/ml) for 2, 6 or 7 h with no other additions (NA) or, at 6 h, with the addition of polyclonal antiserum to TTR (pAb; 2.5 μg /ml) or RA receptor antagonist (RAant; 3.3 μM). The medium was removed from the cells and total RNA was isolated. Using in-house primers for C3 and GAPDH (GAP), RT-PCR was performed. The results are expressed as the C3/GAPDH ratio (arbitrary units) and are means $\pm$ S.E.M. ($n \geq 5$). Significance of differences: * $P = 0.04$ for chylomicrons (Chylo) at 6 h compared with control (Ctl) at 6 h; + $P < 0.001$ for chylomicrons at 6 h compared with chylomicrons at 2 h and 7 h by multiple comparison analysis (ANOVA).

mRNA was analysed by semi-quantitative RT-PCR (Figure 3). In the control cells there was no significant change in C3 mRNA levels over the 7 h incubation period. However, there was a significant increase in C3 mRNA levels following 6 h of incubation with chylomicrons compared with the control at 6 h [C3/GAPDH ratios of 87 ± 14 ($n = 12$) and 45 ± 4 ($n = 5$) respectively; $P < 0.05$]. In addition, C3 mRNA levels after 6 h of chylomicron incubation were also increased when compared with the chylomicron incubation at 2 and 7 h. This increase in C3 mRNA levels at 6 h again suggests that *de novo* synthesis of C3 was involved. This time profile is consistent with the increase in C3 secretion into the medium, which was minimal at 2 h, maximal at 6 h and had reached a plateau at 7 h (no change from 6 h) [27].

Inhibitors of the chylomicron effect were also tested for their effects on C3 mRNA levels at 6 h. We have shown previously

Table 3 Activation by RA of the C3 promoter

The transcriptional activity of the C3 promoter was assessed in 3T3-L1 and 3T3-F442 cells following transient transfection with a C3-promoter-luciferase construct. Cells were stimulated with chylomicrons (50 μg of TG/ml) or RA (3.75 μM) for 36 h following transfection. Subsequently, the cells were lysed and assayed for luciferase activity. The results are expressed in arbitrary units, and are means $\pm$ S.E.M. ($n = 9$). Significance of differences: * $P = 0.009$ compared with control.

Conditions	Luciferase activity (units)	
	3T3-L1	3T3-F442
Control	2.78 ± 1.38	40.81 ± 4.81
+ Chylomicrons	5.38 ± 1.00	55.20 ± 7.07
+ RA	$9.01 \pm 1.86^*$	$69.16 \pm 5.81^*$

that a polyclonal antibody to TTR inhibits the stimulation of C3 by chylomicrons [27]. On addition of a polyclonal antibody to TTR, the chylomicron-induced increase in C3 mRNA levels was blocked (Figure 3). Addition of the RA antagonist also blocked the effect. These results are consistent with the chylomicron stimulation of C3 production acting via gene up-regulation and stimulation of *de novo* protein synthesis, and both TTR and RA appear to be involved in the chylomicron-induced stimulation of C3 production.

Effects on C3-promoter-luciferase reporter gene expression in 3T3 cells

Analysis of the 5' flanking region (1018 bp of exon 1) of the C3 gene has previously identified putative response elements for interferon γ , interleukin-6, oestrogen, glucocorticoids, nuclear factor- κ B and thyroid hormone [39]. Our analysis of this C3 promoter region using the Mac Vector program version 6 (Oxford Molecular Group) indicated that there are also two putative RXR binding half-sites at -268 to -275 and -205 to -212 bp. These sites have not been tested to our knowledge. A C3 promoter (-1807 to $+58$ bp) fused to a firefly luciferase reporter gene was used to test activation of the C3 promoter by RA in

transiently transfected 3T3-F442 and 3T3-L1 cells. As seen in Table 3, a 36 h incubation with chylomicrons resulted in slight activation of the C3 promoter in both 3T3-F442 and 3T3-L1 cells. Incubation with RA ($3.75 \mu\text{M}$) for 36 h had a significant effect in both 3T3-F442 and 3T3-L1 cells as compared with the control [3T3-F442 cells: $(69.2 \pm 5.8) \times 10^3$ and $(40.8 \pm 4.8) \times 10^3$ arbitrary units respectively ($P < 0.05$); 3T3-L1 cells: $(9.0 \pm 1.9) \times 10^3$ and $(2.8 \pm 1.4) \times 10^3$ arbitrary units respectively ($P < 0.05$)]. Oestradiol, which has been shown to stimulate C3 promoter-luciferase activity in hepatocytes [40], also increased C3 promoter activity in preadipocytes, by $22 \pm 7\%$ in 3T3-F442 cells and $92 \pm 11\%$ in 3T3-L1 cells. Stimulation of the C3 promoter for 6 h demonstrated a similar trend, and the addition of the RA receptor antagonist to chylomicrons resulted in the complete loss of activation of the C3 promoter (results not shown). Thus this evidence confirms the involvement of RA in the stimulatory effect of chylomicrons on C3 production in adipocytes.

DISCUSSION

The present results demonstrate that the stimulatory effect of chylomicrons occurs through moderate up-regulation of C3 mRNA and larger increases in *de novo* protein synthesis and secretion of C3, thus resulting in an increase in ASP production. Although antibody blockage of TTR can inhibit the increase in C3 mRNA as well as the secretion of both C3 and ASP [27], when TTR was added alone to adipocytes there was little stimulatory effect [27]. Therefore we proposed that TTR acts as a transporter of a hormonal signal, which we believe is RA. It is of note that RA does not enhance the effect of chylomicrons (suggesting a similar and non-additive mechanism); in addition, an RA antagonist can effectively block the effects of both RA and chylomicrons (individually and together) on gene expression, protein synthesis and secretion of C3. It has been demonstrated previously that increases in protein secretion occur without large changes in mRNA levels, and that RA is capable of prolonging mRNA stability [41]. Thus minimal changes in C3 mRNA levels could result in increases in secreted C3, and retinol/RA is likely to be one of the bioactive components of chylomicrons that stimulate C3.

Although the effect of chylomicrons was comparable for C3 and ASP, stimulation by RA and its inhibition by the RA antagonist were less pronounced for ASP than for C3. In addition, the absolute level of ASP was much lower than that of C3. The concentration of ASP will depend not only on the level of its precursor, C3, but also on the enzymic activity of the conversion process, which involves C3, adipsin, factor B and cell attachment activators. How this overall process, which generates ASP, may be influenced by RA is unknown. Although RA has been shown previously to modulate the expression in adipocytes of adipsin [42], a key enzyme in the ASP pathway, this is the first evidence to demonstrate that RA has a direct regulatory effect on complement C3 in adipocytes, although the role of RA in adipocytes has been studied extensively.

Retinol is an exogenously derived fat-soluble vitamin (vitamin A) that is incorporated into the lipid core of the dietary TG-rich lipoproteins, chylomicrons, in intestinal villus cells. Most of the dietary retinol, as retinyl ester in chylomicrons, is taken up by the liver, where the majority of the retinoid reserves are stored [43]. Retinol is then transported in the circulation bound to retinol-binding protein/TTR and transferred to cells. However, it has been demonstrated that retinyl ester is also transferred from chylomicrons to adipocytes [34] and, in fact, adipose tissue is the

second largest storage site for retinoids in the body [44]. Retinol-binding protein, a plasma transport protein for retinol, has been shown to be synthesized and secreted by adipocytes [44]. In addition, adipocytes express cellular retinol-binding protein, the intracellular protein that is thought to have a primary role in retinol uptake, esterification and oxidation to RA [34]. Within the cells, the retinol is esterified by lecithin:retinol acyltransferase for storage as retinyl ester, or oxidized enzymically to the bioactive forms of RA, i.e. all-*trans*-RA and 9-*cis*-RA [34].

RA regulates the expression of several genes by binding to specific nuclear receptors, RARs (RAR α , RAR β and RAR γ) and RXRs (RXR α , RXR β and RXR γ) (for a review, see [34]). These nuclear receptors are all found in adipocytes, are ligand-dependent and function as transcription factors that recognize specific response elements on DNA. RARs can form homodimers or heterodimers with RXRs, and RXRs can also form homodimers or heterodimers with the thyroid hormone receptor, the peroxisome-proliferator-activated receptor (PPAR) and the vitamin D receptor [34]. Cross-talk has also been demonstrated between thyroid hormone receptors, PPARs and RARs. In addition, it has been reported that RXR can be activated in the RAR/RXR heterodimer in the presence of RAR ligands [45], and as a heterodimer it can bind to imperfect repeats of a hexamer consensus [37].

While RA acutely increases the expression in adipocytes of cellular retinol-binding protein [35], RAR β and RAR γ [38], acyl-CoA oxidase [46], S14 [47], the uncoupling proteins UCP1 and UCP2 [48], phosphoenolpyruvate carboxykinase [36], fatty acid transport protein (FATP-1) [49] and acyl-CoA synthetase [49], it decreases the expression of adipsin [42], lipoprotein lipase [46] and glycerol-3-phosphate dehydrogenase [50]. RA also affects gene expression and/or protein secretion in other cell types. Low-dose RA has been implicated in the stimulation of adipogenesis [34,45], whereas high-dose RA blocks the CCAAT enhancer binding protein β (C/EBP β)-dependent activation (early differentiation signal) of PPAR α and C/EBP α expression, which leads to the inhibition of adipose differentiation [34]. From our data, RA also appears to have a stimulatory effect at low doses and an inhibitory effect at high doses on the production of C3 and, to a lesser extent, ASP. It has been suggested that the diversity of effects depends not only on RA concentration, but also on isomer availability and retinoid receptor subtype expression [34].

In view of the above evidence, we propose that, in the postprandial state, chylomicrons transport dietary TG and retinol/retinyl ester to the adipocyte. The retinol associated with TTR/retinol-binding protein and/or chylomicrons is then presented to the adipocyte [33,34]. This retinol is transferred to the cell and metabolized further to the bioactive form of RA, and/or stored as retinyl ester [34]. Thus we propose that RA can function as a postprandial signal that increases the synthesis and secretion of C3 and, consequently, affects ASP levels. The augmentation in ASP levels stimulates the adipocyte to synthesize and store TG. This is consistent with the *in vivo* evidence that demonstrates a similar temporal profile of ASP production and TG uptake in the micro-environment of the adipose tissue [16,17].

In summary, retinol/retinyl ester is a novel postprandial signal that is transferred from chylomicrons, and partly accounts for the stimulatory effect of chylomicrons on the production of C3 and subsequently ASP. Taken together with the previous observation that insulin is also capable of stimulating C3 and ASP production [23], we believe the effects of insulin, chylomicrons and other factors will all be relevant in regulating ASP production and, consequently, increasing TG storage after a meal. We

hypothesize that the increased activity of the ASP pathway may play a pathophysiological role in the development of obesity, and that the potential regulation by RA may provide possible strategies for future pharmacotherapy.

This work was supported by a grant to K.C. from the Heart and Stroke Foundation. K.C. is a research scholar of Fonds de la Recherche en Santé du Québec. T.S. is the recipient of a Medical Research Council of Canada Doctoral Research Award (9810DRN-1414-58633). We appreciate the help of Dr G. Schwarz, Dr L. Lessard and Dr R. Charbonneau in obtaining adipose tissue, and of Steve Phélis in preparation of the ASP monoclonal antibody. We are grateful to Dr M. J. Kornblatt and Magdalena Maslowska for helpful discussions.

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Received 18 August 2000/12 February 2001; accepted 9 March 2001