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Obesity-inducing diet promotes acylation stimulating protein resistance

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ABSTRACT

Acylation stimulating protein (ASP) is an adipokine derived from the immune complement system that is involved in energy homeostasis and inflammation. ASP acts on and correlates positively with postprandial fat clearance in healthy subjects. However, in obesity, ASP levels are elevated and correlate inversely with fat clearance, indicative of a potential resistance to ASP. Using a mouse model, we hypothesized that, over time, diet-induced obesity (DIO) would result in development of ASP insensitivity, as compared to chow-fed animals as controls. Injection of recombinant ASP in DIO mice failed to accelerate fat clearance to the same extent as in chow-fed mice. DIO mice exhibited higher basal levels of plasma ASP and, after 30 weeks of diet, showed lower ASP receptor (*C5L2*) expression in adipose tissue compared to chow-fed mice. Additionally, *ex vivo* ASP stimulation failed to induce normal Ser^{473}AKT phosphorylation in adipose tissue from DIO mice VS chow-fed controls. These results demonstrate for the first time a state of diet-induced ASP resistance. Changes in the ASP-C5L2 pathway dynamics in obesity could alter the development of obesity and co-morbidities such as atherosclerosis and type 2 diabetes.

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1. Introduction

Acylation stimulating protein (ASP), identical to C3adesArg, is an adipose tissue-derived hormone linked with the immune complement system. Many studies have demonstrated the association of ASP with fat storage and glucose uptake *in vivo* and *in vitro* [1]. In humans, plasma ASP is associated with indices of fat mass and body mass, and correlates with various blood lipid parameters including triglycerides, fatty acids and cholesterol [1]. Administration of ASP enhances *in vivo* postprandial fat clearance in both wildtype and genetically obese mice [2]. While ASP deficient mice have delayed postprandial fat clearance, administration of ASP to these mice normalizes fat absorption [3], further strengthening the relation between ASP and lipid clearance. ASP effects are additive and independent to those of insulin [4].

ASP levels are positively associated with several metabolic pathologies, including obesity, visceral adiposity, atherosclerosis and type 2 diabetes [1]. Recent reports also demonstrate a role for ASP and its recently identified receptor, *C5L2*, in obesity-related chronic inflammation [5,6] and other immune processes such as sepsis and lung inflammation [7,8].

Several studies have evaluated potential functional roles of *C5L2* [9,8,10], although these remains controversial [11]. *C5L2* is a seven-transmembrane serpentine receptor expressed widely in tissues, including white and brown adipose tissue, muscle, liver and immune cells [9]. ASP function is mediated through *C5L2* phosphorylation and β-arrestin interaction, which leads to controlled internalization and recycling/degradation of *C5L2* [12]. The signaling cascade of ASP includes PI3-kinase, PDK, AKT, PLC, PKC, ERK and PLA2 activation [13].

Postprandial generation of ASP within the adipose tissue microenvironment correlates with dietary fatty acid uptake [14]. However, while circulating ASP levels in obesity, type 2 diabetes and cardiovascular diseases are increased, lipid uptake is delayed [15]. High levels of fasting ASP are therefore associated with delayed postprandial triglyceride clearance [16], a paradox analogous to increased fasting insulin in subjects with glucose intolerance. Such *in vivo* evidence suggests a potential interference in ASP signaling leading to a state of ASP resistance.

Published studies support the concept of decreased/increased ASP sensitivity. Sex steroid hormone treatment of adipocytes decreases *in vitro* ASP sensitivity and reduces fat storage, an effect mediated through a reduction in *C5L2* expression and interference with signaling proteins [17]. *C5L2* sensitivity could also be potentially altered through dimerization [18], cell surface density changes [19] and β-arrestin binding [10]. Altogether, these *in vitro* studies are consistent with the potential development of "ASP resistance" *in vivo*.

Although high ASP is a hallmark of obesity and its co-morbidities, no study has yet demonstrated "ASP resistance", nor

Abbrevations: ASP, acylation stimulating protein; AUC, area under the curve; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HF/HS, high-fat high-sucrose; LF, low-fat; TG, triglyceride.

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addressed the potential link between nutrition and ASP sensitivity. Nutrient overload is known to be a key factor in other types of hormonal desensitization, including insulin and leptin resistance. Proof of concept for hormone resistance can be characterized through a functional resistance, where expected physiological effects are not triggered normally upon stimulation, and a molecular resistance, where downstream effectors are not activated or chemically modified to the same extent.

The aim of the present study was to verify the existence of a diet-induced ASP-resistant state *in vivo* by utilizing a rodent model of diet-induced obesity in comparison with chow-fed controls.

2. Materials and methods

2.1. Animals

Male C57Bl/6 wildtype mice were obtained from Charles River Laboratories (Wilmington, PA, USA). At 8 weeks, mice were weight-matched and randomly fed low fat (LF) diet (6.2% kcal fat Harlan Teklad 2918; Harlan, Montreal, Canada) or high fat–high sucrose (HF/HS) diet (58% kcal fat D12331; Research Diets Inc., New Brunswick, NJ, USA) for 20 or 30 weeks and were euthanized at the end of the protocol. All protocols were pre-approved by the Laval University Animal Care Committee and were conducted in accordance with the Canadian Council of Animal Care (CACC) guidelines.

2.2. Recombinant ASP

Recombinant human ASP was prepared and its purity (98%) and endotoxin-free content verified as previously described [10,18].

2.3. ASP quantitation

Mouse plasma ASP was measured using a sandwich ELISA as previously described [20].

2.4. Real time quantitative PCR

C5L2 was quantified using custom primers (Forward: CTGGTGGTGTGGTTCATCAT, Reverse: GCTCACATCCAGGAAGCTGT) using *GAPDH* (Quantitect Primer Assay, Qiagen, Gaithersburg, MD, USA) as housekeeping gene.

2.5. Fat load

One week before the end of the protocol, fasted mice were separated into weight-matched groups and received an intra-peritoneal injection of recombinant ASP (1.5 μg/g body weight) or vehicle (PBS) immediately followed by administration of a fat load (13 μl of olive oil per g of body weight by intragastric gavage). Blood samples were taken at time 0, 1, 2, 3, and 4.5 h after the fat load. Plasma triglycerides (TG) were measured using a commercial colorimetric kit (Roche Diagnostics, Richmond, VA, USA).

2.6. Ex vivo AKT phosphorylation

Fresh gonadal adipose tissue (250 mg) was stimulated *ex vivo* for 30 min with either PBS (control), insulin (100 nM) or ASP (200 nM, 600 nM) in serum-free DMEM/F12 in a CO_2 incubator at 37 °C. Tissue pieces were lysed using Bio-Rad Cell Lysis kit (Bio-Rad, Mississauga, ON, Canada) and phosphorylation of Ser^{473}AKT was assessed with Luminex technology, using Bio-Plex Phosphoprotein and Total assay kits (Bio-Rad, Mississauga, ON, Canada).

2.7. Statistical analysis

Results are expressed as mean ± sem. Groups were compared either using one-way ANOVA, two-way ANOVA with Student–Newman–Keuls post hoc test or t-test using Prism 5.0 software (GraphPad, CA, USA). Statistical significance was set as $p < 0.05$, where * <0.05, ** <0.01, *** <0.001.

3. Results

3.1. ASP effects on fat clearance are reduced following a HF/HS diet

The hallmark physiological effect of ASP is an increase in clearance of postprandial lipids. ASP sensitivity was evaluated *in vivo* with respect to two parameters, (1) a HF/HS vs LF diet and (2) the length of the diet protocol: 20 weeks vs 30 weeks. Mice fed a LF diet for 20 weeks showed a strong response to ASP injection with respect to fat clearance. Following ASP administration, plasma TG following a fat load returned to basal levels more rapidly than PBS-injected controls with an overall reduction in plasma TG of 58% based on Area-under-the curve (AUC) ($p < 0.05$, Fig. 1A). Similarly, even after 30 weeks on LF diet, older mice (weighing an average of 31.3 g from 26.2 g at 20 weeks) were still ASP responsive, with a marked reduction of 49% in postprandial TG profile following ASP injection ($p < 0.05$, Fig. 1B). However, mice fed a HF/HS diet for 20 or 30 weeks (weighing on average 44.5 g and 48.6 g, respectively) failed to respond similarly to ASP stimulation during a fat load. No significant effect could be detected in both groups (Fig. 1C, D).

3.2. HF/HS diet increases circulating ASP but reduces C5L2 adipose tissue expression

Mice administered a HF/HS diet for 20 weeks showed increased ASP levels compared to LF diet fed controls (303 ± 29 vs 226 ± 22 nM, $p = 0.05$) (Fig. 2A). No significant change in *C5L2* expression was detected in gonadal or inguinal fat depots between LF and HF/HS fed mice.

The effect on ASP levels of a HF/HS vs LF diet was heightened after a 30 week regimen (422 ± 56 vs 255 ± 18 nM, $p < 0.05$, Fig. 2A). Further, *C5L2* expression was altered by the 30 week diet: inguinal fat *C5L2* mRNA was reduced while a tendency to decrease in gonadal depot was observed (inguinal $p < 0.01$, gonadal $p = 0.09$, Fig. 2B). Moreover, *C5L2* expression in both gonadal and inguinal fat depots of 30 week HF/HS fed mice was significantly lower than in 20 week HF/HS fed mice ($p < 0.05$ for both).

3.3. Reduced ASP-dependant phosphorylation of Ser473 AKT in obese mice

Fresh adipose tissue was stimulated *ex vivo* by ASP or insulin. The phospho-Ser^{473}AKT/Total AKT ratio was measured, as it is a well-known phosphorylation target involved in both insulin and ASP signaling [13]. Insulin and both concentrations of ASP (200 and 600 nM) induced a strong phosphorylation of Ser^{473}AKT in adipose tissue from mice fed a LF diet during 20 or 30 weeks (Fig. 2C). However, while insulin and ASP still significantly increased phosphorylation of Ser^{473}AKT in 20 week HF/HS fed mice, ASP induction of Ser^{473}AKT phosphorylation was strongly reduced in 30 week HF/HS fed mice.

4. Discussion

In the present study, a potential age and diet-dependent development of ASP resistance was evaluated. While changes in ASP

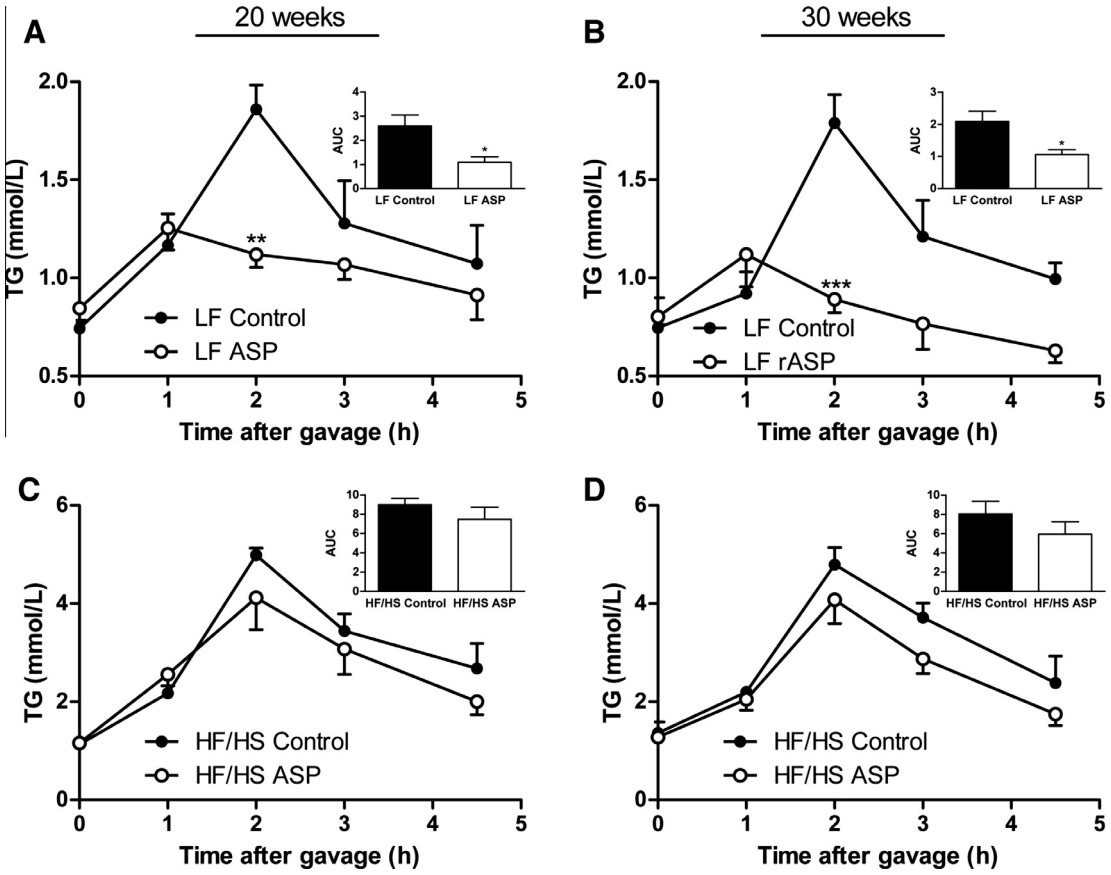

Fig. 1. Functional ASP resistance: Impaired stimulation of triglyceride clearance. A fat gavage with/without previous ASP injection was performed and plasma TG was measured over 4.5 h. Results for the cohorts on a LF diet are shown after a 20 weeks (A) and a 30 weeks protocol (B). Results for the cohorts on a HF/HS diet are shown after a 20 weeks (C) and a 30 weeks protocol (D) Incremental area under the curve for each graph is shown in the insert. Data is presented as TG (mmol/L). Results are expressed as mean ± sem where differences vs Control (PBS) are expressed as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ with $n = 10–12$ per group.

sensitivity have been suggested in the literature, this study provides evidence for a "proof-of-concept" for the development of a diet-induced resistance to ASP *in vivo*.

One advantage of the current study is that diet-induced ASP resistance was described using both functional and molecular approaches. We demonstrated that, in DIO animals, a constant dose of ASP did not properly promote postprandial fat clearance or intracellular signaling in adipose tissue. We hypothesize that the reduction in *C5L2* expression can at least partially account for this desensitization.

The underlying mechanism behind the C5L2 downregulation in adipose tissue in obesity remains to be elucidated. ASP stimulation induces internalization of cell-surface C5L2, followed by both receptor recycling and degradation [12]. Chronically elevated ASP levels, as seen in obesity in both mice (present study) and humans [1], could be directly responsible for diminished cellular sensitivity. The increased-ASP-decreased-C5L2 profile could also be an indirect consequence mediated by other factors. Obesity is associated with increased fatty acids and inflammatory factors, and TNF-α both stimulates ASP production yet downregulates C5L2 expression and cell-surface protein [21,22]. Similarly, fatty acids (as dietary chylomicrons or non-esterified fatty acids) also increase ASP production and decrease C5L2 expression and protein [20,23,24]. In both instances this is associated with decreased ASP−C5L2 pathway functional responses [22,24]. Interestingly, this mechanism of ASP-resistance may be further enhanced directly by ASP via proinflammatory processes, as ASP stimulates cytokine release *in vitro* [25] and *in vivo* [6,26]. Conversely, treatment with rosiglitazone decreases ASP production and increases C5L2 expression in cells, leading to increased ASP binding, potentially indicative of ASP sensitization [20,22], while in humans, rosiglitazone treatment decreases postprandial production of ASP in type 2 diabetics [27].

Further, molecular mechanisms involved in ASP resistance include decreased ASP-induced phosphorylation of AKT, at a level comparable to the demonstrated DIO-induced insulin resistance. AKT is one of the main intracellular targets of the ASP-C5L2 signaling pathway and has been shown to be crucial for ASP-induced triglyceride synthesis in adipocytes [13]. Reduced AKT phosphorylation by ASP in DIO models could be due to several factors related to ASP-C5L2 signaling pathway. First, changes in C5L2 expression, as demonstrated in the present study, most likely affect the ability of ASP to elicit a cellular response. Additionally, inactivating serine phosphorylation of upstream mediators such as PI3-kinase or PDK could also contribute to decreased ASP-dependent AKT phosphorylation. Both PI3-kinase and PDK are important and well-known components of the insulin response as well, which could partly link the development of insulin resistance to the loss of ASP sensitivity via downregulation of shared intracellular response mediators. Further, in addition to AKT, PI3-kinase and PDK, several other signaling components are involved in ASP-mediated stimulation of triglyceride synthesis, including phospholipases C and A2 and MAPK/ERKPLC [13], however the timeframe at which the DIO-induced obesity affects these pathways was not determined.

Interestingly, while the *in vitro* ASP signaling pathway appears to be affected only at 30 weeks, there was already evidence of ASP-resistance *in vivo* at 20 weeks, as evidenced by increases in

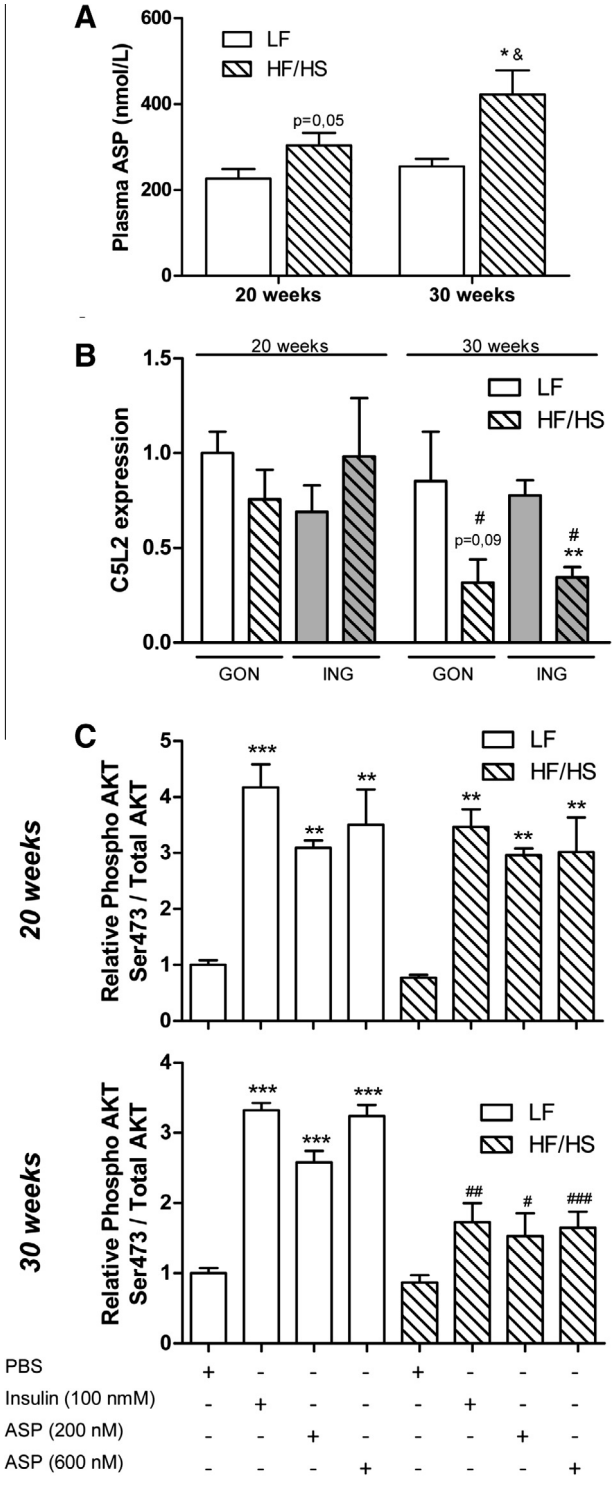

Fig. 2. Molecular ASP resistance: dysfunctional signaling. Plasma ASP levels for all cohorts are shown in panel (A) Gonadal (GON) and inguinal (ING) adipose tissue expression of *C5L2* is shown in panel (B) PBS, insulin and ASP-stimulated phosphorylation of Ser^{473}-AKT in *ex vivo* adipose tissue is shown in panel (C), where the top figure shows results for the 20 week diet protocol and the bottom figure shows results for the 30 week protocol. Results are expressed as ASP nmol/L (A), relative expression (B) or relative phosphorylation (C) Results are expressed as mean ± SEM where differences vs controls are expressed as $* p < 0.05$, $** p < 0.01$, $*** p < 0.001$ with $n = 8–12$ per group (A and B), $n = 4$ (C), and where differences between animals on LF and HF/HS diet for the same treatment are expressed as $^{\#} p < 0.05$, $^{\#\#} p < 0.01$, $^{\#\#\#} p < 0.001$.

plasma ASP and lack of ASP enhanced clearance of postprandial triglyceride. ASP has been shown to enhance the lipoprotein lipase-mediated clearance of dietary triglycerides in adipocytes *in vitro* [28], and this pathway would appear to be affected even as early as 20 weeks.

In a context of obesity and dyslipidemia, the development of ASP resistance can be considered deleterious. Impaired ASP effects on postprandial lipid clearance and reduced *C5L2* expression could contribute to increased circulating lipids and heightened inflammation, and contribute to the DIO-induced metabolic dysfunction which includes insulin resistance.

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References

[1] K. Cianflone, Z.N. Xia, L.Y. Chen, Critical review of acylation-stimulating protein physiology in humans and rodents, Biochimica et Biophysica Acta-Biomembranes 1609 (2003) 127–143.
[2] J. Saleh, J.E. Blevins, P.J. Havel, J.A. Barrett, D.W. Gietzen, K. Cianflone, Acylation stimulating protein (ASP) acute effects on postprandial lipemia and food intake in rodents, International Journal of Obesity 25 (2001) 705–713.
[3] I. Murray, A.D. Sniderman, K. Cianflone, Mice lacking acylation stimulating protein (ASP) have delayed postprandial triglyceride clearance, Journal of Lipid Research 40 (1999) 1671–1676.
[4] V. Van Hermelen, S. Reynisdottir, K. Cianflone, E. Degerman, J. Hoffstedt, K. Nilsell, A. Sniderman, P. Arner, Mechanisms involved in the regulation of free fatty acid release from isolated human fat cells by acylation-stimulating protein and insulin, Journal of Biological Chemistry 274 (1999) 18243–18251.
[5] A. Fisette, M.N. Munkonda, K. Oikonomopoulou, S. Paglialunga, J.D. Lambris, K. Cianflone, C5L2 receptor disruption enhances the development of diet-induced insulin resistance in mice, Immunobiology 218 (2013) 127–133.
[6] M.N. Munkonda, M. Lapointe, P. Miegueu, C. Roy, D. Gauvreau, D. Richard, K. Cianflone, Recombinant acylation stimulating protein administration to c3(-) mice increases insulin resistance via adipocyte inflammatory mechanisms, PLoS ONE 7 (2012).
[7] H.W. Gao, T.A. Neff, R.F. Guo, C.L. Speyer, J.V. Sarma, S. Tomlins, Y.F. Man, N.C. Riedemann, L.M. Hoesel, E. Younkin, F.S. Zetoune, P.A. Ward, Evidence for a functional role of the second C5a receptor C5L2, Federation Of American Societies For Experimental Biology Journal 19 (2005) 1003–1005.
[8] N.J. Chen, C. Mirtsos, D. Suh, Y.C. Lu, W.J. Lin, C. McKerlie, T. Lee, H. Baribault, H. Tian, W.C. Yeh, C5L2 is critical for the biological activities of the anaphylatoxins C5a and C3a, Nature 446 (2007) 203–207.
[9] D. Kalant, R. MacLaren, W. Cui, R. Samanta, P.N. Monk, S.A. Laporte, K. Cianflone, C5L2 is a functional receptor for acylation-stimulating protein, Journal of Biological Chemistry 280 (2005) 23936–23944.
[10] W. Cui, M. Lapointe, D. Gauvreau, D. Kalant, K. Cianflone, Recombinant C3adesArg/acylation stimulating protein (ASP) is highly bioactive: a critical evaluation of C5L2 binding and 3T3-L1 adipocyte activation, Molecular Immunology 46 (2009) 3207–3217.
[11] K. Johswich, A. Klos, C5L2 an anti-inflammatory molecule or a receptor for acylation stimulating protein (C3a-desArg)?, Current Topics in Innate Immunity 598 (2007) 159–180.
[12] W. Cui, M. Simaan, S. Laporte, R. Lodge, K. Cianflone, C5a-and ASP-mediated C5L2 activation, endocytosis and recycling are lost in S323I–C5L2 mutation, Molecular Immunology 46 (2009) 3086–3098.
[13] M. Maslowska, H. Legakis, F. Assadi, K. Cianflone, Targeting the signaling pathway of acylation stimulating protein, Journal of Lipid Research 47 (2006) 643–652.
[14] J. Saleh, L.K.M. Summers, K. Cianflone, B.A. Fielding, A.D. Sniderman, K.N. Frayn, Coordinated release of acylation stimulating protein (ASP) and triacylglycerol clearance by human adipose tissue in vivo in the postprandial period, Journal of Lipid Research 39 (1998) 884–891.
[15] A. Onat, G. Can, R. Rezvani, K. Cianflone, Complement C3 and cleavage products in cardiometabolic risk, Clinica Chimica Acta 412 (2011) 1171–1179.
[16] K. Cianflone, R. Zakarian, C. Couillard, B. Delplanque, J.P. Despres, A. Sniderman, Fasting acylation-stimulating protein is predictive of postprandial triglyceride clearance, Journal of Lipid Research 45 (2004) 124–131.
[17] Y. Wen, H.W. Wang, R. MacLaren, H.L. Lu, X.F. Hu, K. Cianflone, Sex steroid hormones induce acylation stimulating protein resistance in 3T3-L1 adipocytes, Journal of Cellular Biochemistry 105 (2008) 404–413.
[18] P. Poursharifi, M. Lapointe, T. Hebert, K. Cianflone, C5L2 and C5aR interaction in adipocytes and macrophages: insight into adipoimmunology, Obesity 19 (2011) S75.

[19] Y. Tahiri, F. Karpe, G.D. Tan, K. Cianflone, Rosiglitazone decreases postprandial production of acylation stimulating protein in type 2 diabetics, Nutrition and Metabolism 4 (2007).
[20] Y. Gao, D. Gauyreau, K. Cianflone, Hormone and pharmaceutical regulation of ASP production in 3T3-L1 adipocytes, Journal of Cellular Biochemistry 109 (2010) 896–905.
[21] H. Lu, D. Gauvreau, M. Lapointe, X.P. Luo, K. Cianflone, Inflammatory markers and adipokines alter adipocyte-derived ASP production through direct and indirect immune interaction, Experimental and Clinical Endocrinology & Diabetes 121 (2013) 194–200.
[22] R. MacLaren, D. Kalant, K. Cianflone, The ASP receptor C5L2 is regulated by metabolic hormones associated with insulin resistance, Biochemistry and Cell Biology 5 (2007) 11–21.
[23] T. Scantlebury, M. Maslowska, K. Cianflone, Chylomicron-specific enhancement of acylation stimulating protein and precursor protein C3 production in differentiated human adipocytes, Journal of Biological Chemistry 273 (1998) 20903–20909.
[24] Y. Wen, H. Wang, R. MacLaren, J. Wu, H. Lu, K. Cianflone, Palmitate and oleate induction of acylation stimulating protein resistance in 3T3-L1 adipocytes and preadipocytes, Journal of Cellular Biochemistry 104 (2008) 391–401.
[25] F.Q. Tom, D. Gauvreau, M. Lapointe, H. Lu, P. Poursharifi, X.P. Luo, K. Cianflone, Differential chemoattractant response in adipocytes and macrophages to the action of acylation stimulating protein, European Journal of Cell Biology 92 (2013) 61–69.
[26] A. Fisette, P. Poursharifi, K. Oikonomopoulou, M.N. Munkonda, M. Lapointe, K. Cianflone, Paradoxical glucose-sensitizing yet proinflammatory effects of acute asp administration in mice, Mediators of Inflammation (2013). Epub ahead of print.
[27] Y. Tahiri, F. Karpe, G.D. Tan, K. Cianflone, Rosiglitazone decreases postprandial production of acylation stimulating protein in type 2 diabetics, Nutrition Metabolism,Lond 4 (2007) 11.
[28] M. Faraj, A.D. Sniderman, K. Cianflone, ASP enhances in situ lipoprotein lipase activity by increasing fatty acid trapping in adipocytes, Journal of Lipid Research 45 (2004) 657–666.