C5L2 receptor disruption enhances the development of diet-induced insulin resistance in mice

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Introduction

In humans, excess food energy intake results in increased adipose tissue mass, which can lead to lipotoxicity in organs such as skeletal muscle and liver. Such ectopic fat deposition is a well-known cause for further metabolic dysfunctions such as type 2 diabetes and cardiovascular diseases. Strategies aimed at reducing the capacity of adipose tissue to store triglycerides (TGs) are faced with the question of whether this could result in excess lipid overflow into other tissues (Cianflone 2003).

The inflammatory component is another important factor in the development of obesity-related pathologies. Chronic low-grade inflammation has been associated with type 2 diabetes, cardiovascular diseases and obesity, and is evidenced in part through a cross-talk between immune cells and resident tissue cells (Hotamisligil 2006). Macrophages, infiltrated and activated in the adipose tissue, muscle and liver, release several factors such as TNF-α, IL-6, IL-10 or MCP-1 that, in addition to adipokines, alter the inflammatome profile in the organism.

Acylation stimulating protein (ASP) is an adipokine derived from the alternative complement branch of the innate immune system. Proteolytic cleavage of complement C3 by factor B and adipin leads to the formation of C3a, which is rapidly desarginated in circulation by carboxypeptidase B to produce C3adesArg, also termed ASP (Cianflone et al. 2003). ASP has been shown to promote fatty acid uptake and glucose transport in adipocytes. These effects are directly mediated through increased diacylglycerol acyltransferase
activity (Yasrue et al. 1991) and increased translocation of glucose transporters GLUT1 and GLUT4 to the cell surface (Maslowska et al. 1997; Tao et al. 1997). Inhibition of hormone-sensitive lipase (Van Hermel et al. 1999) and facilitation of lipoprotein lipase activity (Faraj et al. 2004) by ASP result in increased uptake and intracellular accumulation of TG in adipocytes. In humans, ASP levels are increased in subjects with obesity, type 2 diabetes and/or cardiovascular diseases (Onat et al. 2011).

CSL2 has been identified as a functional receptor for ASP (Kalant et al. 2003, 2005) and is expressed in multiple tissues such as white and brown adipose tissue, brain, muscle, liver and several types of immune cells, suggesting potential direct effects of ASP on many types of tissues (Kalant et al. 2005). Furthermore, the effects of ASP stimulation have been extensively evaluated in adipocytes but not as thoroughly described in other cell types. In addition to binding ASP, CSL2 also binds the proinflammatory molecule C5a; however, this does not result in an immune response (Scola et al. 2009). Such a process suggests a potential C5a-sequestering role for CSL2, which could lead to anti-inflammatory effects (Fisette and Cianflone 2010). An association of ASP with chronic inflammation in ASP–CSL2 deficient models was indicated by reciprocal links between IL-6 and ASP in previous studies (Paglia lunga et al. 2010; Wernstedt et al. 2006). Studies have previously questioned whether CSL2 binds and/or is activated by ASP and C3a (Johnswich and Klos 2007; Scola et al. 2009). We and others have however demonstrated the role of CSL2 in the signaling of ASP/C3a (Chen et al. 2007; Cui et al. 2009; Kalant et al. 2003, 2005).

ASP deficient (C3KO) and CSL2 deficient (CSL2KO) models have been used to further study the role of ASP in vivo. Complement C3 knockout mice are obligatorily ASP deficient, as C3 is the direct precursor of ASP, while CSL2 knockout mice lack the ASP receptor. A delay in postprandial TG and non-esterified fatty acids (NEFA) clearance is observed in both models (Murray et al. 1999b; Paglia lunga et al. 2007) and is normalized in ASP deficient mice by ASP injection (Murray et al. 1999a; Xia et al. 2004). The disruption of the ASP–CSL2 pathway in these knockout models has also been shown to confer an obesity-resistant phenotype. Both ASP deficient (C3KO) and CSL2KO resulted in similar phenotypes, characterized by decreased fat storage in spite of increased food intake, accompanied by increased energy expenditure (Pagli alunga et al. 2007; Roy et al. 2008; Xia et al. 2002, 2004). The metabolic similarities exhibited by the ASP deficient C3KO mice and by the CSL2KO mice further suggest that CSL2 is a functional ASP receptor. Additionally, we have shown that short-term chronic injection of ASP and CSL2 antibodies induce the same phenotype in wild type mice (Cui et al. 2007).

This evidence suggests that inactivation of the ASP–CSL2 signaling pathway could be a potential therapy for tipping the balance from energy storage to energy usage (Cianflone 2003). The metabolic changes observed in ASP or CSL2 deficient mice point toward a healthier and leaner phenotype (Paglia lunga et al. 2007; Roy et al. 2008; Xia et al. 2002, 2004). However, the delay in postprandial fat clearance and the increased food consumption exhibited by these animal models could also potentially lead to deleterious consequences. In spite of higher energy usage, a redistribution of lipids away from adipose tissue toward other tissues, such as the liver, could potentially lead to ectopic fat deposition. Indices of insulin resistance were found in CSL2 deficient mice fed a diabetogenic diet (Paglia lunga et al. 2007). Additionally, ASP has been shown to increase glucose-stimulated insulin secretion ex vivo, an important component of insulin action and sensitivity (Ahren et al. 2003).

The aim of the present study was to assess the influence of the ASP–CSL2 pathway in the development of insulin resistance using CSL2KO mice. We hypothesized that, under a diabetogenic diet, CSL2 deficient mice would exhibit decreased insulin sensitivity compared to wild type mice due to higher ectopic fat deposition and inflammation. The metabolic and inflammatory states were physiologically evaluated and compared with parameters of glucose handling and insulin sensitivity.

Materials and methods

Animals

Whole-body-C5L2KO mice were obtained and genotyped as previously described (Paglia lunga et al. 2007). All mice were on a C57Bl/6 background and had been backcrossed for at least eight generations. Heterozygous breeding in our internal colony generated CSL2KO and wild type (WT) littermates. All mice were individually housed in a sterile barrier facility with a 12 h light:12 h dark cycle. At 8 weeks, mice were placed on high fat–high sucrose (HF/HS) diet (58% kcal fat D12331; Research Diets Inc., New Brunswick, NJ, USA) for 12 weeks. At the end of the protocol, overnight–fasted mice received an intraperitoneal injection of glucose (2 g/kg body weight) supplemented with radioactive deoxyglucose (0.5 mCi [3H]deoxyglucose/kg of body weight) and were sacrificed exactly 2 h later. Animals were euthanized using a ketamine–xylazine mix. Blood was collected through cardiac puncture and tissues (muscle, liver, abdominal (perigonadal) adipose tissue) were harvested and immediately frozen in liquid nitrogen. Tissues were subsequently transferred to –80 °C and stored for further analysis. All protocols were approved and were conducted in accordance with the CACC guidelines and approved by the Laval University Animal Care Committee.

Plasma analysis

Blood was collected through cardiac puncture. Plasma TG, NEFA and glucose were measured using colorimetric enzymatic kits as follows: plasma TG (Roche Diagnostics, Richmond, VA, USA), NEFA (Wako Chemicals, Richmond, VA, USA), and glucose (Sigma). Mouse IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-17A, eotaxin, G-CSF, GM-CSF, IFN-γ, KC, MCP-1, MIP-1α, MIP-1β, RANTES, TNF-α, ghrelin, GIP, GLP-1, glucagon, insulin, leptin, PAI-1 and resistin were measured using suspension bead array immunoassay kits following manufacturer’s specifications (Bio–Plex Pro Mouse Cytokine Assay 32–plex and Bio–Plex Pro Mouse Diabetes Assay 8–plex, Biorad, Mississauga, ON, Canada) on a Bio–Plex series 100 instrument (Biorad, Mississauga, ON, Canada).

Glucose and insulin tolerance tests

Glucose tolerance test (GTT) was performed on mice (at week 11 of their regimen) following an overnight fast. Blood samples were taken from saphenous vein bleeding at 0, 15, 30, 60, and 90 min after an intraperitoneal glucose injection (2 g/kg body weight). Mice were allowed 5 days to recover. An insulin tolerance test (ITT) was then performed following a 4-h fast. Blood samples were taken from saphenous vein bleeding at 0, 15, 30, 60 and 120 min after an intraperitoneal insulin injection (1 U/kg of body weight). Glucose was measured using colorimetric enzymatic kit (Sigma) while insulin was measured using RIA kit (Linco, St Charles, MO, USA).

Glucose uptake and oxidation

Glucose uptake in abdominal adipose tissue, muscle and liver was evaluated as the accumulation of radioactive [3H] deoxyglucose in tissue lysates. Results were corrected for total protein content (muscle and liver) or weight (adipose tissue) and are expressed as relative deoxyglucose uptake. Glucose oxidation was measured ex vivo as previously described (Roy et al. 2008) with
the following modification: benzethonium hydroxide was added as the CO2 trapping agent, rather than KOH. Results are expressed as pmol of oxidized glucose per gram (g) of protein content.

**HADH activity**

Hydroxyacyl-Coenzyme A dehydrogenase (HADH) maximal (Vmax) enzyme activity was assayed as previously described (Marcus and Hosey 1980; Roy et al. 2008).

**Lipid and glycogen measurements**

Liver and muscle lipids (TG and fatty acids) were extracted from tissue pieces (40 mg) using 2 mL of heptane:isopropanol (3:2). The extract was transferred while the remaining tissue (liver and muscle) was air-dried, dissolved in 0.3 N NaOH and assessed for protein content using the Bradford method (Bio-Rad, Mississauga, ON, Canada). Organic extracts were lyophilized and lipids were redissolved in 10% Triton X-100 aqueous solution. TG and fatty acids were measured using commercial colorimetric kits as described above. Results are expressed as mmol of TG or NEFA per kilogram (kg) of protein. Liver glycogen was measured as previously described (Paglialunga et al. 2010). Results are expressed as μmol of glucose per gram (g) of liver wet weight.

**Real-time quantitative PCR**

Tissue mRNA was extracted, purified and reverse transcribed into cDNA using RNeasy Mini kits or RNeasy Lipid Tissue Mini kit and Quantitect Reverse Transcription kits (Qiagen, Gaithersburg, MD, USA). mRNA for ACACA, ADRA1B, FATP4, FBP1, GAPDH, GLUT2 and GLUT4 genes were measured using Quantitect Primer Assays (Qiagen, Gaithersburg, MD, USA). mRNA for F4-80, CD11c and CD163 was quantified using custom primers (F4-80 forward: CTTTGCTATGGCTTCCAGTC reverse: GCAAGGAGCAGCTTATACGT SEQUENCE, CD11c forward: CTGGATAGCTTTCTCTGCT reverse: GCACACTGTGTCGAACCT, CD163 forward: GGGTCCTCAGGCAACTG reverse: CTGGCTGTCCTCAAGGCT). Relative gene expression was calculated and corrected using GAPDH as the housekeeping gene. All procedures followed manufacturer’s instructions and MIQE guidelines (Bustin et al. 2009).

**Statistical analysis**

Results are expressed as mean ± SEM. Groups were compared using two-way ANOVA with SNK 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, and *** < 0.001.

**Results**

**CSL2KO mice on HF/HS show an exacerbated phenotype of insulin resistance**

CSL2KO mice have fasting glucose similar to the WT controls. However, fasting insulin is almost two-fold higher in CSL2KO (Table 1). A glucose tolerance test showed that CSL2KO mice have similar glucose clearance than WT mice (Fig. 1A). However, this clearance rate was obtained with much higher insulin levels, as shown in Fig. 1B (p < 0.001). In addition, a state of pronounced insulin resistance was confirmed through an insulin tolerance test (Fig. 1C), where CSL2KO failed to respond as efficiently to insulin as WT controls (p < 0.05). Furthermore, glucagon levels following the GTT (Post-GTT) were significantly higher in CSL2KO mice (Table 1), confirming the diabetic state. Meanwhile, plasma leptin and PAI-1 concentrations, both strongly associated with insulin resistance (Kahn and Flier 2000; Ma et al. 2004), were significantly higher in CSL2KO mice vs WT controls (Table 1).
C5L2 is critical for appropriate absorption, storage and oxidation of energetic substrates

Previous studies have shown that ASP and C5L2 deficient mice display altered substrate partitioning. In the present study, we examined glucose metabolism in the tissues of the “metabolic triad”. Interestingly, deoxyglucose uptake was reduced in abdominal adipose tissue (−42%, p < 0.05) and skeletal muscle (−49%, p < 0.05) of C5L2KO mice and increased in the liver (+41%, p < 0.05), all vs WT controls (Fig. 2A). Furthermore, ex vivo C5L2KO liver glucose oxidation was increased two-fold (p < 0.001) while the rate-limiting enzyme of fatty acid oxidation (HADH) showed a significantly lower activity (p < 0.01) (Fig. 2B). Adipose tissue GLUT4 levels did not change significantly in C5L2KO mice while muscle GLUT4 expression was reduced and liver GLUT2 expression increased, following the trends seen in deoxyglucose absorption. Meanwhile, skeletal muscle lipid content of C5L2KO mice was increased, as both TG (p < 0.05) and fatty acids (p < 0.05) were found in higher concentration (Fig. 2C). Liver TG content was also increased (p < 0.01), but fatty acid concentrations were lower (Fig. 2D, p < 0.05), suggesting less reliance on fat as an energy source. Gene expression mRNA analysis showed that both the adipose tissue (p < 0.01) and muscle (p = 0.07) of C5L2KO mice exhibit higher FATP4 mRNA than WT controls while there is a trend for reduction in the liver (p = 0.06) (Fig. 2E). Finally, liver glycogen was significantly lower in C5L2KO mice (p < 0.05) (Fig. 2D). FBP1 mRNA was increased in both adipose tissue (p = 0.06) and the liver (p < 0.05) of C5L2KO mice vs WT.
controls. ACACA (p < 0.05) and ADRA1B (p < 0.05) mRNA were in turn higher the skeletal muscle of C5L2KO mice vs WT controls.

Disruption of C5L2 modulates the systemic inflammatory phenotype

We evaluated macrophage infiltration and polarization in abdominal adipose tissue, skeletal muscle and liver. While M1 macrophages are associated with the secretion of proinflammatory molecules, M2 macrophages mostly show an anti-inflammatory cytokine secretion profile. For all three tissues, markers of total macrophages (F4-80), M1 subtype macrophage (CD11c) and M2 subtype macrophage (CD163) increased at least threefold in C5L2KO mice vs WT counterparts, as seen in Fig. 3A. M1 and M2 macrophages markers increase in a similar ratio in the adipose tissue and skeletal muscle, but M1 marker increase ratio is higher in the liver. Plasma concentrations of several pro- and anti-inflammatory cytokines were evaluated: IL-4, IL-6, IL-9, IL-10, IL-12(p40), IL-12(p70), KC, MIP-1α, MCP-1, TNF-α, IFN-γ and RANTES. Interestingly, all cytokine levels were elevated in C5L2KO mice (Fig. 3B–M).
Table 1
C5L2KO and WT mice basal parameters.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>WT (n = 8)</th>
<th>C5L2KO (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (weeks)</td>
<td>21</td>
<td>21</td>
</tr>
<tr>
<td>Fasting glucose (mg/dL)</td>
<td>82.6 ± 6.7</td>
<td>93.1 ± 3.9</td>
</tr>
<tr>
<td>Fasting insulin (ng/mL)</td>
<td>1.35 ± 0.19</td>
<td>2.18 ± 0.26'</td>
</tr>
<tr>
<td>Post-GTT TG (mmol/L)</td>
<td>1.05 ± 0.18</td>
<td>1.29 ± 0.09</td>
</tr>
<tr>
<td>Post-GTT NEFA (mmol/L)</td>
<td>0.68 ± 0.05</td>
<td>0.61 ± 0.04</td>
</tr>
<tr>
<td>Post-GTT triglycerides (mg/dL)</td>
<td>0.31 ± 0.06</td>
<td>2.49 ± 0.54</td>
</tr>
<tr>
<td>Post-GTT leptin (ng/mL)</td>
<td>0.83 ± 0.17</td>
<td>2.93 ± 0.35'</td>
</tr>
<tr>
<td>Post-GTT CRP (mg/mL)</td>
<td>14.1 ± 5.5</td>
<td>34.3 ± 4.6</td>
</tr>
</tbody>
</table>

Basal characteristics of C5L2KO mice vs WT controls.

* Age, plasma TG, NEFA, fasting glucose, insulin, postprandial triglycerides, CRP and leptin levels were compared by t-tests where p < 0.05.

** Age, plasma TG, NEFA, fasting glucose, insulin, postprandial triglycerides, CRP and leptin levels were compared by t-tests where p < 0.001.

Discussion

In the present study, we aimed at evaluating the effects of C5L2 disruption on the development of insulin resistance. This study is the first to demonstrate a deleterious metabolic effect of ASP–C5L2 pathway disruption. On standard chow low-fat diet or a high-fat only diet, the disruption of ASP–C5L2 pathway has been previously shown to induce beneficial effects (Cianflone 2003; Pagliualunga et al. 2007; Roy et al. 2008; Xia et al. 2002, 2004). However, the joint presence of elevated fat content and sucrose in the diet pushes the balance toward a stronger negative adaptation in C5L2KO mice, which do not handle substrate overload properly under these circumstances when compared to wild type controls.

Here we show that C5L2 disruption exacerbates the development of insulin resistance in mice through two different pathways. First, C5L2KO mice demonstrate substrate partitioning typical of insulin resistant mice: reduced glucose uptake in the muscle and adipose tissue, increased glucose absorption by the liver, associated with the subsequent consequential changes in substrate oxidation. This partitioning is not deleterious per se in low-fat diet fed animals, but could worsen the condition of animals fed a calorie dense diet, as shown in this study. Redistribution of substrates in excess of tissue oxidation capacities – such as in the liver or skeletal muscle – can result in ectopic fat deposition and is known to lead to local and systemic insulin resistance (Kahn and Flier 2000). Such negative consequences of substrate partitioning have been shown in several knockout models (Goldberg et al. 2009; Kim et al. 2001; Kotani et al. 2004), while other examples exhibit a sufficient increase in energy expenditure to overcome the repartitioning and avoid ectopic fat deposition (Franckhauser et al. 2008; Smith et al. 2000). As ASP was demonstrated to be implicated in glucosed-stimulated insulin release from pancreatic cells (Ahren et al. 2003), a dysfunctional ASP–C5L2 pathway could therefore unbalance this role and trigger a compensatory mechanism.

Further, the presence of a chronic state of low-grade inflammation has been established in diet-induced obesity and this can also contribute to the development of insulin resistance (Hotamisligil 2006). Molecules such as TNF-α and MCP-1, secreted by infiltrating macrophages and adipocytes, have been shown to induce insulin resistance through several mechanisms (Hotamisligil 2006). We show in this study that C5L2KO mice exhibit a more pronounced state of chronic inflammation that wild type controls, a phenotype that likely contributes to the more severe insulin resistance. The proinflammatory effects of C5L2 disruption could be due to several processes: the absence of ASP-mediated inflammatory effects in metabolic and immune cells; the increased oxidative stress due to higher substrate oxidation; the lack of C5a sequestration by C5L2, which could be an ASP-independent process; or the absence of potential C5a receptor and C5L2 heterodimerization (Fisette and Cianflone 2010). It is interesting to note that C5a have been shown to play a role in the polarization of macrophages (Langer et al. 2010).

In conclusion, targeting ASP–C5L2 interaction to induce substrate repartitioning and potentially reduce the incidence of obesity has been previously suggested (Cianflone 2003). Our study demonstrates that in the presence of a high-fat–high-sucrose diet, a dysfunctional C5L2 receptor worsens the state of insulin resistance in mice models through metabolic and proinflammatory changes.

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References


Murray, I., Sniderman, A.D., Cianflone, K., 1999b. Mice lacking acylation stimulating protein (ASP) have delayed postprandial triglyceride clearance. J. Lipid Res. 40, 1671–1676.


