Research Article

Paradoxical Glucose-Sensitizing yet Proinflammatory Effects of Acute ASP Administration in Mice

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Acylation stimulating protein (ASP) is an adipokine derived from the immune complement system, which stimulates fat storage and is typically increased in obesity, type 2 diabetes, and cardiovascular disease. Using a diet-induced obesity (DIO) mouse model, the acute effects of ASP on energy metabolism and inflammatory processes in vivo were evaluated. We hypothesized that ASP would specifically exert proinflammatory effects. C57Bl/6 wild-type mice were put on a high-fat-high-sucrose diet for 12 weeks. Mice were then subjected to both glucose and insulin tolerance tests, each manipulation being preceded by recombinant ASP or vehicle (control) bolus injection. ASP supplementation increased whole-body glucose excursion, and this was accomplished with reduced concomitant insulin levels. However, ASP did not directly alter insulin sensitivity. ASP supplementation induced a proinflammatory phenotype, with higher levels of cytokines including IL-6 and TNF-α in plasma and in adipose tissue, liver, and skeletal muscle mRNA. Additionally, ASP increased M1 macrophage content of these tissues. ASP exerted a direct concentration-dependent role in the migration and M1 activation of cultured macrophages. Altogether, the in vivo and in vitro experiments demonstrate that ASP plays a role in both energy metabolism and inflammation, with paradoxical whole-body glucose-sensitizing yet proinflammatory effects.

1. Introduction

The past decades of obesity research have seen a change of paradigm. Adipose tissue was once labeled as a passive storage organ, while obesity comorbidities were predominantly linked with energy metabolism disorders. Currently, it is widely recognized that adipose tissue also plays a major endocrine role and that progressive metabolic diseases such as type 2 diabetes and atherosclerosis manifest a strong immune component. Moreover, molecules secreted by the expanding adipose tissue modulate systemic inflammation and contribute heavily to the pathology of the aforementioned diseases [1].

Acylation stimulating protein (ASP), identical to C3adesArg, is a protein generated through the alternative complement pathway of the immune system [2]. C3a/C3adesArg is rapidly produced during acute immunological responses such as bacterial infection, which triggers the classical or lectin pathways of the complement system. However, in the absence of acute immune response, spontaneous activation of the alternative pathway of complement, mediated through adipin, factor B, and complement C3 convertase, leads to the generation of C3a. C3a is rapidly desarginated by carboxypeptidase B or N to generate C3adesArg, termed ASP based on its specific metabolic effects. ASP acts through its only known receptor, C5L2. This 7-transmembrane receptor is widely expressed, with expression in tissues such as liver, muscle, adipose tissue, immune cells, and brain, and C5L2 shares significant homology with other complement receptors such as C3a receptor and C5a receptor [3].

In vitro, the functional role of ASP/C3adesArg has been extensively studied on adipocytes. Its primary effect is the
promotion of fat storage, accomplished by increasing diacyl-
glycerol acyltransferase activity [4] and GLUT4 translocation
[5, 6], which drive fatty acid and glucose uptake into cells
and provide the substrates for triglyceride synthesis. These
effects have been shown to be independent and additive to
those of insulin [7]. In adipocytes, ASP action is mediated
through activation of PI3kinase, phospholipases C and A2,
Akt phosphorylation, and protein kinase C [8].

The presence of C5L2 is required for ASP response in
adipocytes [9–12], but in other cell types, the interaction of
ASP with C5L2 remains controversial. However, C5a is also
a ligand for both C5L2 and C5aR [13–15]. While C3a binds
C3a receptor (C3aR), initiating a potent proinflammatory
signaling cascade, C3adesArg does not bind C3aR, and it has
canonical been considered as an inert fragment, indicative
of complement system activation, and subsequent proteolytic
cleavage of C3a [16]. However, C3adesArg has also been
shown to be implicated in several immune processes, with
less potency than C3a [17–20]. For example, C3adesArg
retains indirect chemotactic properties on hematopoietic
cells [19] and can also induce cytokine secretion by cultured
macrophages and modulate liver regeneration [20].

ASP has been shown to possess a metabolic role in both
humans and rodents. Acute postprandial production of ASP
within the adipose tissue bed has been demonstrated in
human studies, and this acute production directly correlates
with dietary fatty acid uptake into adipose tissues [21, 22].
By contrast, ASP levels are chronically increased in obesity,
type 2 diabetes, and cardiovascular diseases in humans [23],
and higher circulating levels of fasting ASP in humans are
associated with a delayed clearance of postprandial triglyc-
eride [24]. This suggests that acute ASP responses may be
very different from the metabolic profile associated with
chronically elevated ASP, and the exact consequences of acute
versus chronic ASP in these diseases remain unclear.

The aim of the present study was to assess the effects
of acute ASP administration on in vivo glucose metabolism
and rapid inflammatory responses in a diet-induced obesity
(DIO) mouse model. We hypothesized that ASP could mod-
ulate both adipose inflammation and alter systemic glucose
disposal.

2. Materials and Methods

2.1. Animals. Male C57Bl/6 wild-type mice were obtained
through breeding in our internal colony. All mice were
individually housed in a sterile barrier facility with a 12 h light
12 h dark cycles. 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. All mice
were weighed and randomly separated into two groups of
equal body weight at week 11. Throughout the study, treated
mice received a total of three ASP doses (high physiological)
intraperitoneally (1.5 μg/g body weight), and control mice
received three PBS (vehicle) injections at the following times:
(i) before glucose tolerance test (GTT) early week 11, (ii)
ITT late week 11, and (iii) GTT sacrifice week 12, with a
minimum of 5 days recovery between each injection. Blood

was collected through cardiac puncture, and tissues 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 Canadian Council of Animal Care
(CCAC) guidelines and approved by the Laval University
Animal Care Committee.

2.2. Recombinant ASP. Recombinant human ASP was pre-
pared as previously described, using a Ni²⁺-NTA–Sepharose col-
umn for initial purification via binding to the 6XHis-tag
sequence at the amino-terminal, followed by HPLC purifi-
cation as described in detail elsewhere [25]. No denaturing
agents were used at any step in the purification to avoid
ASP inactivation. Recombinant ASP purity was assessed with
mass spectrometry and was endotoxin-free as evidenced by
the Limulus amebocyte lysate (LAL) endotoxin assay (Lanza,
Walkersville, MD).

2.3. Plasma Analysis. Blood was collected through cardiac
puncture. Plasma triglycerides, non-esterified fatty acids
(NEFA), and glucose were measured using colorimetric enzym-
ic kits as follows: plasma triglyceride (Roche Diagnostics,
Richmond, VA, USA), NEFA (Wako Chemicals, Richmond,
VA, USA), and glucose (Sigma, Saint Louis, MO, USA). IL-6,
IL-10, G-CSF, GM-CSF, KC, MCP-1, MIP-1α, TNF-α, insulin,
leptin, and PAI-1 were measured using suspension bead array
immunoassay kits following the manufacturer’s specifications
(Bio-Plex Pro Mouse Cytokine Assay 23-plex and Bio-Plex
Pro Mouse Diabetes Assay 8-plex, Biorad, Mississauga, ON,
Canada) on a Bio-Plex series 100 instrument (BioRad, Missis-
sauga, ON, Canada), and information on intra- and interassay
CVs IS provided by the manufacturer and is freely available.

2.4. Glucose and Insulin Tolerance Tests. A glucose tolerance
test (GTT) was performed on mice at week 11 of their diet
after an overnight fast. Following an intraperitoneal glucose
injection (2mg/g body weight) supplemented with/without
ASP (1.5 μg/g body weight), blood samples were taken at
0, 15, 30, 60, and 90 min. Mice were allowed 5 days to
recover. An insulin tolerance test (ITT) was then performed
following a 4-hour fast. Blood samples were taken at 0, 15,
30, 60, and 90 min after an intraperitoneal insulin injection
(1mU/g of body weight) supplemented with/without ASP
(1.5 μg/g body weight) as indicated. Glucose was measured
using a colorimetric enzymatic kit (Sigma, Saint Louis, MO,
USA), while insulin was measured using a RIA kit (Linco, St.
Charles, MO, USA), and information on intra- and interassay
CVs is provided by the manufacturer and is freely available.

2.5. Tissue Glucose Uptake. At the end of the 12-week diet
protocol, overnight-fasted mice received an intraperitoneal
injection of glucose (2mg/g body weight) supplemented with
radioactive deoxyglucose (0.5 μCi (3H) deoxyglucose/g of
body weight) and ASP (1.5μg/g body weight) and were euth-
anized exactly 2 hours later. Radioactive glucose uptake in
visceral adipose tissue, muscle, and liver was evaluated using
(3H) deoxyglucose tracer uptake in tissue homogenates. Raw
using custom primers (Table 1). Relative gene expression was assessed using qRT-PCR experiments (MIQE guidelines [26]). All procedures followed the manufacturer's instructions. Briefly, confluent RAW 264.7 cells were harvested and resuspended in serum-free media, and 1 × 10⁶ cells were added to each insert. Adipocyte-conditioned media was obtained by incubating 3T3-L1 differentiated adipocytes overnight in serum-free DMEM/F12. 3T3-L1-conditioned media or serum-free medium control was then added to the lower chambers, with or without ASP (50 nM or 200 nM). Cells were incubated for 20 hours at 37°C in a CO₂ incubator. Macrophages that migrated through the membrane were quantified following the manufacturer's instructions.

2.9. Macrophage Polarization. RAW 264.7 cells were seeded in 25 cm² flasks (1 × 10⁶ cells per flask) in DMEM/F12 with 10% FBS medium. Cells were incubated for 24 h to permit attachment and cultured to reach 80% confluence. Flasks were then subjected to a combination of a polarization treatment with/without a fixed ASP concentration. Polarization treatments were as follows: Control treatment consisted of serum-free media; M1-polarization treatment consisted of 100 ng/mL LPS and 100 ng/mL of IFN-γ; M2-polarization treatment consisted of 10 ng/mL of IL-4 and 10 ng/mL of IL-13. Concentrations of ASP used in conjunction with each treatment were 0 nM (baseline), 50 nM (low physiological), and 200 nM (high physiological). Cells were incubated 6 hours at 37°C in a CO₂ incubator. After incubation, cells were immediately lysed, and mRNA was extracted.

2.10. Statistical Analysis. Results are expressed as mean ± SEM. Groups were compared using the appropriate statistical test, either one-way ANOVA with linear trend post hoc test, 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 *P < 0.05, **P < 0.01, and ***P < 0.001 and where 0.05 < P < 0.1 was considered a tendency.

3. Results

3.1. ASP Injections Did not Alter Body Weight or Food Intake. Mice were injected with high-physiological doses of ASP at the indicated time periods, as described in the methods. No change in body weight or food intake between ASP-treated animals and controls was recorded during the 12-week treatment period.

3.2. ASP Alters Substrate Partitioning. An intraperitoneal glucose tolerance test (GTT) with/without ASP was performed. The recombinant ASP dose was chosen to represent a high-physiological concentration of endogenous ASP, such as seen in severely obese individuals [22]. Glucose and insulin levels were monitored throughout a 90-minute timeframe after the injection (Figure 1). A more efficient glucose clearance was achieved with acute ASP supplementation (Figure 1a, P < 0.05). Concomitant insulin levels showed a statistical tendency to be lower in the ASP-treated mice (Figure 1b, P = 0.08). In order to assess if ASP acutely influenced insulin sensitivity, an intraperitoneal insulin tolerance test (ITT) was performed in the same animals, supplemented or not with ASP. No difference in insulin-mediated glucose response was

| Table 1: Real-time qPCR primers. |
|-------------------------------|
| F4-80 forward                  | CTTTGAGTATGGCGTCCAGTC |
| F4-80 reverse                  | GCAAGGAGGGAGCAAGTCTGTG |
| CD11c forward                  | CTGGATACCTTCTGTTGCTG |
| CD11c reverse                  | GCCACGTGCTGCTGAGCTC |
| CD163 forward                  | GGTCTATCCAGGGCAGCAC |
| CD163 reverse                  | CTGGCTGTTCCGTCAAAGGCT |
| IL-6 forward                   | GAGGATACCTCCAACAGACC |
| IL-6 reverse                   | AAGTGCATCATGTTTTGATA |
| KC forward                     | TCTCCGTACTGCTGAGGAC |
| KC reverse                     | CCACACTCAAGAATGGTCGC |
| MCP-1 forward                  | ATGGGATACCATCTTGTG |
| MCP-1 reverse                  | CCTGCTGTTACAGTGGCC |
| MIP-1α forward                 | GTGGAATCTTCCCAGGTAG |
| MIP-1α reverse                 | ACCATGACACTCTGCAACA |
| PAI-1 forward                  | GCCAGGGTGACATCAAAAT |
| PAI-1 reverse                  | GCCCTCTCATCCTGCTAA |
| TNF-α forward                  | CATCTTCTAAAAATGAGTGCAA |
| TNF-α reverse                  | TGGGAGTGACAAAGTGACAAC |

Quantitative real-time PCR primer sequences used in evaluation of expression.

2.6. Tissue Lipid. Adipose tissue, liver and muscle triglycerides, and NEFA were extracted from tissues using heptane : isopropanol (3 : 2). The organic 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. Triglycerides and NEFA were measured using commercial colorimetric kits as described above. Results are expressed as μmoles of triglycerides or NEFA per gram (g) of protein.

2.7. Real-Time Quantitative PCR. Tissue or cell mRNA was extracted, purified, and reverse transcribed into cDNA using RNeasy Mini Kits or RNeasy Lipid Tissue Mini Kits and QuantiTect Reverse Transcription Kits (Qiagen, Gaithersburg, MD, USA). mRNA for F4-80, CD11c, CD163, IL-6, KC, MCP-1, MIP-1α, PAI-1, and TNF-α was quantified using custom primers (Table 1). Relative gene expression was calculated and corrected using GAPDH (QuantiTect Primer Assay, Qiagen, Gaithersburg, MD, USA) as housekeeping gene. All procedures followed the manufacturer's instructions and minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines [26].

2.8. Macrophage Migration. Macrophage migration was assessed using QCM Chemotaxis 5 μM 24-well cell migration assay (Millipore, Billerica, MA, USA) following the manufacturer's instructions. Briefly, confluent RAW 264.7 cells were
detected in mice treated acutely with/without ASP (data not shown).

A GTT with \(^3\)H deoxyglucose supplemented with/without ASP was used to evaluate ASP effects on glucose uptake in specific tissues. Acute ASP treatment altered glucose uptake in a tissue-specific fashion; tissues relying mostly on active transport showed increased or tended to increase glucose uptake (adipose tissue, \(P = 0.08\), and skeletal muscle, \(P < 0.01\)), while the liver, relying mainly on passive transport, exhibited lower glucose uptake (\(P < 0.01\)), as shown in Figure 2(a).

Acute ASP treatment did not alter muscle or liver triglyceride (data not shown). However, cellular NEFA content was increased in skeletal muscle (\(P < 0.05\)) and reduced in liver (\(P < 0.05\)), as seen in Figure 2(b). Circulating NEFA was decreased as well (\(P < 0.05\)) (Table 2).
3.3. ASP Increased Plasma Cytokine Concentrations and Altered Liver and Adipose Tissue Cytokine Expression. The ASP bolus also induced acute changes in adipokine and cytokine profiles. Plasma leptin was reduced, while several inflammation-related cytokines including IL-6, IL-10, PAI-1, TNF-α, KC, and MIP-1α were significantly increased (Figure 3(a), Table 2). mRNA levels for these same cytokines were evaluated in liver and adipose tissue, with increased expression of IL-6, KC, TNF-α, and MCP-1 in both tissues and increased MIP-1α and PAI-1 in liver (Figure 3(a)). A global inflammatory phenotype is clearly induced by acute ASP injection in obese mice, mediated by both adipose tissue and liver changes.

3.4. Macrophage Infiltration and Polarization Is Influenced by ASP. Macrophage infiltration and polarization were evaluated in adipose tissue, skeletal muscle, and liver. As shown in Figure 3(b), macrophage marker F4/80 was not significantly altered by ASP treatment, although it tended to increase in the skeletal muscle (P = 0.07). No change was seen in anti-inflammatory M2 polarization marker (CD163). However, in all tissues, proinflammatory M1 polarization marker was significantly higher or tended to increase (adipose tissue, P < 0.05, skeletal muscle, P = 0.07, liver, P = 0.10), suggesting a preferential activation of infiltrating macrophages to an M1 phenotype with acute ASP treatment. In order to further investigate the direct role of ASP in the physiological changes observed in mice, in vitro effects of ASP on macrophage migration and polarization in RAW 264.7 macrophages were evaluated. ASP showed dose-dependent chemotactic migration properties when incubated alone with macrophages (Figure 4(a)), and these effects were further additive to those of 3T3-L1 adipocyte-conditioned media. ASP by itself had very limited capacity to induce macrophage M1 or M2 phenotype polarization (Figures 4(b) and 4(c)). However, RAW 264.7 macrophages treated with INF-γ and LPS to stimulate M1 polarization, as assessed by CD11c mRNA expression (Figure 4(b)), showed further M1 polarization with ASP treatment, in a dose-dependent fashion (Figure 4(b)). By contrast, while treatment with IL-4 and IL-13 increased M2 polarization, as assessed through CD163 mRNA expression (Figure 4(c)), ASP did not further enhance this effect.

4. Discussion

In the present study, the inflammatory role of acute high-physiological ASP administration in a context of established obesity was evaluated. Previous studies have focused either on the metabolic role of ASP in adipocytes or the immune role of ASP/C3adesArg in immune cells. This study examines a broader, more integrative physiological role of ASP. This study
clearly demonstrated that ASP/C3adesArg acutely enhances glucose-insulin clearance mechanisms in spite of its acute paradoxical in vivo and in vitro proinflammatory effects.

One advantage of the current study is that acute ASP effects rather than chronic effects were evaluated. In previous studies, effects of chronic administration of ASP were evaluated in either C3KO or wild-type mice [27, 28], demonstrating effects on decreased insulin sensitivity, weight gain, and lipid/glucose metabolism. However, it is difficult to establish whether these effects are direct or indirect or a consequence of resistance induced by chronic stimulation. Similar concerns are an issue in knockout mice models where the proinflammatory effects [27, 29] may result from indirect disruptions in the immune system. Specifically, C3KO mice lack ASP but also lack an active complement system; C5L2KO mice lack ASP/C5L2 interaction but also lack C5a/C5aR interaction which could disrupt the balance in C5a/C5aR-mediated effects.

The results shown in this study are not consequent to changes in body weight or food intake, which could have
been confounding factors. We can therefore directly relate the changes observed to acute bolus ASP-mediated effects. The dose of ASP used was high physiological-pathological, as it theoretically could increase circulating ASP levels in obese mice by up to tenfold (from 400 nM to 4.2 μM) and would mimic the high concentration of ASP generated following an acute immune activation of the complement system or seen in obese individuals. The most striking results obtained are the rapid, global increases in plasma inflammatory cytokines and the M1 polarization of tissue macrophages upon acute ASP treatment. We show additional in vitro evidence that supports a direct role for ASP in macrophage recruitment and M1 activation that is additive to classic cytokine effects. Notwithstanding the demonstrated proinflammatory ASP effects, acute ASP enhances plasma glucose excursion in DIO mice, with increased uptake into skeletal muscle and adipose tissue and reduced liver glucose uptake, and this effect is mediated in the presence of lower circulating insulin levels. While this could point towards increased insulin sensitivity, we also demonstrated that a single acute dose of ASP does not directly alter insulin sensitivity. We therefore speculate that the known in vitro activating effect of ASP on GLUT4 translocation in adipocyte and muscle cells [6] could explain the increase in muscle and adipose tissue glucose uptake, while passive concentration-dependent uptake in the liver, relying mostly on GLUT2 transporters, would indirectly be lowered.

Our study, while demonstrating intriguing novel properties of ASP, is also consistent with previously published results. Chronic ASP supplementation decreased plasma NEFA, as in the present study [28]. An acute ASP bolus, on the other hand, has been shown to increase glucose excursion following a fat load [30], while C5L2−/− (ASP receptor) deficient mice showed delayed glucose clearance [12]. Additionally, the potential chemoattractive role proposed for ASP [19, 20] has been demonstrated specifically in this study. He et al. also showed that high levels of ASP increased TNF-α and IL-6 liver expression [20]. We demonstrate here that this phenotype is also present in other tissues as well as in the circulation and extends to other major cytokines. By contrast, leptin levels were acutely decreased by ASP bolus. This effect could be potentially linked with the reduction of insulin secretion, a well-known stimulator of leptin release.

Inflammation and macrophage recruitment are inherent components of several biological systems related to tissue injury and infections and are beneficial in the short term. At the same time, the capacity of immune cells to exert their respective functions is modulated by their metabolic status. To label ASP acute effects as deleterious because of the proinflammatory capacities is likely an oversimplification of a potentially complex process. Certain cytokines exhibit both proinflammatory and glucose-sensitizing effects, while others have dual roles (that are either concentration dependent or time dependent) in immunity. Adiponectin is a well-known adipokine that improves insulin sensitivity but has been shown to exert both anti-inflammatory and proinflammatory effects, depending on the context [31]. IL-6 is another prime example of a cytokine with paradoxical effects; IL-6 increases glucose excursion through a short-term insulin-sensitizing process in spite of its role in inflammation [32, 33]. Long-term exposure to IL-6 may then, paradoxically, induce insulin resistance [34].

Humans with obesity, type 2 diabetes, and cardiovascular disease have been shown to exhibit higher chronic plasma ASP levels [2]. These morbidities are associated with macrophage infiltration and M1 polarization within several tissues. However, even in the absence of chronically increased ASP, adipose tissue acutely increases local ASP production postprandially [21, 22]. Thus, the response to an acute increase versus chronically elevated levels may differ and may depend on the concomitant diet (such as high-fat and high-sucrose diet). While it has been suggested that C3adesArg is simply an inactive immune by-product representative of complement activation, we clearly demonstrate in this study a direct role of ASP within these inflammatory processes. The relative contribution of acute ASP-stimulated inflammation in obesity-related diseases remains to be investigated. As with several signaling pathways in obesity, it could be that chronic substrate overload leads to excessive activation of the ASP signaling pathway, eliciting a deleterious response that could exacerbate the development of comorbidities through a low-grade proinflammatory phenotype transition [3].

5. Conclusion

The present study sheds new light on the dual role of ASP in energy metabolism and inflammation. ASP exhibits both substrate storage and proinflammatory properties that have been strongly associated with several pathologies. Effectively, neutralizing a hormone that can be both produced locally and acts locally represents a strong pharmaceutical challenge. A TNF-α inhibitor, for example, failed at altering insulin sensitivity in a human cohort [35]. Additional studies on C5L2, the only known receptor for ASP, are needed to better understand ASP-C5L2 signal transduction and to assess the potential of C5L2 as a pharmaceutical target in obesity and metabolic syndrome.

Abbreviations

ASP: Acylation stimulating protein
C3aR: Complement C3a receptor
DIO: Diet-induced obesity
GAPDH: Glyceraldehyde 3-phosphate dehydrogenase
GM-CSF: Granulocyte colony-stimulating factor
G-CSF: Granulocyte macrophage colony-stimulating factor
GTT: Glucose tolerance test
HF/HS: High-fat/high-sucrose
ITT: Insulin tolerance test
KC: Keratinocyte-derived chemokine
MCP-1: Monocyte chemoattractant protein-1
MIP-1α: Macrophage inflammatory protein-1α
NEFA: Nonesterified fatty acids
PAI-1: Plasminogen activator inhibitor-1
TG: Triglyceride.
Conflict of Interests

The authors have no conflict of interests to declare.

Author’s Contribution

A. Fisette designed the study, acquired data, analyzed results, and wrote the paper. P. Poursharifi supported a training scholarship (Obesity Network). K. Oikonomopoulou is a recipient of an NSERC postdoctoral fellowship. K. Cianflone holds a Tier 1 Canada Research Chair in Adipose Tissue. The would like to acknowledge the help of Jennifer Dumais and Erika Turmel, CRIUCPQ, for animal handling, and of Mélanie Cianflone, for paper preparation.

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