Substance P decreases fat storage and increases adipocytokine production in 3T3-L1 adipocytes

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Miegou P, St-Pierre DH, Lapointe M, Pourshariﬁ P, Lu H, Gupta A, Cianﬂone K. Substance P decreases fat storage and increases adipocytokine production in 3T3-L1 adipocytes. Am J Physiol Gastrointest Liver Physiol 304: G420–G427, 2013. First published December 20, 2012; doi:10.1152/ajpgi.00162.2012.—Obesity, inﬂammation, and insulin resistance are closely linked. Substance P (SP), via its neurokinin 1 receptor (NK1R), mediates inﬂammatory and, possibly, neuroendocrine processes. We examined SP effects on lipid storage and cytokine production in 3T3-L1 adipocytes and preadipocytes. Expression of the high-affinity SP receptor NK1R has been shown to bind to neurokinin-2 and -3 receptors with lower afﬁnity. In animal models, NK1R knockout (KO) mice showed neither increased food intake nor weight gain compared with their wild-type littermates (14).

Therefore, this study was undertaken to examine the effects of SP on lipid storage, cytokine production, and insulin-related genes in mature adipocytes.

MATERIALS AND METHODS

Reagents

DMEM-Ham’s F-12 medium (DMEM-F12), PBS, bovine calf serum, and FBS were supplied by Gibco (Burlington, ON, Canada).
Table 1. Target genes in custom mouse RT² Profiler PCR array

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>National Center for Biotechnology Information Reference No.</th>
<th>Official Full Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pparg2</td>
<td>NM_011146</td>
<td>Peroxisome proliferator-activated receptor-γ</td>
</tr>
<tr>
<td>Cebpα</td>
<td>NM_007678</td>
<td>CCAAT/enhancer-binding protein-α</td>
</tr>
<tr>
<td>Cebpβ</td>
<td>NM_009883</td>
<td>CCAAT/enhancer-binding protein-β</td>
</tr>
<tr>
<td>Dgα1</td>
<td>NM_001046</td>
<td>Diacylglycerol O-acyltransferase 1</td>
</tr>
<tr>
<td>Cd36</td>
<td>NM_007643</td>
<td>CD36 antigen</td>
</tr>
<tr>
<td>Fapt4</td>
<td>NM_024406</td>
<td>Fatty acid-binding protein 4, adipocyte</td>
</tr>
<tr>
<td>Cav3</td>
<td>NM_007617</td>
<td>Caveolin 3</td>
</tr>
<tr>
<td>Adipoq</td>
<td>NM_009605</td>
<td>Adiponectin, C1q and collagen domain-containing</td>
</tr>
<tr>
<td>Gapdh</td>
<td>NM_008084</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
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IBMX, dexamethasone, penicillin-streptomycin, and trypsin were obtained from Life Technologies (Carlsbad, CA). SP (human sequence) was generously provided by Peptidec Technologies (Montréal, Qc, Canada). Peptide purity (>95%) and identity were confirmed by analytical reverse-phase HPLC using a C18 3-μm column and by liquid chromatography-mass spectrometry. Concentrated peptide solutions (1 mg/ml in PBS) were divided into aliquots and stored at -80°C. The SP concentrations had been shown previously to elicit responses in vitro (1, 10, 28).

**Effect of SP on 3T3-L1 Adipocyte Differentiation**

The 3T3-L1 preadipocytes (American Type Culture Collection, Manassas, VA) were routinely cultured in DMEM-F12 supplemented with 10% bovine calf serum (37°C, 5% CO2) and subdivided at <70% confluence. Preadipocytes were differentiated to adipocytes by standard methods, as described in detail previously (20).

The effect of SP on 3T3-L1 differentiation was evaluated using a modification of the differentiation procedure described above. Two-day postconfluent (day 0) preadipocytes were treated with the standard differentiation medium supplemented with SP (10 nM) for 2 days. At each medium change thereafter, every 2 days according to the standard differentiation procedure, the regular differentiation medium was supplemented with 10 nM SP. Cells were visually assessed by microscopy, with images obtained at the indicated time intervals.

**Effect of SP on Mature Adipocyte Functions**

**Fatty acid uptake.** Mature 3T3-L1 differentiated adipocytes were preincubated for 2 h in serum-free DMEM-F12 and treated with hormones for 1 h (200 nM). Fatty acid uptake and incorporation were evaluated in the constant presence of the hormones, as previously described (20).

**Adipokine secretion.** Adipocytes were incubated for 24 h with the indicated hormones, and the cell culture medium was collected. Adiponectin, MCP-1, and keratinocyte-derived chemokine (KC) levels were assessed using mouse Duoset ELISA assays (R & D Systems, Minneapolis, MN). Complement C3 levels were evaluated using a mouse complement C3 ELISA kit (Kamiya Biomedical, Seattle, WA).

**Assessment of gene expression by RT-quantitative PCR assay.** Mature 3T3-L1 differentiated adipocytes were treated with 10 nM SP for 24 h. RNA was extracted using a RNeasy Mini Kit and RNase-Free DNase Set treatment according to the manufacturer’s specifications (Qiagen, Mississauga, ON, Canada), and 1-μg samples of total RNA were converted to cDNA with random primers in a total volume of 10 μl. The RT² First Strand Kit (SABiosciences, Frederick, MD) was used to prevent genomic DNA contamination. Real-time RT-quantitative PCR array analyses were performed in 96-well plates of a Custom Mouse RT² Profiler PCR Array, which contained mouse genomic DNA contamination and reverse transcription controls (Table 1). The array was designed to simultaneously quantify mRNA expression of the indicated and housekeeping genes (SABiosciences). For assessment of NK1R (a SP receptor), insulin receptor substrate-1 (IRS-1), and...
Sclk24 (GLUT4) gene expression, RNA was reverse-transcribed using the Quantitect reverse transcription kit (Qiagen). cDNA products were used as templates for the PCR assays using a SYBR Green PCR master mix and specific primers designed against mouse NK1R, IRS-1, and GLUT4 genes (NM_009313, NM_010570, and NM_009204, respectively) and mouse glyceraldehyde 3-phosphate dehydrogenase (NM_008084) as the housekeeping gene (Qiagen). Results were normalized to the housekeeping gene. A real-time thermal cycler (CFX96 real-time PCR, Bio-Rad Laboratories, Mississauga, ON, Canada) was used as previously described (20).

**Assessment of Akt phosphorylation at Ser473 by direct cell-based ELISA.** Akt phosphorylation at Ser473, as well as total Akt protein, was measured using the fast-activated cell-based ELISA Akt profiler kit (FACE kit, Active Motif, Carlsbad, CA) according to the manufacturer’s instructions. 3T3-L1 adipocytes were treated with insulin (50 nM), SP (10 nM), or both in 96-well plates for the indicated incubation times, and then cells were fixed. Each well was incubated with a primary antibody specific for phosphorylated (Ser473) or total Akt overnight at 4°C. After the cells were washed with PBS, they were incubated first with secondary horseradish peroxidase-conjugated antibody and then with developing solution. The levels of Akt phosphorylation were calculated relative to total Akt protein (ratio of phosphorylated to total protein) and total cell number in each well according to the manufacturer’s instructions.

**Experimental Procedures for Mouse and Human Adipose Tissue and Cultured Adipocytes**

**Animals.** All protocols were conducted in accordance with Canadian Council on Animal Care guidelines and preapproved by the Laval University Animal Care Committee and the Laval Hospital Research Institute committee. Adult male mice were fed standard chow (containing 24.5 g of protein, 51 g of carbohydrate, and 4 g of fat/100 g) ad libitum. The animals were euthanized, and epididymal fat depots were collected and immediately placed in Krebs-Ringer buffer at pH 7.4.

**Primary mouse adipose tissue culture assay.** Epididymal fat depots were minced into small (<10-mg) pieces and placed in serum-free medium in 24-well plates. After 2 h, explants were treated with/without 10 nM SP; tissue culture media were collected after 24 h and stored at −80°C for analysis of complement C3 levels with a mouse complement C3 ELISA kit.

**Isolation of primary mouse adipocytes.** After dissection, adipose tissues were rinsed immediately in Krebs-Ringer buffer, and fibrous tissues and blood vessels were carefully dissected and removed. The remaining adipose tissues were minced into small pieces and digested with 0.25% collagenase in 2% BSA for 45 min at 37°C under continuous shaking. The dispersed tissues were filtered through a nylon mesh sheet (250-μm pore size) and centrifuged, and the floating mature adipocytes were collected and cultured for 24 h (as described for 3T3-L1 adipocytes). MCP-1 secretion and nonesterified fatty acid (NEFA) release in primary SP-treated adipocyte culture medium were evaluated as described above for MCP-1 or as previously described (20) for NEFA.

**Isolation of human adipose tissues.** Human adipose tissues were obtained at the time of bariatric surgery, as previously described (17, 18), and gene expression data are reported in Gene Expression Omnibus profiles. Results are presented for plasma insulin, NEFA, and complement C3 and the following genes: the SP receptor NK1R (NM_015727), adiponectin (NM_004797), CEBPα, (NM_004364), CEBPβ (NM_008194), and peroxisome proliferator-activated receptor-γ (PPARγ, NM_005037).

**Statistical Analysis**

Each experiment was performed in triplicate and repeated at least three times. Values are means ± SE. Groups were compared by t-test, one- or two-way ANOVA followed by Dunnett’s post hoc test in the presence of several groups, or regression analysis for correlations using GraphPad Prism (GraphPad Software, San Diego, CA) for graphs and statistical analyses. Statistical significance was set as P < 0.05.

**RESULTS**

**SP Decreases Fatty Acid Storage in 3T3-L1 Mature Differentiated Adipocytes**

The effect of SP on fatty acid uptake and incorporation into neutral lipid (TG) was investigated in real-time in

![Image](https://example.com/image.png)

**Fig. 2.** SP receptor neuropeptide 1 receptor (NK1R) is expressed in adipocytes and preadipocytes, and SP interferes with differentiation. 3T3-L1 preadipocytes were differentiated with/without SP supplementation (10 nM) in the differentiation medium and evaluated by microscopy at days 0, 2, 4, 6, and 8. Representative micrographs are shown. Inset: RNA from 3T3-L1 preadipocytes and mature adipocytes was extracted, and gene expression of NK1R was evaluated by real-time PCR. Gene expression is presented relative to expression of the housekeeping gene GAPDH for an average of 3 separate determinations.

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3T3-L1 mature differentiated adipocytes. As indicated in Fig. 1A, SP treatment over 2 h decreased fatty acid incorporation, which contrasts with the stimulatory effect of 50 nM insulin (168 ± 2%, P < 0.01). Calculation of the area under the curve (Fig. 1B) indicates a dose-dependent decrease in fatty acid uptake in response to 10 nM SP (ANOVA linear trend P < 0.02 to a maximum of 31 ± 7%, P < 0.05). Treatment with comparable concentrations of SP (1 to 10 nM) had previously been shown to elicit responses in vitro (1, 10, 28).

SP Increases Lipolysis in Primary Mouse Adipocytes

The effect of SP on lipolysis was evaluated in primary mouse adipocytes and found to increase NEFA release by 138 ± 13% (P < 0.05; Fig. 1C). As expected, insulin (positive control) decreased NEFA release by 51 ± 16% (P < 0.05).

SP Decreases mRNA Expression of Adipogenic and Lipogenic Factors in 3T3-L1 Mature Adipocytes

After 24 h of treatment, SP induced a significant decrease in expression of the differentiation-related transcription factors PPARγ type 2 (−64 ± 2%, P < 0.001) and CEBPα (−65 ± 2%, P < 0.001), as well as the lipid uptake/storage genes fatty acid-binding protein-4 (FABP4, −59 ± 2%, P < 0.001) and diacylglycerol O-acyltransferase-1 (−45 ± 2%, P < 0.01), in 3T3-L1 adipocytes. On the other hand, SP upregulated expression of CD36 (+181 ± 2%, P < 0.01), a fatty acid transporter (Fig. 1D).

SP Decreases Differentiation of 3T3-L1 Adipocytes

As treatment of mature differentiated 3T3-L1 adipocytes demonstrated changes in transcription factors, the effect of chronic SP on preadipocyte differentiation was evaluated. As shown in Fig. 2 (inset), 3T3-L1 preadipocytes and mature

Fig. 3. SP effects on secretion of adipocytokines in 3T3-L1 adipocytes, mouse adipose tissue, and primary adipocytes. A–C: 3T3-L1 adipocytes were treated with 1 and 10 nM SP for 24 h, and complement C3, monocyte chemoattractant protein-1 (MCP-1), and keratinocyte-derived chemokine (KC) secretion was evaluated in cell culture medium. Values are means ± SE. *P < 0.05 vs. CTL (by ANOVA). D and E: mouse adipose tissue was dissected, and primary adipocytes were prepared by collagenase digestion. Adipose tissue and primary adipocytes were treated with 10 nM SP for 24 h, and complement C3 and MCP-1 secretion was evaluated in cell culture medium. Values are means ± SE. *P < 0.05 vs. CTL (by t-test).
adipocytes express the SP receptor NK1R. 3T3-L1 preadipocytes were differentiated according to standard procedure with/without chronic supplementation with SP. As demonstrated by microscopy in Fig. 2, chronic supplementation with SP decreased differentiation, with a delay in overall development of lipid droplets within the treated cells.

**SP Increases Proinflammatory Adipokine Secretion in 3T3-L1 Adipocytes and Mouse Primary Adipocytes**

As SP has been shown to have effects on cytokine secretion in other cells (25), the effect of SP on adipokine secretion was evaluated in 3T3-L1 adipocytes. SP induced significant dose-dependent increases in complement C3 secretion (126 ± 13% and 148 ± 15%, respectively, \( P = 0.04 \) by ANOVA; Fig. 3A). Furthermore, SP (1 and 10 nM) stimulated increases in MCP-1 (132 ± 10% and 156 ± 16%, respectively, \( P < 0.05 \); Fig. 3B) and KC (109 ± 12% and 148 ± 18%, respectively, \( P < 0.05 \) by ANOVA; Fig. 3C) secretion in 3T3-L1 adipocytes.

In mouse epididymal adipose tissue, SP (10 nM) significantly stimulated C3 secretion (152 ± 25%, \( P = 0.037 \)) compared with the control treatment (Fig. 3D). Also, SP stimulated MCP-1 secretion from mice primary adipocytes by 496 ± 42% (\( P < 0.02 \)) compared with nonstimulated cells (Fig. 3E).

**SP Downregulates IRS-1, GLUT4, and Adiponectin mRNA Expression and Blocks Insulin-Mediated Action in 3T3-L1 Adipocytes**

Gene expression of key factors of the insulin-signaling cascade was evaluated in mature adipocytes in response to treatment with SP. As presented in Fig. 4A, 10 nM SP decreased IRS-1 and GLUT4 mRNA expression by −82 ± 2% (\( P < 0.01 \)) and −76 ± 2% (\( P < 0.01 \)), respectively, compared with control. Furthermore, SP also blocked the insulin-mediated stimulation of fatty acid uptake and storage in 3T3-L1 adipocytes (Fig. 4B). This insulin-blocking effect was, at least partly, mediated through interference of insulin-mediated Akt phosphorylation at Ser473 (Fig. 4C; \( P < 0.001 \), insulin vs. insulin + SP, by 2-way ANOVA). After 24 h of incubation, secretion and mRNA expression of adiponectin were also evaluated. Although no change in adiponectin secretion was observed (Fig. 4D), adiponectin mRNA expression was significantly reduced (−86 ± 8%, \( P < 0.001 \)) after 24 h of treatment (Fig. 4E).

**In Human Omental Adipose Tissue, SP Receptor NK1R Gene Expression Correlates With Protein and Gene Expression of Metabolic-Related Factors**

Gene expression of NK1R correlates positively with plasma insulin, NEFA, and complement C3 in human omental adipose
tissue (Fig. 5, A–C), but not in subcutaneous adipose tissue (data not shown). Furthermore, NK1R expression inversely correlates with adiponectin, CEBPα, CEBPβ, and PPARγ gene expression (Fig. 5, D–G), as well as expression of a number of other genes related to lipid storage and insulin signaling (data not shown).

DISCUSSION

The salient novel findings in the present study are that, in mature adipocytes, SP inhibits fat uptake and increases lipolysis and that such effects are associated with changes in gene expression of relevant adipogenic and lipogenic markers. Adipocytes and preadipocytes express the SP receptor NK1R, and during preadipocyte differentiation, chronic SP treatment decreases lipid droplet accumulation. We also show that SP increases adipocytokine secretion in 3T3-L1 cultured adipocytes and primary ex vivo mouse adipocytes. Furthermore, SP treatment leads to a decreased expression of factors involved in differentiation and the insulin signaling cascade and blocks insulin-mediated action via a mechanism that involves, at least partly, inhibition of Akt phosphorylation.

Finally, in human adipose tissue, expression of NK1R correlates with plasma insulin, NEFA, complement C3, and various genes related to differentiation, insulin signaling, and lipid storage.

The present data are consistent with a previous report that SP decreases insulin-stimulated glucose uptake in primary adipocytes (14) and point to a potential role in insulin resistance. Interestingly, the SP-mediated decrease in transcription factors in adipocytes contrasts with the increased PPARγ expression in human monocytes (1), suggesting that SP effects are tissue-specific. SP increased CD36 mRNA but decreased FABP4 mRNA gene expression. While both proteins are involved in fatty acid transport (CD36 as a membrane fatty acid transporter and FABP4 as a fatty acid-binding protein), only CD36 plays a dual role and facilitates chronic inflammation and has been suggested as a biomarker for peripheral blood mononuclear cell activation and inflammation in type 2 diabetes patients (27). Together, these findings suggest multiple mechanisms through which SP may contribute to insulin resistance (16) and highlight the potential for interaction between lipid storage pathways and immune pathways.

Fig. 5. Correlation of SP receptor NK1R gene expression with plasma protein concentrations and human omental adipose tissue gene expression. Human omental adipose tissue gene expression of NK1R correlates positively with plasma insulin (A), NEFA (B), and complement C3 (C) and inversely with adiponectin (D), CEBPα (E), CEBPβ (F), and PPARγ (G) gene expression.
While there are no studies in mature adipocytes, a number of previous studies in immune cells demonstrated inflammatory-related effects of SP, which enhance immune cell activation and recruitment (8, 19), leading to further release of proinflammatory cytokines (4) and amplification of the inflammatory response. For example, SP stimulates MCP-1 secretion from mast cells and pancreatic acinar cells (7, 24, 25). Our results extend these effects, showing that SP stimulates MCP-1 secretion from 3T3-L1 adipocytes. Furthermore, we observe that SP stimulates secretion of KC, which is the mouse homolog of human IL-8 and might function as a major proinflammatory chemokine in mice. These results are consistent with those of Karagiannides et al. (13), who showed that human mesenteric preadipocytes respond to SP with increased IL-8 secretion in an NF-κB-dependent manner.

One novel and interesting finding reported here is that SP stimulates complement C3 secretion in adipocytes, and expression of NK1R in human omental adipose tissue correlates with plasma complement C3. Adipocytes are known to be an important source of complement C3. Increased plasma C3 levels have been identified recently as a more specific marker of the inflammatory processes underpinning myocardial infarction than C-reactive protein (6). The association of C3 with myocardial infarction is more significant than any other association of traditional risk factors with myocardial infarction (22). Furthermore, increased serum C3 is a stronger marker of insulin resistance than C-reactive protein (21).

In addition to its effects on adipocytokines, SP also down-regulates adiponectin gene expression in adipocytes, and in human adipose tissue, expression of NK1R inversely correlated with adiponectin mRNA. Although no measurable changes could be detected in adiponectin concentrations, this may be related to the half-life of the secreted protein. Our gene expression data are consistent with increased adiponectin receptor mRNA levels in mesenteric fat and liver from NK-1 KO compared with wild-type mice (14). Furthermore, SP induces decreases in mRNA expression of IRS-1 and GLUT4, two important components in insulin sensitivity, and blocks insulin-mediated effects on fatty acid uptake and storage via interference with Akt phosphorylation. Hence, SP might contribute to insulin resistance via multiple pathways: decreased adiponectin gene expression, increased adipocytokine secretion, and interference with insulin-mediated action. Higher SP levels were found in obese individuals with type 2 diabetes (9), as well as in obese children (2), than in controls, suggesting a role for SP in obesity and related dysfunctions. It should be noted that the levels of SP used in the present study are comparable to those used in other studies (1, 10, 28) and to physiological levels (2, 9). This could partly explain the association of SP with chronic inflammation and type 2 diabetes mellitus (9) and the lack of inflammatory response in NK1R KO compared with wild-type mice (3).

We have demonstrated effects of SP on differentiation, fat storage, lipolysis, secretion of inflammatory factors, and interference with insulin action coupled to increased expression of NK1R in omental adipose tissue from hyperinsulinemic subjects. Our results provide evidence that could link the gut-derived hormone SP to adipocyte metabolism and inflammation. Together, these findings support a role for SP in chronic inflammation, adipocyte dysregulation, and insulin resistance.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

P.M., D.H.S.-P., and K.C. are responsible for conception and design of the research; P.M., M.L., P.P., and H.L. performed the experiments; P.M. and K.C. analyzed the data; P.M. and K.C. interpreted the results of the experiments; P.M. and K.C. prepared the figures; P.M. and K.C. drafted the manuscript; P.M., D.H.S.-P., M.L., P.P., H.L., A.G., and K.C. edited and revised the manuscript; P.M., D.H.S.-P., M.L., P.P., H.L., A.G., and K.C. approved the final version of the manuscript.

REFERENCES


