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Substance P mediates pro-inflammatory cytokine release form mesenteric adipocytes in Inflammatory Bowel Disease patients

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Abstract

Background & Aims—Substance P (SP), neurokinin-1 receptors (NK-1Rs) are expressed in mesenteric preadipocytes and SP binding activates proinflammatory signalling in these cells. We evaluated the expression levels of SP (Tac-1), NK-1R (Tacr-1), and NK-2R (Tacr-2) mRNA in preadipocytes isolated from patients with Inflammatory Bowel Disease (IBD) and examined their responsiveness to SP compared to control human mesenteric preadipocytes. The Aim of our study is to investigate the effects of the neuropeptide SP on cytokine expression in preadipocytes of IBD vs control patients and evaluate the potential effects of these cells on IBD pathophysiology via SP-NK-R interactions.

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Methods—Mesenteric fat was collected from control, Ulcerative colitis (UC) and Crohn's disease (CD) patients (n=10-11 per group). Preadipocytes were isolated, expanded in culture and exposed to substance P. Colon biopsies were obtained from control and IBD patients.

Results—Tacr-1 and -2 mRNA were increased in IBD preadipocytes compared to controls, while Tac-1 mRNA was increased only in UC preadipocytes. SP differentially regulated the expression of inflammatory mediators in IBD preadipocytes compared to controls. Disease-dependent responses to SP were also observed between UC and CD preadipocytes. IL-17A mRNA expression and release increased after SP treatment in both CD and UC preadipocytes, while IL-17RA mRNA increased in colon biopsies from IBD patients.

Conclusions—Preadipocyte SP-NK-1R interactions during IBD may participate in IBD pathophysiology. The ability of human preadipocytes to release IL-17A in response to SP together with increased IL-17A receptor in IBD colon opens the possibility of a fat-colonic mucosa inflammatory loop that may be active during IBD.

Keywords

Substance P; Cytokines; preadipocytes; Interleukin-17

INTRODUCTION

SP is an endecapeptide [1] member of the tachykinin family of peptides and a product of the pre-pro-tachykinin-A (Tac1) gene [2]. SP signals via binding to three G-protein coupled neurokinin receptors (NK-1R-2R-3R), with highest affinity for NK-1R [2]. SP is expressed in numerous tissues and organs, including the gastrointestinal tract [2, 3]. SP is also expressed in cells of the immune system, and it functions both as a neurotransmitter and an immune modulator in many disease states, including several intestinal diseases with an inflammatory phenotype [2, 4].

IBD, that includes UC and CD, are complex diseases of unknown aetiology. Their pathophysiology involves complex interactions between genetic, microbial and immune factors [5]. Our group and others have shown that SP and NK-1R have a role in the pathophysiology of intestinal inflammation, including IBD [6-8]. NK-1R expression is increased in the intestinal mucosa of mice with intestinal inflammation [9] as well as IBD patients [7, 10, 11]. Studies employing NK-1R knock out mice [12, 13], and SP receptor antagonists [14-17], show that SP, via NK-1R, play a dual role in the development of colitis. SP acts as a proinflammatory peptide in acute intestinal inflammation, but also enhances proliferation and mucosal healing during chronic colitis [13, 16, 18, 19], by activating distinct protective signaling pathways [14, 19, 20]. The mechanism involved in the proinflammatory NK-1R – associated responses includes interactions of SP with NK-1R on epithelial and inflammatory cells [10, 21, 22] and release of cytokines [17, 23, 24] that modulate colitis, and colitis-associated motility [3], primarily by activating NF-κB-dependent pathways [6].

A potential role for adipose tissue in IBD pathophysiology is suggested by clinical studies associating increased Body Mass Index (BMI) with the development of active CD and the requirement of patients for hospitalization [25]. Fat accumulation surrounding the inflamed

intestine ("creeping fat") during CD represents a hallmark of the disease [26, 27]. Histological examination of the mesenteric fat of patients with "creeping fat" demonstrated inflammatory changes [26] and alterations of adipokine levels in the circulation of IBD patients [28]. These data combined with the emergence of fat as an endocrine organ [29], suggest a role of intra-abdominal fat in IBD pathophysiology. Previously, we demonstrated the presence of NK-1R in human mesenteric preadipocytes [30], along with activation of inflammatory [30], anti-apoptotic [31], and metabolic [32, 33] pathways following SP treatment. We also reproduced the "creeping fat" phenotype in the intracolonic trinitrobenzylsulfonic acid (TNBS) mouse colitis model that was associated with increased proinflammatory cytokine expression in these depots [30]. However, modulation of expression of SP and NK-1R in adipose tissue during IBD has never been examined, and the responsiveness of IBD preadipocytes to SP has not been determined.

Here, we compared for the first time the effects of SP treatment on cytokine production in human mesenteric preadipocytes isolated from a substantial number of control, UC and CD patients. In these cells, we also compared the levels of expression of the Tac1 and NK-1R, NK-2R, and NK-3R genes. Initially, we demonstrate differential cytokine release from preadipocytes isolated from IBD patients compared to controls. We show that human mesenteric preadipocytes isolated from UC and CD patients release different levels express higher levels of NK-1R and NK-2R, but not NK-3R. We also found that human mesenteric preadipocytes express Tac-1 mRNA whose expression was elevated in UC, but not CD preadipocytes. Further, we present evidence that UC and CD preadipocytes display differential responses after treatment with SP compared to cells from control patients. Our data also demonstrate IBD-disease dependent changes in SP-induced inflammatory stimulation of human preadipocytes, including increased IL-17A transcription, while IL-17RA mRNA expression is higher in colonic biopsies of both UC and CD patients compared to controls.

METHODS

Human subjects

Mesenteric fat tissues from IBD (11 UC, 11 CD) and non-IBD patients (adenocarcinoma surgery, other gastrointestinal complications, or vascular surgery, n=10), males and females, were used. The control patients were either of Hispanic or (mainly) of Caucasian descent, mixed males females, and had an average BMI of 26.86. Their pathologies included 4 Adenocarcinoma, 2 Polyposis Coli, 1 Whipple, 1 Diverticulitis, 1 Idiopathic Motility Disorder, and 1 Tubular Adenoma case. The UC and CD patients we also mixed male and female populations with an average BMI of 27.23 and 24.12, respectively. The protocol is approved by the UCLA Institutional Review Board for Human Research (IRB#11-001527-AM-00003). All subjects were fasted for at least 10 hrs prior to surgery and provided informed consent. Tissues from Cedar's Sinai were obtained after informed consent in accordance with procedures established by the Cedars-Sinai Institutional Review Board, IRBs 3358 and 23705. Tissues from Chicago were obtained in accordance with procedures established by the University of Chicago Institutional review board, IRB 12960. Colon biopsies were collected from patients undergoing colonoscopy for colon cancer screening or

IBD disease activity monitoring. Samples were obtained, immediately frozen, and used for RNA isolation.

Isolation and cell culture of human preadipocytes

2-5 grams of mesenteric fat tissue from each patient were minced to pieces. Samples were then placed into 50 ml tubes containing collagenase solution (1 mg/1 ml of PBS, 3 ml solution/1 g tissue) and minced to a fine consistency. After vortexing the tubes placed in a 37°C shaking water bath (100 rpm) for 40 min. The solution was vortexed and filtered through a sterile 100μm nylon mesh (Fisher Scientific Inc, Hampton, NH). The homogenates were centrifuged at 1000 rpm for 10 min. The pellet was then re-suspended in 10 ml of erythrocyte lysis buffer (catalog# A1049201, Gibco-Invitrogen), placed in a 37°C shaking water bath for 5 min at 100 rpm, and then centrifuged at 1000 rpm for 10 min. The pellet was re-suspended in 10 ml plating medium (DMEM, 0.1 mM penicillin, 0.06 mM streptomycin, 10% HI-fetal bovine serum [FBS], pH 7.4), vortexed, plated onto 100 mm dishes, and incubated at 37°C.

Culture of Human Preadipocytes

After 20 hrs cells were washed 3x with 10 ml PBS and 1 ml trypsin solution (Invitrogen, Carlsbad, CA) was added. Trypsin was inactivated with 5 ml plating medium and the cells were centrifuged at 1000 rpm for 10 min. After resuspension in plating medium the cells were plated at 5x104 cells /cm2 in plating medium and incubated at 37°C until confluence. Previous studies have demonstrated that this isolation procedure yields >99% pure preadipocyte populations [34]. Cells were then sub-cultured 3 or 4 times to ensure removal of macrophages. No ADAM8, F4/80, or macrophage inflammatory protein-1a mRNA, markers of macrophages, were detected by Affymetrix array analysis of human mesenteric preadipocytes prepared using this protocol. Preadipocytes at passages 3-4 were then exposed to 10^{-7}M SP for 8 hours in 3mL of Human Maintenance Medium (DMEM/F-12 with 23mM HEPES, 25mM $NaHCO_3$, 0.1 mM penicillin, 0.06 mM streptomycin, 10mg/L Transferin, 0.3mM Biotin and 2mM L-Glutamine). Medium and RNA of the cells were collected for analysis. Protein lysates were also collected from preadipocytes in plating medium.

Real-time PCR

RNA was isolated from human mesenteric preadipocytes and colon biopsies using the Trizol method. 1 μg of RNA was reverse-transcribed into cDNA as previously described [33] and incubated with dual fluorogenic probes (Applied Biosystems, Foster City, CA).18s (Hs03928990_g1) was used as endogenous control and was also detected using dual labeled fluorogenic probe (5’-FAM/3’-MGB probe, Applied Biosystems, Foster City, CA). Target mRNA (all from Applied Biosystems) levels were quantified using a fluorogenic 5'-nuclease PCR assay as described in using a 7500 Fast Real-Time PCR sequence detection system (Applied Biosystems, Foster City, CA). Cycle conditions were subject to change for higher efficiency as different targets require. The primers used were Hs00243225_m1 (Tac1), Hs00185530_m1 (Tacr1), Hs00169052_m1 (Tacr2), Hs00357277_m1 (Tacr3), Hs01064648_m1 (IL-17RA), Hs00994305_m1 (IL-17RC) and for normalization Hs03928990_g1 (Human Eukaryotic 18S rRNA).

Western immunoblots

Proteins were collected from human mesenteric preadipocytes of control, UC and CD patients (n=4 per group) in RIPA TRITON X100 (Boston Bioproducts BP-116TX) with protease and phosphatase inhibitors (Sigma-Aldrich). 30μg of protein were loaded on a 10% polyacrylamide gel and electrophoresed for 1.5 hrs. The proteins were transferred on PVDF membranes, and membranes were blocked for 1 hr at RT in LI-COR blocking buffer (LI-COR Biosciences, Lincoln, NE). The membranes were blotted with a rabbit NK-1R primary antibody, at a dilution of 1:100, O/N at RT (Santa Cruz Biotechnology Inc, sc-15323, Santa Cruz, CA). Secondary goat anti-rabbit antibody (1:15000, LI-COR Biosciences, cat# 926-32211) was added for 1 hr at RT. Loading was normalized using a mouse β-actin primary antibody (1:1000, Santa Cruz Biotechnology Inc., cat# sc-81178) and a goat anti-mouse secondary antibody (1:15000, LI-COR Biosciences #926-68170). Bands were visualized and quantified using Odyssey IR Imaging System (LI-COR Biosciences).

mRNA multiplex analysis

Total RNA was isolated as described above and inflammation-related gene expression was analyzed using the 42-plex FlexScript LDA inflammatory panels 3 and 4 (Luminex, Austin, TX). 20 ng of total RNA were loaded in each well and following treatments described in the company manual were performed (FlexScript LDA). The plate was run using Bio-plex 3D suspension array system (Bio-Rad, Hercules, CA). In addition to total RNA concentration, data were normalized to endogenous controls (GAPDH, B2M, β-actin) included within the gene panels.

Multiplex cytokine and phospho-protein immunoassays

Human mesenteric preadipocytes were isolated and plated as described above and media were collected at the end of the 8 hour exposure to SP. Cytokine concentrations in human preadipocyte media were determined using the Bio-Plex Pro™ Human Cytokine 27-Plex, Group I, with Magnetic Beads (Bio-Rad, Hercules, CA) and the final data were obtained and analyzed via the Bio-plex 3D Suspension array system (Bio-Rad, Hercules, CA). In addition to loading volume results were normalized for cell plating number and total protein.

Immunohistochemistry

Paraffin-embedded whole fat sections from UC and control patients (n=4) were mounted on slides. SP staining was detected using an anti-SP rabbit polyclonal antibody (AB1566, Millipore, Darmstadt, Germany) and the EnVision+ System-HRP Labelled Polymer Anti-Rabbit kit (DAKO, Carpinteria, CA). The staining was performed at the Translational Pathology Core, UCLA following a standard procedure described in Millipore's manual for the primary antibody treatment (1:100 Ab dilution, pretreatment with Citrate pH 6.0, antigen retrieval).

Determination of endotoxin levels

Aliquots from the SP preparations used in our treatments were diluted in cultured media as above to match the concentration and conditions represented in our study. The Pierce LAL Chromogenic endotoxin Quantification kit (Thermo Scientific, Rockford, IL) was used for

the quantitative measurement of endotoxin levels using E. coli 0111-B4 endotoxin as standard. Endotoxin measurements in all treatment preparations were below detection levels (data not shown).

Statistical Analysis

Results were analyzed using the Prism professional statistics software program (Graphpad Software Inc., San Diego, CA). Analyses of variances (ANOVA, one-way) as well as Mann-Whitney (for comparisons between two groups) were used for intergroup comparisons. $P < 0.05$ was considered significant.

RESULTS

Human mesenteric preadipocytes isolated from CD patients demonstrate distinct mediator release compared to controls

We have isolated preadipocytes from mesenteric fat depots of 10 control, 11 UC and 11 CD patients and expanded them in culture without prior freezing or external stimulation. At the end of the 2nd passage fresh medium was added to preadipocytes and collected after 8 hrs for multiplex cytokine analysis. Analysis of the 27 cytokines showed changes in the release of mediators from human preadipocytes during IBD compared to controls (Figure 1A-F, *$p<0.05$, **$p<0.01$, #$p<0.1$, n=4-11). Preadipocytes isolated from CD patients demonstrated statistically significant increases in the release of IL-1β, IL-9, and IL-17 (Figure 1A, B, and F)and significant decreases in the release of IL-10, IL-12 (trend in CD), and IL-13 (Figure 1C-E) compared to controls. Of the 27 mediators included in our panel none exhibited differential secretion in preadipocytes from UC patients compared to control and only IL-1β and IL-17 were increased (strong trend for IL-1β, $p=0.07$), while substantial differences were observed between preadipocytes isolated from UC and CD patients (IL-9, IL-10, IL-12, IL-13, and b-FGF, data not shown), suggesting disease-dependent changes in these cells. Patients that their cytokine values fell outside the standard curve, were excluded.

Human mesenteric preadipocytes from UC patients express higher levels of Tac1 compared to cells isolated from control and CD patients

SP levels are modulated in the intestine during IBD [35, 36]. However, there is no information whether human preadipocytes express Tac-1 mRNA, the gene encoding for SP. To address this, we isolated RNA from preadipocytes from 11 control, 10 UC and 11 CD patients and examined mRNA expression levels of Tac1 mRNA. We found that human mesenteric preadipocytes express Tac1 mRNA. We also found increased mRNA levels of Tac1 in preadipocytes isolated from UC patients (Figure 2A, $p<0.05$, n=9-11, extreme outliers were determined using the Grubbs test) compared to control cells. There was no significant difference (or trend) in Tac1 mRNA levels between preadipocytes from CD and control patients. To verify SP expression levels between control, UC, and CD patients we performed immunohistochemistry in whole fat tissue isolated from these patients using an anti-SP antibody and observed increased expression of SP-positive cells in UC and CD compared to control patient sections of (Figure 2E).

NK-1R and NK-2R mRNA expression is increased in human mesenteric preadipocytes from IBD patients

Here, we examined the expression levels of NK receptors in preadipocytes of control, UC and CD patients. Densitometric analysis of western immunoblots demonstrates that Tacr-1 protein levels were increased in human mesenteric preadipocytes isolated for CD patients while there was a strong trend for increase in UC patient preadipocytes (Figure 2D, $p<0.05$, n=4). At the mRNA level human preadipocytes isolated from UC and CD patients expressed higher levels of the Tacr-1 receptor compared to controls (Figure 2B, $p<0.05$ and <0.01 for UC and CD, respectively, n=10-11, one extreme outlier was determined via Grubbs test). The levels of Tacr-2 were significantly higher in preadipocytes from CD patients (Figure 2C, $p<0.05$, n=10-11) compared to controls, although a trend for increased expression in preadipocytes from UC patients was evident.

SP induces inflammation-associated cytokine mRNA expression in human mesenteric preadipocytes isolated from IBD patients

Previous studies from our group have demonstrated the ability of SP to induce inflammation-associated responses in preadipocytes and influence the metabolic responses of mice via the activation of intracellular signaling pathways in fat tissue [30, 32]. Here we exposed human mesenteric preadipocytes from control, UC and CD patients to SP and examined their individual responses in the production of cytokines that may affect IBD pathophysiology. We observed that in preadipocytes from UC patients, SP increased the mRNA expression of IL-1β, IL-12B, RANTES, IL-17A, IL-15, VEGFA, PDGFA, IFNγ, CXCL9, and MCP-1 (Figure 3B, *$p<0.05$, **$p<0.01$, #$p<0.1$, n=8) while the expression of CXCL10 and IL-4 was significantly decreased (Figure 3C, $p<0.05$, n=8). In preadipocytes isolated from CD patients SP treatment increased IL-12A, IL-17A, and CXCL10 mRNA levels (Figure 3D, *$p<0.05$, #$p<0.1$, n=7) and decreased IL-4 and TGFβ mRNA levels (Figure 3E, *$p<0.05$, #$p<0.1$, n=7). In control patient preadipocytes, IL-2, RANTES, and LTB mRNA expression (Figure 3A, *$p<0.05$, #$p<0.1$, n=10) was reduced in response to SP treatment.

SP induces differential, IBD disease-dependent, cytokine mRNA expression in human mesenteric preadipocytes

We next investigated whether higher NK receptor levels in IBD patient preadipocytes (Figure 2) reflect altered responsiveness to SP. Thus, we compared the fold difference changes in cytokine mRNA expression in isolated preadipocytes from control and IBD patients to examine whether their responsiveness to SP is significantly altered in IBD. We observed that several cytokines responded in a disease-dependent manner. The vast majority of responses demonstrated higher fold changes in cytokine expression in preadipocytes isolated from IBD patients compared to control. More specifically, we show 5 mediators that increase in response to SP only in preadipocytes isolated from UC patients (Figure 4A, $p<0.05$ for IL-2, and IL-15; $p<0.01$ for IL-17, VEGFA, and RANTES; IL-17 also has a strong trend towards increased expression in CD patients, $p<0.1$, n=7-8), 3 mediators that are increased only in preadipocytes from CD patients (Figure 4A, $p<0.05$ for LTB; $p<0.01$ for IL-12A and CXCL10 and all mediators also show a trend for increase in UC, $p<0.1$,

n=7-8), while for IL-12B, MCP-1, and CXCL9 we only observed a strong trend for increase in UC patient preadipocyte RNA ($p<0.1$, n=7-8). A mini heat map describing these changes in response to SP treatment (including the strong trends towards change, $p<0.1$) is also provided and includes the strong trends towards changes (Figure 4B).

SP induces inflammation-associated cytokine release in human mesenteric preadipocytes isolated from IBD patients

At the protein level, in preadipocytes isolated from UC patients SP treatment induced release of IL-2, IL-17A, RANTES, PDGF-BB, MIP-1β, and CSF-2 (Figure 5B, *$p<0.05$, #$p<0.1$, n=7) and inhibited the release of IL-6, IL-12p70, IL-13, VEGF, and Eotaxin (Figure 5C, *$p<0.05$, #$p<0.1$, n=7). Moreover, in CD preadipocytes, SP induced the release of IL-1β, IL-2, IL-15, IL-17A, b-FGF, and MIP-1β (Figure 5D, *$p<0.05$, #$p<0.1$, n=7) and inhibited the release of IL-7, IL-8, IL-10, IL-12p70, IL-13, and MIP-1α (Figure 5E, *$p<0.05$, #$p<0.1$, n=7). Treatment of preadipocytes isolated from control patients with SP produced milder responses at both the mRNA and protein secreted levels. In preadipocytes isolated from control patients, IL-2, IL-17A, TNFα, and IFNγ release (Figure 5A, #$p<0.1$, n=10) were reduced after SP treatment. These results indicate that mesenteric preadipocytes from CD, UC and control subjects respond to SP by releasing different proinflammatory cytokines at the mRNA and protein level.

SP induced cytokine secretion differs significantly, in human mesenteric preadipocytes from IBD patients compared to controls

We further analyzed the data included in Figure 5 in order to signify potential disease-based differences in SP responsiveness at the protein level. Of the 27 cytokines tested in supernatants from SP-exposed UC and CD preadipocytes 6 cytokines showed significantly increased secretion in both UC and CD (Figure 6A, IL-1b, IL-2, IL-15, IL-17A, bFGF, and MIP-1b, *$p<0.05$, **$p<0.01$, n=7-11) and 4 showed increased, or a trend towards increased release only in UC (Figure 6A, RANTES, PDGF-BB, TNFα, and CSF-2, *$p<0.05$, #$p<0.1$, n=7-11). Secretion of IL-13 and Eotaxin in the supernatants of SP-exposed preadipocytes was either significantly decreased or had a trend towards significant decrease in both UC and CD (Figure 6A, *$p<0.05$, **$p<0.01$, #$p<0.1$, n=7-11) compared to controls. IL-12 was significantly decreased in SP-treated UC preadipocytes (Figure 6A, **$p<0.01$, n=7-11), while IL-8 and IL-10 (Figure 6A, $p<0.05$) were significantly decreased in CD preadipocytes after SP treatment, compared to controls. A mini heat map depicting these changes in response to SP treatment (including the strong trends towards change, $p<0.1$) is also provided (Figure 6B). A modified Venn diagram (Figure 7) was created to summarize the mRNA and protein responses of preadipocytes from UC and CD patients compared to control patients to SP exposure and highlight the similarities and differences between the two diseases. IL-17A was the sole mediator among 24 common molecules (between mRNA and protein panels used) that was increased after SP exposure of CD and UC preadipocytes both at the mRNA and protein panels.

Preadipocytes express increased IL-17A mRNA during IBD while IL-17 receptor A (IL-17RA) mRNA levels are increased in colonic biopsies of IBD patients

Based on the proximity of mesenteric fat and the inflamed intestine during IBD, adipocyte-derived products may reach the involved areas and affect the course of IBD. Moreover, our results showed that IL-17A is the only mediator modulated consistently following SP stimulation at both the mRNA and protein levels in both CD and UC preadipocytes. As also shown in Figure 1F, mesenteric preadipocytes from IBD patients produce higher IL-17 protein levels even in the absence of SP stimulation. To investigate whether this change may potentially be important in the regulation of responses in the intestine during IBD we examined for the presence of IL-17 receptors in human colonocytes and compared their levels in colonic biopsies of IBD and control patients. We first verified that IL-17RA (the high affinity receptor for IL-17A) is expressed in significant amounts in NCM460 human colonic epithelial cells (data not shown). Furthermore, we examined the IL-17RA expression levels in colonic biopsies of control and IBD patients. We analyzed 19 non-IBD, 23 UC and 30 CD colonic biopsies and we observed that IL-17RA is increased in biopsies from UC and CD patients compared to the control samples (Figure 8, $p=0.0693$ and $p<0.05$, respectively). Expression levels of IL-17RA are shown separately for each patient, and the mean of the control samples was used as a cut-off value to signify high expression levels. Seven of the 19 of the controls, 14 of the 23 UC and 19 of the 30 CD (36.84%of the control, 60.86% of the UC and 63.33% of the CD) are above that cutoff value, signifying increased IL-17RA mRNA levels during IBD.

DISCUSSION

We found that mesenteric preadipocytes from control and IBD patients demonstrate differential mediator secretion patterns even after days in culture (Figure 1). Our results also indicate that SP exert potent anti-inflammatory effects in preadipocytes from control patients compared to the mainly proinflammatory stimulation in cells from patients with IBD. This stark discrepancy indicates that components of the mesenteric fat depots have acquired disease-dependent characteristics adding to the complexity of the factors that may contribute to its pathophysiology. Most importantly, our results (Figure 2) demonstrate that human mesenteric preadipocytes express Tac1 mRNA as well as both NK-1 and NK-2 receptors. Our findings also show that preadipocytes isolated from IBD patients respond to SP in a considerably different manner from controls with profound differences in the responses between preadipocytes isolated from UC and CD patients (Figures 3 and 5) possibly due to intrinsic characteristics these cells acquired during the course of IBD. Further analysis lead to the identification of an IBD-specific cytokine response pattern after exposure to SP (Figures 4 and 6). Overall, and despite the variability in patient backgrounds and potential treatments, we observed significant and quite consistent differences in the inherent ability of preadipocytes from patients from different disease groups to respond to SP. These responses can be pro and anti-inflammatory (at both the transcription and secretion levels), suggesting the potential involvement of mesenteric adipose tissue in disease manifestations and activity in the different phases of colitis.

We have shown that NK-1R is present in mouse mesenteric adipose tissue and its expression is regulated during colitis [30]. However, this is the first evidence that expression of NK-1R is regulated differentially by IBD in preadipocytes, leading to specific UC and CD-dependent inflammatory SP responses. Previous observations suggested a possible role of "creeping fat" in the pathophysiology of CD alone; however, this is the first evidence that mesenteric adipose tissue may be involved in the pathophysiology of UC as well. This is an important observation since only CD, but not UC is associated with a "creeping fat" phenotype, suggesting different mechanisms for mesenteric fat activation in these two disease sates. Increased *Tacr-1* mRNA expression in IBD preadipocytes likely involves activation of NF-κB and binding of this transcription factor to sites at the promoter region of *Tacr-1*, shown to be important for its transcription [37]. SP alone can also increase transcription of *Tacr-1* in control mesenteric preadipocytes as we previously showed [30].

The diverse differential responses described here in human mesenteric preadipocytes in response to SP during IBD are both pro- and anti-inflammatory. Many of these molecules have been implicated in IBD pathophysiology and their levels depend on disease activity and/or different cell populations involved in this group of diseases. For example, IL-1β polymorphisms are linked with IBD disease activity and phenotype, and IL-1β levels are elevated in serum and colonic biopsies of IBD and non-IBD colitis patients [38]. IL-12 and IL-15 are highly expressed in IBD, and both cytokines represent potential therapeutic targets [39, 40]. Antibodies against the p40 subunit of IL-12/23 are currently in clinical trials for IBD treatment [41]. IL-8 is increased in colonic intestinal epithelium in IBD, and is a potent neutrophil attractant [42]. IL-2 polymorphisms seem to predispose to UC, and knock-out animals for IL-2 or IL-10 are known to develop colitis [43, 44]. IL-13 seems to have a protective role against colitis and its levels are decreased in IBD colon biopsies of pediatric UC patients [45, 46]. VEGF is also considered a susceptibility factor for IBD linking angiogenesis with the development of colitis [47]. Biologic factors targeting TNFα are the most widely used and effective treatment for IBD currently [48]. Several of the cytokines demonstrating IBD-dependent responses to SP treatment in our study [Eotaxin [49], PDGF-BB, bFGF [50],CXCL9 [51], CXCL-10 [52], MIP-1β (CCL4) [53], RANTES (CCL5) [54], MCP-1 (CCL2) [30], CSF2 (GM-CSF) [55, 56], LTB [57] have been implicated in IBD pathophysiology. Collectively, the multitude of responsive mediators to SP in mesenteric preadipocytes from IBD patients highlights the potential magnitude of the involvement of mesenteric preadipocytes in IBD pathophysiology via regulation of inflammatory responses that may affect the involved intestine.

Mediators described here to be affected by SP treatment in human mesenteric preadipocytes from both UC and CD patients have been implicated in the regulation of innate and adaptive immunity. In addition to their similarities the combination of affected mediators by SP is mainly reminiscent of changes in macrophage responses in UC (IL-1β, IL-12, IL-15, CXCL10, GM-CSF, RANTES, MIP1, IFNγ) and mostly resemble dendritic cell changes observed in CD (IL-1β, IL-2, IL-12, IL-15, CXCL10, MIP1) patient preadipocytes [58, 59]. In both these cases, the described changes in response to SP can affect T cell function (via IFNγ, IL-10 and IL4) [60, 61]. Interestingly, a considerable number of SP-induced mediators in preadipocytes isolated from both UC and CD patients (IL-1β, IL-12, IL-13, CCL2, CCL4)

described here are downstream targets of IL-17A activation in macrophages, T helper cells, and intestinal epithelial cells [62]. Collectively, treatment of human preadipocytes isolated from UC and CD patients with SP leads to the generation of responses that may be linked to inflammation, cellular development and proliferation, tissue development, connective tissue development and function, hematological tissue development and function , showing the potential involvement of SP and its signaling on preadipocytes on several aspects of IBD pathophysiology.

It is important to note that of all the inflammatory mediators that we were able to screen through in this study, IL-17A was the only one that exhibited consistent IBD-associated changes in mRNA expression and protein secretion levels in preadipocytes in response to SP treatment (schematically described in Figure 7) after SP stimulation compared to controls. Previous studies indicated that IL-17 is regulated by SP in intestinal inflammatory T cells [63], while an IL-23R haplotype, involved in the induction of IL-17A expression, is a risk factor for IBD [5]. Moreover, IL-17 levels are increased in colonic biopsies from UC and CD patients [64]. Here, we present evidence that human mesenteric preadipocytes are a novel source of IL-17A with higher expression during UC and CD. In addition, human mesenteric preadipocytes from IBD patients have elevated NK-1R receptors and demonstrate increased expression and secretion of IL-17 in response to SP and its receptor, IL-17RA, shows increased levels in colonic biopsies of IBD patients (Figure 8). Understandably, fat may not be the only source of IL-17 during colitis and may not reflect the cause behind the changes observed in the expression of IL-17RA in the gut during IBD. However, fat represents a novel reserve of IL-17 during the disease and may affect the progress of IBD by altering intestinal responses via interactions with IL-17RA.

This observation along with the identification of IL-17 as the most consistently regulated mediator in human mesenteric preadipocytes isolated from IBD patients in response to SP, suggest a potential role for this neuropeptide in the regulation of inflammatory changes in the intestine during IBD (both UC and CD) via modulation of IL-17 expression in the adjacent mesenteric preadipocytes. Furthermore, our observations introduce the preadipocytes as a novel cellular population with immune properties likely involved in the regulation of intestinal inflammation during IBD.

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Abbreviations

SP	Substance P
NK-R	neurokinin-1 receptor

IL	Interleukin
IBD	Inflammatory Bowel Disease
CXCL	Chemokine (C–X–C motif) ligand

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Synopsis

Preadipocytes from IBD patients have acquired disease-dependent characteristics that lead to the changes in the release of inflammation-associated mediators even after days in culture. Our results are the first to demonstrate differential disease-dependent responses in human preadipocytes after SP treatment. This observation along with the identification of IL-17 as the most consistently regulated mediator in human mesenteric preadipocytes isolated from IBD patients in response to SP, suggest a potential role for this neuropeptide in the regulation of inflammatory changes in the intestine during IBD (both UC and CD) via modulation of IL-17 expression in the adjacent mesenteric preadipocytes. In addition, this is the first study to indicate that human preadipocytes make IL-17.

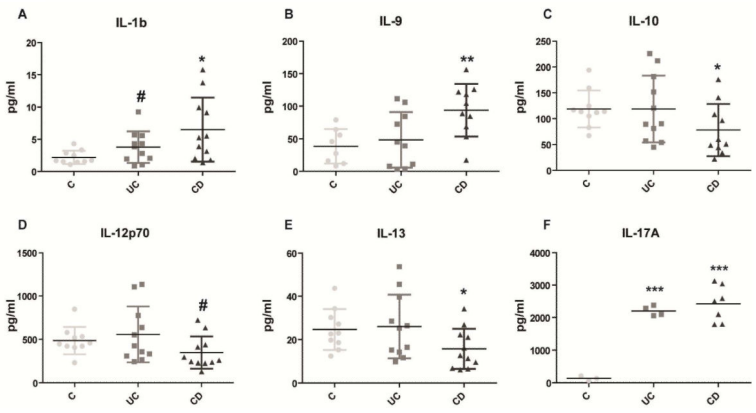

Figure 1.

Preadipocytes isolated from CD patients demonstrate different patterns of cytokine release compared to cells isolated from healthy controls (n=4-11). Mesenteric preadipocytes were isolated from control UC and CD patients and conditioned media were collected at the third passage. Multiplex analysis of a 27 human cytokine-containing panel revealed that preadipocytes isolated from CD patients secrete higher levels of *(A)* IL-1β, *(B)* IL-9, and *(F)* IL-17 while the levels of *(C)* IL-10, *(D)* IL-12, and *(E)* IL-13 were significantly lower compared to controls. In preadipocytes from UC patients strong trends towards an increase were observed for *(A)* IL-1β and significant increases in *(F)* IL-17 compared to controls. *p<0.05, **p<0.01, ***p<0.001, #p<0.1

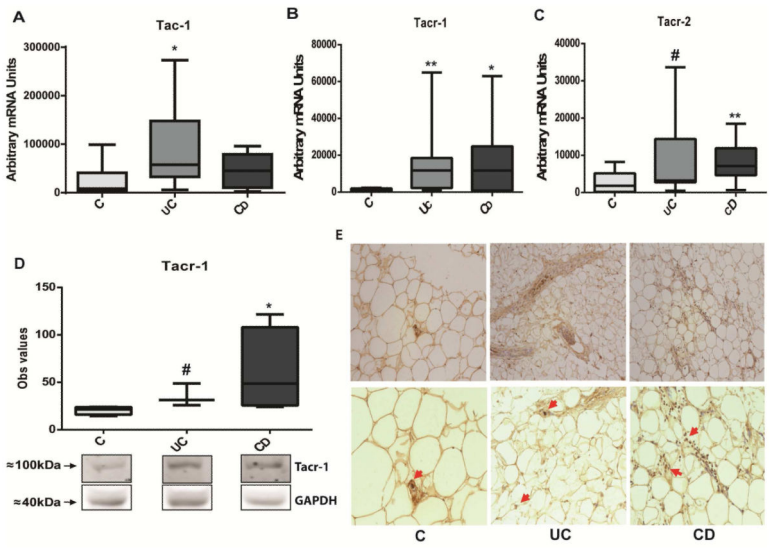

Figure 2.

Regulation of SP and its receptors NK-1R and NK-2R during IBD. Human mesenteric preadipocytes were isolated from control, UC and CD patients and cultured until the third passage when total RNA and protein were collected. (A) Real time PCR analysis shows that Tac1 (SP mRNA precursor) mRNA levels increase in human mesenteric preadipocytes during UC but not during CD. Similar observations were made at the mRNA level for both (B) Tacr-1 and (C) Tacr-2 receptors (trend in UC). (D) Western blot analysis shows that NK-1R protein levels increase during UC (trend) and CD in human preadipocytes while (E) immunohistochemical staining with anti-SP antibody demonstrated increased number of SP positive cells in whole mesenteric fat depots of UC and CD patients compared to controls. *p<0.05, **p<0.01, #p<0.1.

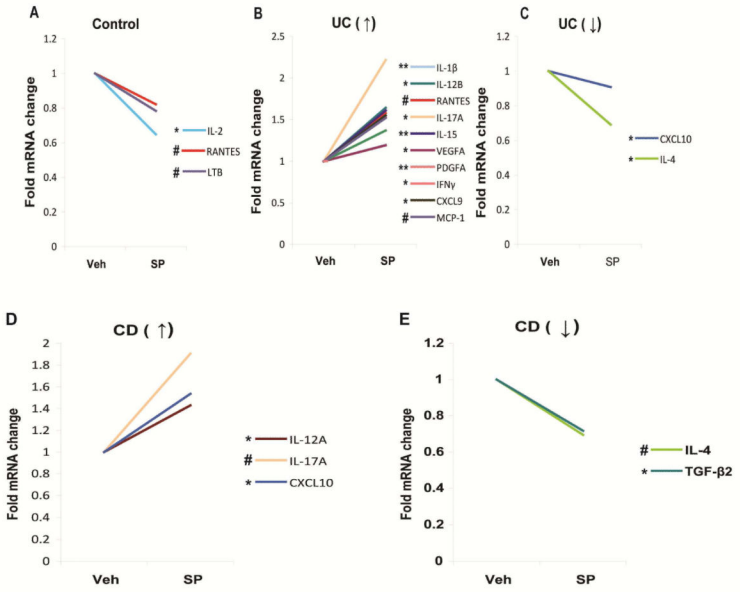

Figure 3.

SP mediates cytokine mRNA expression from human mesenteric preadipocytes isolated from IBD patients. Human mesenteric preadipocytes were isolated from control and IBD patients as described above and treated with SP for 8hrs. Total RNA was collected and cytokine mRNA levels were determined using real-time PCR. (A) SP decreased cytokine mRNA levels in mesenteric preadipocytes from control patients while it both (B) increased and (C) decreased cytokine expression in preadipocytes from UC patients. Preadipocytes isolated from CD patients also demonstrated both (D) increased and (E) decreased cytokine mRNA expression after SP treatment. Despite the similarities in the responses to SP between UC and CD patient preadipocytes, they also demonstrated disease-dependent responses. Values are expressed as fold changes from untreated, *p<0.05, **p<0.01, #p<0.1.

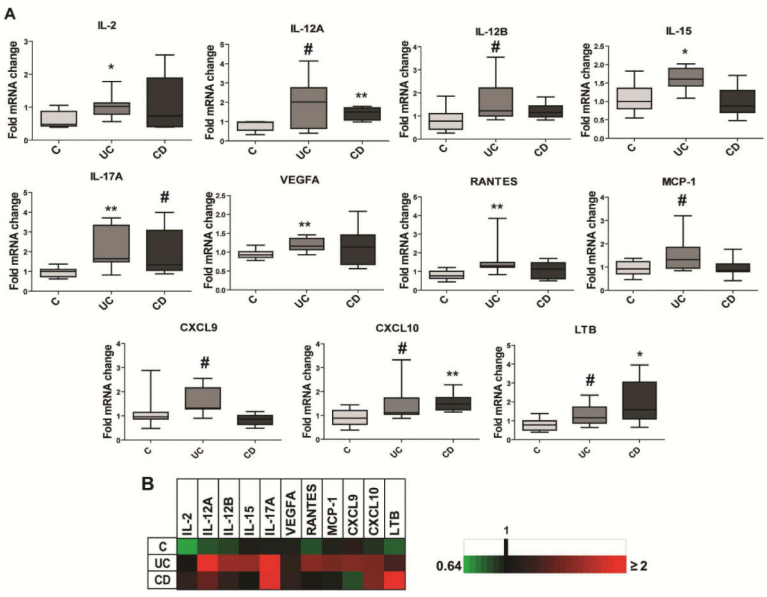

Figure 4.

Human mesenteric preadipocytes isolated from IBD patients demonstrate increased responsiveness in cytokine mRNA expression compared to preadipocytes isolated from control patients. Mesenteric preadipocytes were isolated from control and IBD patients and exposed to SP for 8hrs. Cytokine mRNA was measured using multiplex FlrxScript panels (42-plex) and the fold change in response to SP treatment was calculated for control, UC and CD patient-derived preadipocytes. (A) SP treatment of UC and/or CD patient preadipocytes significantly increases the mRNA expression of IL-2, IL-12A, IL-12B, IL-15, IL-17A, VEGF, RANTES, MCP-1, CXCL9, CXCL10, and LTB compared to SP treated preadipocytes from control patients. (B) Heat map depicting the changes in cytokine mRNA expression in SP treated IBD and control patient preadipocytes. *p<0.05, **p<0.01, #p<0.1.

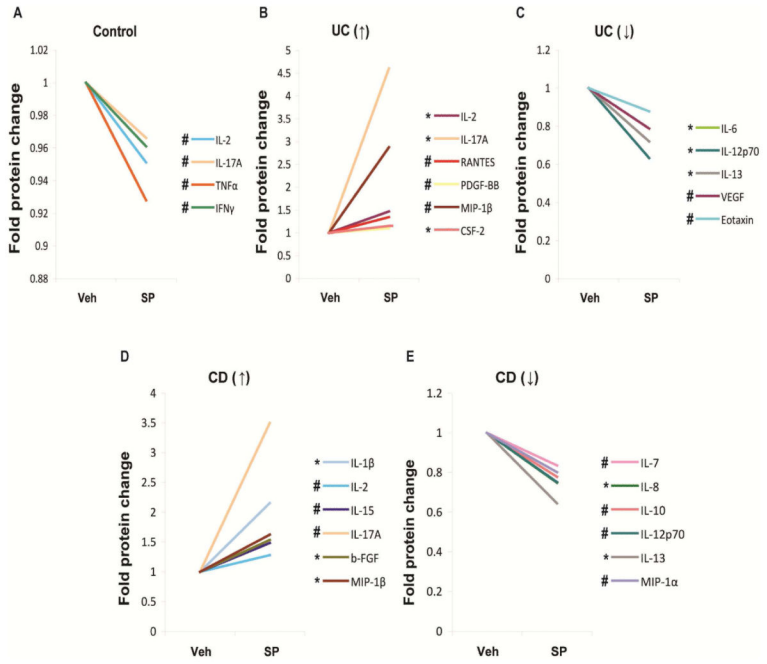

Figure 5.

Human mesenteric preadipocytes from control and IBD patients demonstrate different patterns of cytokine release in response to SP treatment. Conditioned media were isolated from human mesenteric preadipocytes treated with SP as described above. (A) In media from control patients SP treatment decreased (strong trends) the release of cytokines from mesenteric preadipocytes. In media isolated from UC patients SP both (B) promotes and (C) inhibits the release of cytokines from preadipocytes. In media isolated from CD patients, SP also (D) induces the release of several inflammation-associated cytokines and (E) inhibits the release of a number of cytokines from mesenteric preadipocytes. The patterns of cytokine release between UC and CD patient conditioned media demonstrate similarities (IL-2, IL-17A, MIP-1β) but also differences suggesting disease-specific contributions of preadipocytes in the course of these pathological conditions. *$p<0.05$, #$p<0.1$.

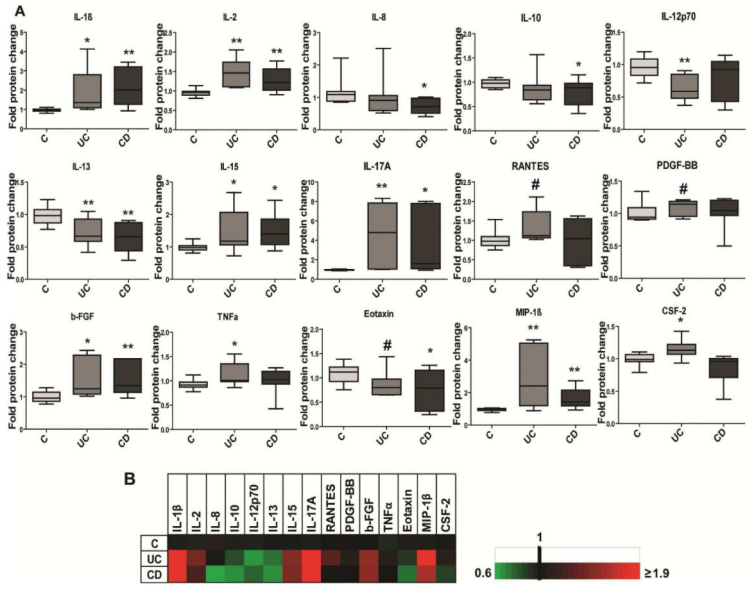

Figure 6.

Human mesenteric preadipocytes isolated from IBD patients demonstrate increased responsiveness in cytokine release compared to preadipocytes isolated from control patients. Mesenteric preadipocytes were isolated and cultured as described above. Conditioned media were isolated after SP treatment and cytokines were measured using a multiplex magnetic assay kit (27-plex). (A) SP treatment induces significantly higher fold release of IL-1β, IL-2, IL-15, IL-17A, RANTES, PDGF-BB, b-FGF, TNFα (down regulated in control), MIP-1β, and CSF-2 and significantly inhibits fold release of IL-8, IL-10, IL-12p70, IL-13, and Eotaxin in preadipocytes isolated from UC and/or CD compared to control patients. (B) Heat map depicting the changes in cytokine release from SP treated IBD and control patient preadipocytes. *p<0.05, **p<0.01, #p<0.1.

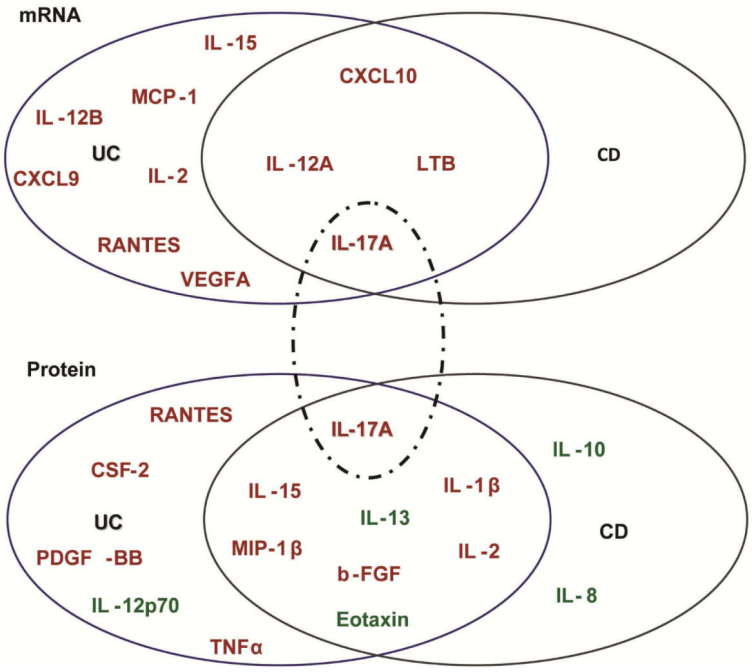

Figure 7.

Venn diagram summarizing the mRNA and protein responses of preadipocytes from UC and CD patients compared to control patients to substance P exposure and highlight the similarities and differences between the two diseases. IL-17A is depicted as the mediator demonstrating the most consistent responses (both in CD and UC and at both the mRNA and protein levels) to SP treatment in preadipocytes from IBD compared to control patients.

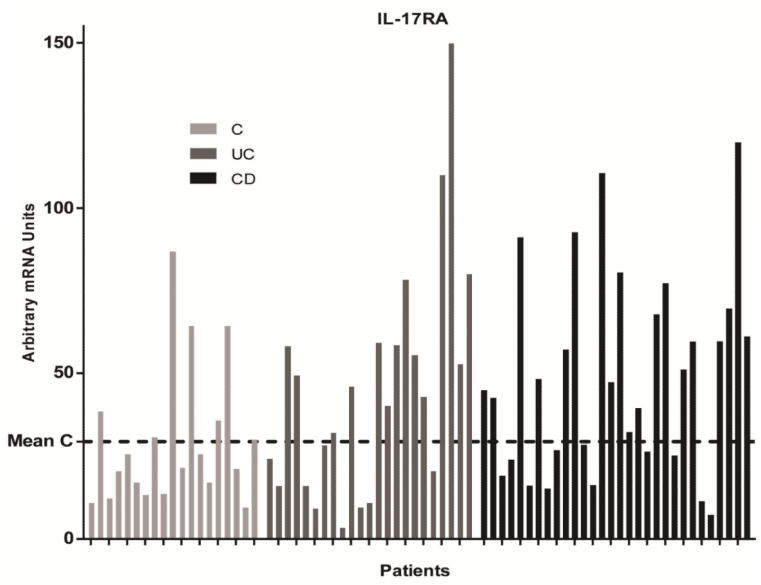

Figure 8.

Increased IL-17R mRNA expression in human colonic biopsies during IBD. Colonic biopsies were obtained from patients during colonoscopy and total RNA was isolated. Real time PCR shows elevated IL-17RA mRNA levels in UC and CD biopsies compared to controls.