Induction of colitis causes inflammatory responses in fat depots: Evidence for substance P pathways in human mesenteric preadipocytes

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Intracolonic administration of trinitrobenzene sulfonic acid in mice causes inflammation in the colon that is accompanied by increased expression of proinflammatory cytokines and of the substance P (SP), neurokinin 1 receptor (NK-1R) in the proximal mesenteric fat depot. We also investigated whether human mesenteric preadipocytes contain NK-1R and examined the functional consequences of exposure of these cells to SP as it relates to proinflammatory signaling. We found that human mesenteric preadipocytes express NK-1R both at the mRNA and protein levels. Exposure of human mesenteric preadipocytes to SP increased NK-1R mRNA and protein expression by 3-fold, and stimulated IL-8 mRNA expression and protein secretion. This effect was abolished when these cells were pretreated with the specific NK-1R antagonist CJ 012,255. Moreover, human mesenteric preadipocytes transfected with a luciferase promoter/reporter system containing the IL-8 promoter with a mutated NF-κB site lost their ability to respond to SP, indicating that SP-induced IL-8 expression is NF-κB-dependent. This report indicates that human mesenteric preadipocytes contain functional SP receptors that are linked to proinflammatory pathways, and that SP can directly increase NK-1R expression. We speculate that mesenteric fat depots may participate in intestinal inflammatory responses via SP–NK-1R-related pathways, as well as other systemic responses to the presence of an ongoing inflammation of the colon.

inflammatory bowel disease

Several groups have shown that in certain inflammatory conditions, including Crohn's disease, the adipose tissue is infiltrated by a significant number of immune cells that could contribute to the production of inflammatory cytokines (1, 2). Moreover, it has been demonstrated that a number of hormones and cytokines (which include leptin, adiponectin, and IL-6) called adipokines are released by adipocytes (as well as other nonimmune cells) within white adipose tissue (WAT) (3). Patients with Crohn's disease have accumulation of intraabdominal fat that is associated with increased PPAR-γ and TNF-α, synthesized, at least in part, by adipocytes (4). Thus, it becomes evident that adipocytes of WAT may play a role in the generation of the inflammatory responses observed under these conditions. However, whether exposure of the colonic mucosa to a proinflammatory agent can cause inflammatory responses in mesenteric fat depots has not been shown.

Substance P (SP), originally identified by Chang and Leeman (5), is an 11-aa peptide expressed in the central nervous system, afferent sensory neurons, and inflammatory cells, among others (6–12). Moreover, SP is shown to be an important mediator of neurogenic inflammation (13, 14), acting via its high-affinity neurokinin 1 receptor (NK-1R). In the intestine, SP mediates motility (15), mucosal permeability (16), and epithelial ion transport and proliferation (17, 18). NK-1R is present in several cell types including neurons, epithelial cells, and various types of immune cells (9, 10, 19–21). Its expression is up-regulated during acute and chronic enterocolitis (19, 22, 23) as well as during inflammation of the bronchi, liver, skin, bladder, and dorsal horn neurons (24–28). Moreover, the mechanism by which NK-1R is increased in inflammatory states involves activation of NF-κB after exposure of NK-1R-bearing cells to proinflammatory cytokines (29–31). Studies using NK-1R antagonists (16) or NK-1R knockout mice (32) demonstrated that interaction of SP with this receptor is essential for the generation of inflammatory responses in the intestine during *Clostridium difficile*-toxin-induced enteritis. Such responses also involve SP-induced activation of NF-κB (33, 34) and mitogen-activated protein kinases (18, 35), with the subsequent release of proinflammatory cytokines in various organs (36–38).

Here we examined whether induction of acute experimental colitis has any effect on the inflammatory state as well as the expression of NK-1R in the mesenteric fat tissue. Furthermore, we investigated whether cells, such as preadipocytes, that are not classical inflammatory cell types express NK-1R and whether they exhibit responses similar to those of other NK-1R-expressing cells after exposure to SP. We report that human mesenteric and omental preadipocytes contain functional SP receptors that are linked to proinflammatory pathways. We also show that SP directly increases expression of NK-1R and IL-8 in these cells in an NF-κB-dependent manner.

Results

Intracolonic Trinitrobenzene Sulfonic Acid (TNBS) Administration Increases the Expression of the Inflammatory Cytokines TNF-α, IL-6, Monocyte Chemoattractant Protein 1 (MCP-1), and Keratinocyte Chemoattractant (KC) in the Mesenteric Fat Depots of CD1 Mice. To examine whether colonic inflammatory changes are associated with inflammatory responses of the surrounding fat tissue, we induced TNBS colitis in CD1 mice. After 48 h, animals were killed and the mesenteric fat depot, as well as part of the colon, were removed. The colonic sections were observed under the light microscope, and the histological score was assessed. In agreement with previous studies (39), colon obtained from TNBS-treated mice had a significantly higher macroscopic damage score ($n = 8$; data not shown) and histological scores than vehicle-treated controls (data not shown). When the mesenteric fat depots from the same animals were observed, more inflammatory infiltrate was evident in TNBS-treated animals compared with control, vehicle-treated mice (Fig. 1). The histological changes observed were venular dilatation and congestion, neutrophil margination and diapedesis, and perivascu-

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Abbreviations: NK-1R, NK-1 receptor; SP, substance P; TNBS, trinitrobenzene sulfonic acid.

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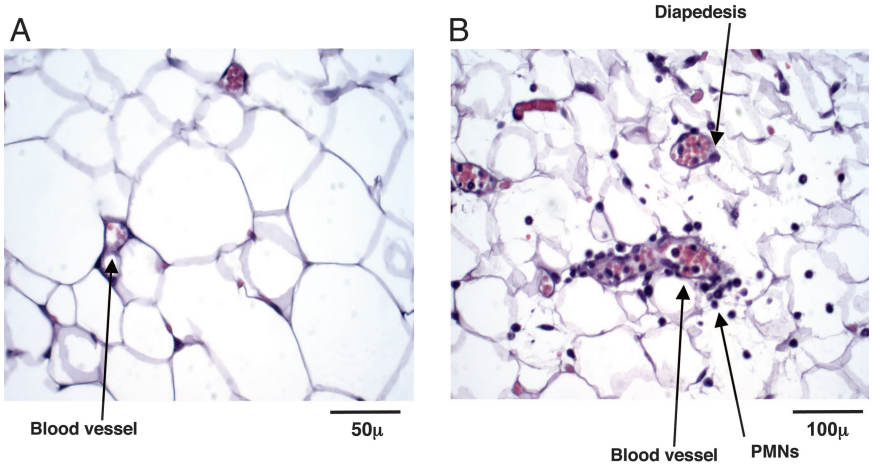

Fig. 1. Intracolonic TNBS administration causes inflammation in mesenteric fat tissue. CD1 mice (seven to eight mice per group) were treated either with TNBS or saline for 48 h. The animals were then killed, the mesenteric fat was placed in formalin, and 10-μm-thick sections of tissue were stained with hematoxylin/eosin and observed by light microscopy. Mesenteric fat depots isolated from TNBS-treated animals exhibited venular congestion with adhesion of polymorphonuclear (PMNs) leukocytes, diapedesis (transmigration), and radial infiltration of PMNs into the perivenular adipose tissue (*B*). Depots isolated from saline-treated controls were normal, devoid of inflammatory cell infiltrate (*A*). (Scale bars: *A*, 50 μm; *B*, 100 μm.)

lar accumulation of neutrophils in the adipose tissue (Fig. 1). RNA extracts obtained from both treatment groups were subjected to real-time RT-PCR analysis and a significant increase in the expression of the proinflammatory cytokines TNF-α, IL-6, MCP-1, and KC was observed in fat depots of TNBS- vs. vehicle-exposed mice (Fig. 2 *A*, *B*, *C*, and *D*, respectively). Thus, TNBS-induced inflammatory changes in the colonic mucosa are also reflected in the surrounding fat.

We next investigated whether mesenteric fat tissue expresses NK-1R and compared the levels of NK-1R mRNA and protein in mesenteric fat isolated from TNBS-exposed vs. control mice. RNA extracts from the mesenteric fat depots of mice with TNBS colitis showed increased NK-1R mRNA levels compared with those of control mice (Fig. 3*A*). A significant increase in the expression of NK-1R protein in depots from mice with TNBS colitis was also observed when tissue lysates were subjected to SDS/PAGE electrophoresis and Western blot analysis by using an antibody directed against NK-1R (Fig. 3*B*). Thus, SP may potentially be involved in the increased inflammation observed in the mesenteric fat depot during TNBS-induced colitis in CD1 mice.

NK-1R Is Present in Human Mesenteric Preadipocytes and Increased by SP Treatment. To determine whether human mesenteric preadipocytes bind SP specifically, we examined ^{125}I BHSP binding to cultured primary human mesenteric preadipocytes in the presence or absence of 10 μM unlabeled SP. We observed that ^{125}I BHSP bound to human mesenteric preadipocytes and that $\approx$80% of radiolabeled SP binding could be displaced by an excess of unlabeled SP (Fig. 4*A*), indicating that SP binding is specific. Furthermore, when we treated primary human mesenteric preadipocytes with SP for 4 h, we observed increased NK-1R mRNA expression, an effect that was completely abolished in cells pretreated with the specific NK-1R antagonist CJ 012,255 (Fig. 4*B*). Up-regulation of NK-1R by its ligand was also observed at the protein level when whole preadipocyte cell lysates were subjected to SDS/PAGE, and subsequent Western blot analysis was performed by using a specific anti-NK-1R antibody (Fig. 4*C*). Thus, SP in these cells stimulates the expression of its own receptor.

SP Treatment Stimulates ERK1/2 Phosphorylation in Primary Human Mesenteric Preadipocytes in a Time-Dependent Manner. Previous studies have demonstrated that SP signaling leads to activation of ERK1/2 through transactivation of EGFR (40, 41). To show that binding of SP to NK-1R activates ERK1/2, we stimulated primary human mesenteric preadipocytes with SP for various times and used Western blot analysis to detect ERK1/2 phosphorylation in lysates obtained from these cells. Using a specific

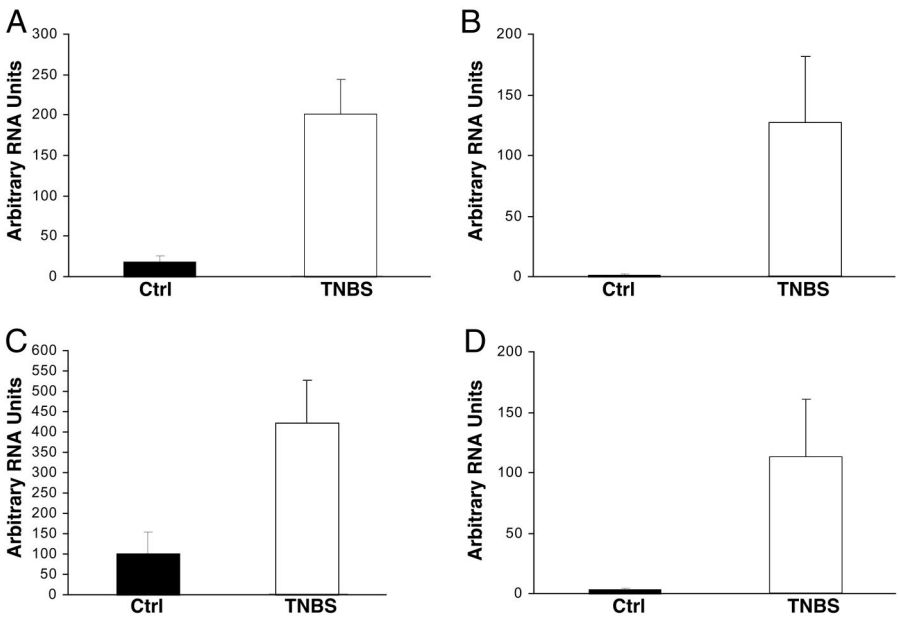

Fig. 2. Intracolonic TNBS administration increases the expression of inflammatory cytokines in mesenteric fat depots. CD1 mice (seven to eight mice per group) were treated either with TNBS or saline for 48 h, and RNA was isolated from the mesenteric fat proximal to the inflamed regions. Real-time PCR analysis of mesenteric depots showed that compared with saline-exposed mice, TNBS-exposed mice had significantly increased TNF-α (*A*), IL-6 (*B*), MCP-1 (*C*), and KC (*D*) mRNA levels.

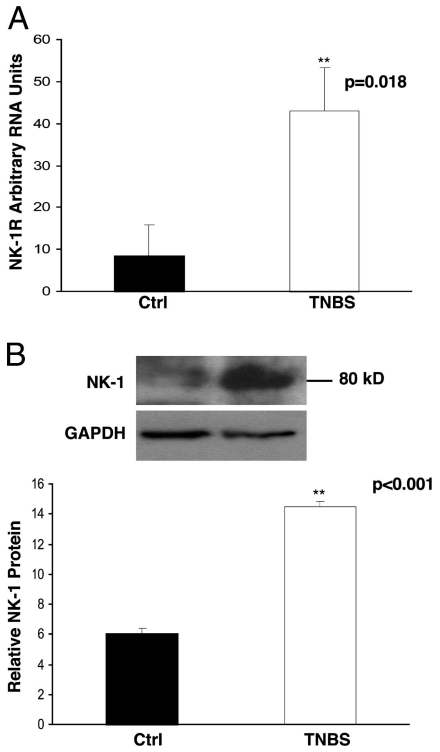

Fig. 3. Intracolonic TNBS administration increases NK-1R mRNA and protein expression in mesenteric fat depots. TNBS was administered to CD1 mice intracolonically. After 48 h, RNA and protein lysates were collected from the mesenteric fat proximal to the inflamed regions and subjected to real-time PCR and Western blot analysis for NK-1R mRNA ($n = 8$ mice per group) (*A*) and protein (*B Upper*), respectively. We found increased NK-1R mRNA expression in the depots obtained from TNBS-exposed versus saline-treated mice (*A*). Similar results were obtained when tissue lysates were tested by Western blot analysis using an antibody directed against human NK-1R (*B Upper*; representative of five independent experiments). The calculated molecular mass shown on the right is estimated by the migration of molecular mass standards run simultaneously. kDa indicates kilodaltons, where $k = 1{,}000$. (*B Lower*) Densitometry analysis of blots shown in *B Upper* ($n = 5$ per group).

anti-phospho-ERK1/2 (T202/Y204) antibody, we observed that addition of SP led to ERK1/2 phosphorylation within 10 min of exposure, confirming NK-1R activation (Fig. 4*D*).

SP Increases the Expression of IL-8 mRNA and Protein Secretion in Human Mesenteric Preadipocytes. SP is established as a strong inducer of the proinflammatory cytokine IL-8 in various cell types (33, 42–44). To investigate whether NK-1R activation leads to increased IL-8 production in human mesenteric preadipocytes, we treated primary cultures with SP (10^{-7} M) in the presence or absence of the NK-1R antagonist CJ 012,255 (10^{-6} M). After 4 h, cells were collected and processed for RNA purification and IL-8 mRNA measurements, while IL-8 protein was determined in cell conditioned media. We found that SP stimulation significantly increased IL-8 protein (Fig. 5*A*) and mRNA levels (Fig. 5*B*), whereas pretreatment with CJ 012,255 completely abolished these responses (Fig. 5 *A* and *B*). A similar response was also obtained in omental primary human preadipocytes (data not shown). Moreover, SP-induced increase in IL-8 mRNA expression in primary human mesenteric preadipocytes was increased with both 10^{-7} and 10^{-8} M SP, with peak expression after 4 h of treatment with 10^{-8} M SP (Fig. 5 *C* and *D*, respectively). It should be noted that the lower concentration of SP (10^{-8} M) was even more effective, perhaps indicating a bell-shaped dose–response effect.

SP-Induced IL-8 Expression in Human Mesenteric Preadipocytes Is NF-κB-Dependent. The involvement of NF-κB in the regulation of SP-induced IL-8 expression previously has been suggested (33, 43). We used two separate approaches to investigate a possible role for NF-κB in the SP-induced increase in IL-8 expression observed in human mesenteric preadipocytes. First, we exposed primary mesenteric preadipocyte cultures to SP for various times and collected total protein. SDS/PAGE electrophoresis and subsequent Western blot analysis of the lysates showed a time-dependent decrease in IκBα protein levels evident after 15 min of exposure (Fig. 6*A*). Because IκBα is a negative regulator of NF-κB activity, by binding and sequestering the latter to the cytoplasm, down-regulation of its protein levels by SP treatment as shown here suggests that SP can participate in the regulation of NF-κB activity and subsequent IL-8 expression.

Second, to demonstrate a direct involvement of NF-κB in the regulation of IL-8 expression by SP in human mesenteric preadipocytes, we transiently transfected primary cell cultures with a luciferase reporter construct containing either the full-length human IL-8 promoter or an IL-8 promoter construct that contained targeted mutations in the NF-κB binding site, as previously described (45). Our results showed that SP (10^{-8} M) significantly stimulated IL-8 promoter-driven luciferase activity, whereas the

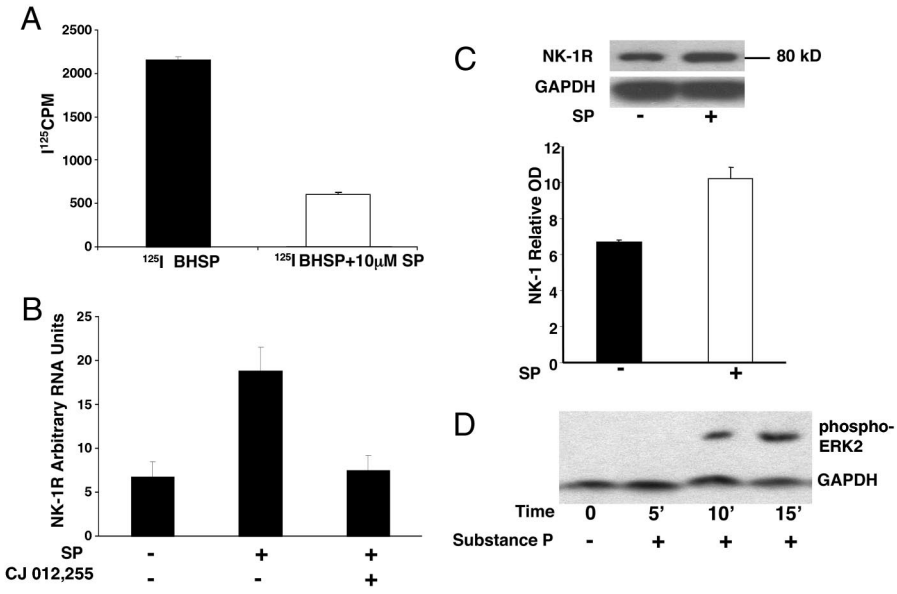

Fig. 4. NK-1R is present in human mesenteric preadipocytes and increased by SP treatment. Human mesenteric preadipocytes express functional NK-1 receptors. (*A*) Primary human mesenteric preadipocytes bind ^{125}I BHSP and binding is diminished in the presence of excess cold SP competitor ($n = 6$ per group). (*B*) Real-time PCR analysis of RNA isolated from human mesenteric preadipocytes shows that these cells express NK-1R, and its expression is increased by exposure to SP for 4 h. SP-induced expression of NK-1R is abolished when the cells are pretreated for 20 min with the specific NK-1R inhibitor CJ 012,255 ($n = 6$ per group). (*C*) Western immunoblot analysis showing increased NK-1R expression by SP at the protein level ($n = 3$ independent experiments per group). The calculated molecular mass shown on the right is estimated by the migration of molecular mass standards run simultaneously. kDa indicates kilodaltons, where $k = 1{,}000$. (*D*) Western blot analysis of protein lysates from human mesenteric preadipocytes showing activation of ERK2 by SP after 10 min of exposure, indicating that NK-1R is functional (representative of four independent experiments).

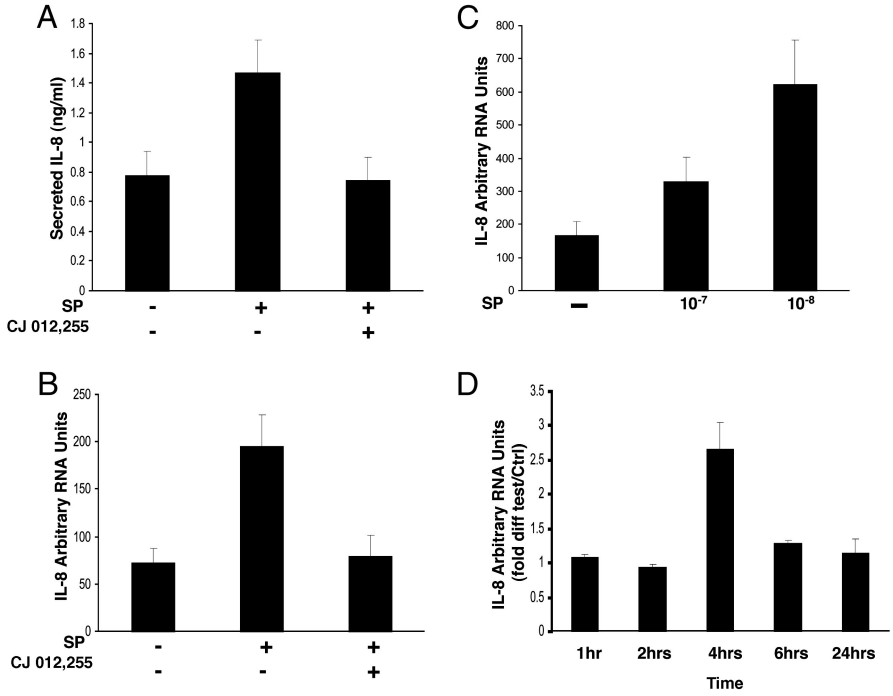

Fig. 5. SP increases IL-8 mRNA and protein expression in human mesenteric preadipocytes. (*A*) ELISA assays were performed on supernatants from human mesenteric preadipocytes treated with SP for 4 h with or without prior CJ 012,255 treatment for 20 min. SP addition caused a significant increase in the secretion of IL-8 by these cells, an effect that was abolished by the specific NK-1R inhibitor CJ 012,255 (n = 8 per group). (*B*) Real-time PCR analysis of RNA extracts from the same cells produced similar data. (*C*) When the cells were exposed to two different concentrations of SP, maximum IL-8 mRNA expression was achieved at a dose of 10^{-8} M (n = 6 per group). (*D*) In cells treated with SP for various time intervals (1, 2, 4, 6, and 24 h), maximum IL-8 mRNA expression was observed after 4 h of treatment (n = 6 per group).

NF-κB mutant construct showed a diminished response to SP (Fig. 6*B*). Moreover, exposure of human mesenteric preadipocytes to the NK-1R antagonist CJ 012,255 diminished SP-induced IL-8 promoter activity (Fig. 6*B*). Thus, NF-κB activation is a major requirement for SP-induced, NK-1R-dependent IL-8 gene transcription in human mesenteric preadipocytes.

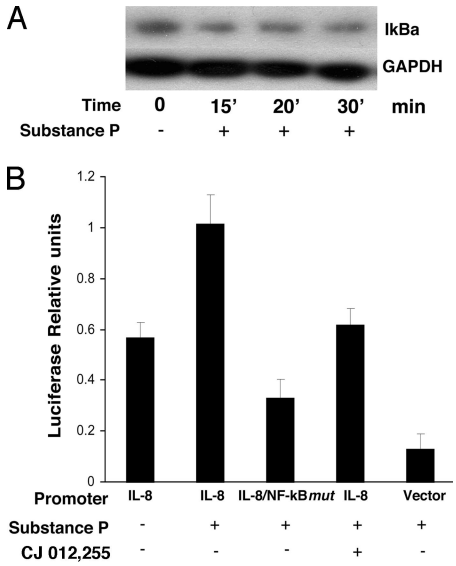

Fig. 6. SP-induced IL-8 expression in human mesenteric preadipocytes is NF-κB-dependent. Induction of SP expression in human mesenteric preadipocytes is NF-κB-dependent. (*A*) SP treatment of human mesenteric preadipocytes leads to decreases in IκBα protein levels within 15 min of exposure, as is evident in cell lysates subjected to Western blot analysis (representative of four independent experiments). (*B*) Transient transfection of human mesenteric preadipocytes with an IL-8 promoter-reporter construct (pGL2/IL-8) and subsequent treatment with SP for 4 h leads to increased IL-8 promoter activity, as measured by luciferase activity assay. Transfection with the same vector with an inactivated NF-κB site in the IL-8 promoter abolishes SP-induced activity of the promoter. Similarly, IL-8 promoter activity is lost when the cells are pretreated with CJ 012,255 for 20 min before the addition of SP (n = 5 per group).

Discussion

We found profound inflammatory changes in the proximal mesenteric fat depot in an animal model for Crohn's disease induced by intracolonic administration of TNBS (Fig. 1). Inflammatory changes in mesenteric fat depots 2 days after induction of colitis are associated with increased expression of mRNAs of several inflammatory cytokines (Fig. 2) as well as increased expression of SP and its high-affinity receptor at the mRNA and protein levels (Fig. 3). Consistent with this observation, previous studies demonstrated that patients with Crohn's disease accumulate intraabdominal fat, characterized by increased expression of PPAR-γ and TNF-α (4). These results suggest that mesenteric depots could participate in the inflammatory response via release of proinflammatory cytokines, and that fat hypertrophy and wrapping of the bowel are factors contributing to the development and progression of Crohn's disease (1, 4).

We next studied SP-related pathways in human mesenteric preadipocytes *in vitro* and found that isolated human preadipocytes express a functional NK-1R (Fig. 4) that upon SP exposure releases the potent neutrophil chemoattractant, IL-8 (Fig. 5), via an NF-κB-mediated pathway (Fig. 6). These results strongly indicate that activation of the SP–NK-1R system in mesenteric fat depots may play an important role in the pathophysiology of intestinal inflammation.

The presence of functional NK-1R in fat depots or isolated preadipocytes has not previously been recognized. Our report of increased NK-1R expression in the mesentery of mice after TNBS-induced colitis is consistent with previous work showing increased expression of NK-1R in tissues with intestinal inflammation in animals and humans (19, 22, 23, 46–48). Although the identity of cells expressing NK-1R in mesenteric fat depots has not been examined, our results showing expression of NK-1R in human preadipocytes suggest that mouse preadipocytes might be a likely source. However, other immune cells such as macrophages, known to be present in fat depots (49), can be another source of NK-1R, because activated macrophages express a functional NK-1R (37).

Moreover, preadipocytes, which account for 15–50% of the cells in fat depots (50), have gene-expression profiles closer to those of macrophages than fat cells and may even be able to transdifferentiate into macrophages (51). The mechanism of NK-1R up-regulation in the mesenteric depots during TNBS-induced colitis might, at least in part, involve increased expression of the adipokines KC, TNF-α, and IL-6 (Fig. 2 *A–D*), known to activate the NF-κB (52–54). Along these lines, several studies indicate that the transcription factor NF-κB represents a major regulator of NK-1R gene expression (29, 30). Our *in vitro* experiments with human mesenteric preadipocytes also indicate that SP induces expression of NK-1R (Fig. 4 *B–D*). The likely mechanism of SP-induced NK-1R expression may also be related to SP-induced NF-κB activation (33, 43), either directly or indirectly via release of proinflammatory cytokines that activate this transcription factor.

We found that exposing isolated preadipocytes to SP stimulates transcription of the potent cytokine IL-8 (Fig. 5) by interacting with NK-1R expressed on the cell surface (Fig. 4*A*). We also demonstrated that SP–NK-1R-induced IL-8 secretion is mediated via activation of NF-κB, in line with prior observations indicating that this peptide stimulates NF-κB-dependent IL-8 transcription in different cell types (33, 43). Because SP is produced by sensory neurons that innervate the intestine, and its production is increased during inflammation, it is possible that mesenteric preadipocytes are exposed to SP and, through up-regulation of NK-1R and increased IL-8 production, contribute to the recruitment of the inflammatory infiltrate.

Our results may be relevant to the pathophysiology of inflammatory bowel disease and, in particular, Crohn's disease, where mesenteric obesity is directly associated with the development of the disease (4). If permanent fat tissue components such as the preadipocytes can respond to SP and produce proinflammatory cytokines, increased fat mass around the area of inflammation could contribute to the pathogenesis of the disease. This notion is also supported by studies that have demonstrated that mesenteric fat is the main source of TNF-α in the mucosa of patients with Crohn's disease (4). Interestingly, several studies have demonstrated that obesity as well as TNF-α stimulate monocyte chemoattractant protein 1 (MCP-1) and macrophage inflammatory protein 1 alpha (MIP-1α) from fat tissue preadipocytes (55–57), which might direct the infiltration of macrophages into fat depots. This process may be facilitated by the expression of adhesion molecules, such as intracellular adhesion molecule (ICAM-1), on the surface of mesenteric preadipocytes. In addition, secretion of macrophage colony-stimulating factor (M-CSF) within adipose tissue (58) may stimulate further maturation and differentiation of macrophages. Furthermore, macrophages and preadipocytes can both express PPAR-γ, which is essential for preadipocyte differentiation and macrophage maturation (59). Thus, preadipocytes may contribute to intestinal inflammation in two ways: first, as shown in our data, by the production of proinflammatory cytokines in response to SP, and second, by increasing the inflammatory infiltrate through recruiting macrophages and possibly by their own transdifferentiation into macrophages. The abundance of cytokine expression in the mesenteric depots during colitis suggests that secreted cytokines, likely to enter the circulation, may have additional generalized responses at distant sites in the body.

Materials and Methods

TNBS-Induced Colitis. TNBS colitis was induced in age- and weight-matched CD1 mice as previously described by us with minor modifications. The macroscopic damage score (0–10) was assigned by two independent investigators using previously described parameters (60) (see *Trinitrobenzene Sulfonic Acid (TNBS)-Induced Colitis* in *Supporting Text*, which is published as supporting information on the PNAS web site).

Isolation of Mesenteric Fat. Forty-eight hours after TNBS administration, mice were killed and their visceral area was cut open for mesenteric fat removal. Using surgical scissors and forceps, we removed the fat that was attached to the bowel from the anus and 4 cm up toward the cecum. Mesenteric fat was then placed in 5-ml polystyrene round-bottom tubes and stored at −80°C until use.

Isolation of Preadipocytes from Human Subjects and Treatments. Fat tissue was resected during gastric bypass surgery for the management of obesity from subjects who had given informed consent, and mesenteric and omental preadipocytes were isolated as previously described by us (61). Cells were subcultured five or six times to ensure removal of macrophages [which do not divide (62) and are resistant to trypsin] (for details, see *Isolation of Preadipocytes from Human Subjects and Treatments* in *Supporting Text*).

Cell Treatments. While growing, the cells were kept in αMEM plus 10% FBS until they were 95% confluent. Cells were washed with sterile PBS before SP treatment and then the appropriate amount of SP was added for the required time (see *Results*). In the experiments where the NK-1R antagonist CJ 012,255 (Pfizer) was used, cells were pretreated with the antagonist for 20 min before the addition of SP. Compound CJ 012,255 is a structurally related analog of the parent compound CJ-11974 (Ezlopitant), shown to be highly specific for inhibiting the binding of [^{3}H] SP to the human NK-1 receptor, with little to no affinity for the NK-2 or NK-3 receptors (63).

Specific Displaceable Binding of ^{125}I Bolton–Hunter SP (BHSP) Assay. Predadipocytes were plated on 12-well plates and allowed to grow until they were 80% confluent. The cells were washed three times with chilled (4°C) Hank's buffered saline solution (HBSS) plus 0.1% BSA and then blocked for 1 h with chilled (4°C) HBSS plus 0.1% BSA. After blocking, the cells were treated with either 62 pM ^{125}I BHSP or 62 pM ^{125}I BHSP plus 10 μM SP for 1 h at 4°C. The cells were washed three times with chilled PBS and then treated with 0.5 M NaOH for 30 min at room temperature. The base solution containing the lysed cells was read for 10 min in a Wallac 1470 gamma counter. The labeled SP was prepared by using ^{125}I Bolton–Hunter reagent (PerkinElmer) and SP (Bachem) in a conjugation reaction as described by Gaudriault and Vincent (64). The values obtained from the samples containing both labeled and unlabeled SP represent the nonspecific binding. The difference between the nonspecific binding and the values of the wells containing labeled SP represent specific SP binding.

Western Immunoblotting. For immunoblots, proteins (15–30 μg) were separated by electrophoresis in a 10% polyacrylamide gel. Protein samples were mixed with sample buffer (3×; Cell Signaling Technology) and denatured by boiling. Samples were electrophoresed at 100–150 V for 1.5 h or longer until the dye migrated to the bottom of the gel. The separating gel was equilibrated in transfer buffer (20 mM Tris·HCl/150 mM glycine/20% methanol/0.1% SDS) for 10 min. The proteins were then transferred to poly(vinylidene difluoride) membranes (PVDF, Millipore) at 4°C. All membrane incubations were carried out at room temperature with rocking. The membranes were blocked for 1 h at room temperature in blocking buffer [TBS/5% nonfat dry milk (Bio-Rad)/0.1% Tween 20] and then incubated with primary antibodies against NK-1R (rabbit polyclonal antibody directed against amino acids 325–407 of the C terminus of the human NK-1R), IκBα (Santa Cruz Biotechnology), and ERK1/2 (Cell Signaling Technology) in blocking buffer, overnight at 4°C. Horseradish peroxidase-conjugated secondary antibodies in blocking buffer were used (Santa Cruz Biotechnology). The proteins were visualized by using SuperSignal West Pico chemiluminescent substrate (Pierce). The membranes were exposed to x-ray films from 10 sec to 5 min.

Determination of mRNA Levels Using Real-Time Quantitative RT-PCR (TaqMan Assay). For mouse NK-1R detection, RNA isolated from whole fat tissue homogenates were reverse-transcribed into cDNA by using the TaqMan One-step RT-PCR kit (Applied Biosystems 4309169), where 1 μg of the RNA was mixed with oligo(dT) primers and incubated at 70°C for 2 min, followed by addition of TaqMan Master Mix and a 1-h incubation at 42°C. The real-time reaction contained 5 μl of the cDNA along with the 5′ and 3′ primers for NK-1R (f-5′-tgcccttccacatcttcttc-3′, r-5′-ttccagcccctcataatcac-3′) and SYBR Green PCR master mix (Applied Biosystems). Human TBP (TATA-box binding protein) was used as an endogenous control and was detected by using dual-labeled fluorogenic probe (5′-FAM/3′-MGB probe, Applied Biosystems). mRNA levels were quantified by using a fluorogenic 5′-nuclease PCR assay (65) with a GeneAmp 5700 sequence detection system (ABI/PerkinElmer). Duplicate reactions of each standard or sample were incubated for 2 min at 50°C, denatured for 10 min at 95°C, and subjected to 40 cycles of annealing at 55°C for 20 sec, extension at 60°C for 1 min, and denaturation at 95°C for 15 sec.

For all other genes described in this work, 100 ng of RNA isolated from either mouse whole fat tissue or human mesenteric and omental primary preadipocyte cultures was incubated with dual fluorogenic probes (Applied Biosystems) and mouse GAPDH along with human TBP were used as endogenous controls, respectively. The detection and quantification reactions were performed by mixing the RNA and probes with the TaqMan One-Step RT-PCR Master Mix reagents (Applied Biosystems) and using the GeneAmp 5700 sequence detection system, as described above (except for the first step, where the incubation was for 30 min at 48°C).

Transient Transfection of Primary Human Mesenteric Preadipocytes. Primary human mesenteric preadipocytes were transfected with IL-8 promoter-reporter vector, either intact or mutated at the NF-κB binding site (45) using the Effectene transfection reagent method (Qiagen). Briefly, 2×10^{-5} cells per well in six-well plates were washed with 1× PBS. Each vector (0.5 μg) was mixed with buffer EC, enhancer, Effectene reagent, and medium (as described in the handbook), incubated at room temperature for 20 min, and added to the cells. The cells were washed again 16 h later. Fresh medium along with 10^{-8} M SP was added for 3 h (CJ-12255, where present, was preincubated with the cells 20 min before the addition of SP). The cells were cotransfected with the pRL-TK vector, a promoter-vector for Renilla luciferase. Lysis of the cells and calculation of luciferase activity was performed by using the dual-luciferase reporter assay system (Promega). Firefly luciferase activity was expressed relative to Renilla luciferase activity.

Statistical Analysis. A two-sample *t* test (assuming either equal or unequal variances according to estimates) was used to analyze data between two groups. ANOVA was used for intergroup comparisons when more than two groups were tested.

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