Pro-inflammatory role of vasopressin through V1b receptors in hapten-induced experimental colitis in rodents: implication in IBD

Running head: Vasopressin in experimental colitis

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**ABSTRACT**

**Background.** Vasopressin and its receptors modulate several gut functions, but their role in intestinal inflammation is unknown. Our aims were to determine (i) the localization of V1b receptors in human and rodent colon, (ii) the role of vasopressin and V1b receptors in experimental colitis using two approaches: V1b−/− mice and a selective V1b receptor antagonist, SSR149415, and, (iii) the mechanisms involved.

**Methods.** V1b receptors were localized in normal and inflamed colon from humans and rats. Experimental colitis was induced in rats and mice and some groups were treated before or after colitis induction with oral SSR149415 (3-30 mg/kg). Other groups of mice were submitted to dehydration to increase vasopressin plasma levels, prior to colitis induction. Body weight, damage scores, MPO and TNF-α tissue levels were determined. Finally, colonic segments of wild-type and V1b−/− mice were mounted in Ussing chambers and paracellular permeability in response to vasopressin was studied.

**Results.** V1b receptors were expressed in enterocytes and ganglia cells of the enteric nervous system of human and rat intestine. Expression levels were independent from inflammatory status. Colitis was less severe in rodents treated by either preventive or curative SSR149415 and in V1b−/− mice. TNBS induced a strong mortality in dehydrated animals, that were reversed by preventive SSR149415 or mast cell stabilizer. Vasopressin significantly increased paracellular permeability in WT, but not in V1b−/− mice. Preincubation of colon tissues with SSR149415 abolished the vasopressin effect. Similarly, vasopressin had no effect in colonic preparations from WT mice pretreated with mast cell stabilizers.

**Conclusions.** Vasopressin, through V1b receptor interaction, has pro-inflammatory properties linked to mast cell activation and downstream alterations of the colonic epithelial barrier.
These findings underline the potential interest of V1b receptor blockers in gut inflammatory diseases.

Keywords: colitis; inflammation; vasopressin; epithelial barrier
INTRODUCTION

Vasopressin is a peptide neurohormone synthesized in hypothalamic neurons and in the gastrointestinal tract (18). It acts through a family of three G-protein coupled receptors referred to as V1a, V1b/V3 and V2 (28). Main roles of vasopressin are directed towards water reabsorption through V2 receptors regulating aquaporin-2 expression in the kidney (11), and vascular tone by V1a receptor activation (7). Besides, vasopressin is involved in physiological adaptations to stress, through the stimulation of the hypothalamo-pituitary-adrenal axis, leading to ACTH release by posterior pituitary, this effect involving V1b receptors (24).

There is also growing interest in the potential therapeutic applications of vasopressin antagonists (10).

The gastrointestinal tract is a potent target of vasopressin, since all receptor subtypes are expressed throughout the gut in humans, which can be activated by either circulating or local vasopressin (30). Some studies have reported that vasopressin stimulates water absorption from rat and human colon (5, 27). As in the kidney, this effect is linked to the regulation of aquaporin-2 expression by enterocytes (19). Vasopressin also regulates the excitation contraction coupling in intestinal smooth muscle cells (46), and acts in brain to mediate colonic motor alterations triggered by stress (6). In addition, one study pointed out that in rat colon, vasopressin infused in blood for three days induces myofibroblast growth in pericryptic spaces, increases aquaporin-2 expression and enhances Na⁺ accumulation (9).

With respect to inflammation, numerous studies have focused on the effects of vasopressin in septic shock. The main cause of death in this context is related to multiple organ dysfunction syndrome, in which intestinal barrier breakdown appears to be a primary event leading to endotoxemia (16). The effects of vasopressin on inflammation are controversial. By acting on V2 receptors, vasopressin dampens the inflammatory response to an uropathogenic E. coli (8).
and lowers the secretion of IL-6 in the lung, in response to LPS-induced pulmonary inflammation (3). By contrast, V1 receptors seem to be linked to pro-inflammatory properties of vasopressin. Indeed, V1-like receptors have been found in human peripheral blood mononuclear cells (2), and vasopressin can promote IFN-γ release by lymphocytes (40). Moreover, endogenous vasopressin released during a septic shock is responsible for gastrointestinal mucosal damage, this effect being markedly lowered by a V1a receptor antagonist (42). Interestingly, intestinal epithelial barrier breakdown is considered as pivotal in the pathogenesis of inflammatory bowel diseases (IBD) (47). Barrier integrity lies in part on the continuous epithelial lining of the gut, mainly regulated by intercellular tight junctions, and numerous studies have emphasized abnormalities of the junctional complex during IBD (20, 26, 45). Moreover, stressful life events are known to exacerbate inflammation during IBD, through mechanisms involving epithelial barrier impairment (36). This is of potential interest herein, since vasopressin is a well-defined mediator of stress. However, despite this background, no study has investigated so far the involvement of vasopressin and vasopressin receptors in gut inflammatory states as IBD or in animal experimental models. Therefore, the aims of our study were (i) to compare the expression of V1b receptors in human and rat colonic tissues under normal and inflammatory conditions, (ii) to determine a possible local involvement of vasopressin and V1b receptors in experimental colitis using V1b deficient mice, (iii) to evaluate the anti-inflammatory properties of a selective non-peptide V1b receptor antagonist, SSR149415 (37) and finally (iv) to explore the mechanisms through which vasopressin may participate to the inflammatory response.
MATERIAL AND METHODS

Human samples

Human colonic biospecimens were obtained from several commercial providers (Asterand, Royston, UK; Indivumed, Hamburg, Germany; Cytomyx, Cambridge, UK; BioCat, Heidelberg, Germany), and represent samples from patients (male & female) diagnosed with inflammatory bowel disease (Ulcerative colitis, n=11; Crohn’s disease, n=8) as well as samples without pathological finding from GI tract tumor patients (control tissue n=10). The surgical biospecimens were granted for research by informed consent.

Chemicals

The V1b receptor antagonist, SSR149415 (37) was synthesized at Sanofi-Aventis R&D, France. It displays nanomolar affinity for the V1b receptor in human and murine tissues, and is 800 and 2000 times more potent on V1b than on murine V1a and V2 receptor, respectively (37). It was dissolved in 0.6% methylcellulose in water and administered to rats or mice by oral gavage. Vasopressin was from Bachem (Weil am Rhein, Germany). Doxantrazole and Sodium cromoglycate were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France).

Animals

All protocols have been approved by the Animal Care and Use Committee of the different institutions. Male Wistar rats (200-250g) were purchased from Elevage Janvier (Le Genest-St-Isle, France). V1b KO mice (Avpr1b<sup>tm1Dgen</sup>) where purchased from Deltagen (San Mateo, CA). In this model, avpr1b gene was disturbed by insertion of the bacterial lacZ vector such that the endogenous gene promoter drives expression of beta-galactosidase. Mice were backcrossed to C57Bl/6J genetic background and were bred at Sanofi-Aventis transgenic service by He x He mating. KO mice were compared with their WT littermates. Genomic
DNA for PCR genotyping analysis was isolated from tail biopsies for all mice with DNeasy 96 Tissue kit (Macherey-Nagel, Düren, Germany) using a 96-well plate format on a Hamilton Microlab Start automated robotic system.

Experimental colitis in rats and mice

DNBS-induced colitis in rats

Male Wistar rats, weighing 200-250 g were used in this study. They were provided free access to food and water. Under light anaesthesia, a rubber catheter was inserted by the rectal route into the colon so that the tip was about 8 cm proximal to the anus (approximately at the level of the splenic flexure). DNBS (2,4-dinitrobenzene sulfonic acid, ICN Biomedicals, Aurora, OH, USA, 60 mg/kg in 50% ethanol) was then instilled into the lumen of the colon through the rubber catheter (total volume = 0.25 ml). Animals were used for subsequent experimental procedures on day 6 after DNBS treatment, at a time when the intestinal inflammatory process is maximal. In control experiments, the animals received 0.25 ml 0.9% NaCl alone. Body weight was observed over this 8-day period. Each group of rats consisted of 8-11 animals. SSR149415 was administered daily, at the same time, for 7 days, starting the day before the induction of colitis.

TNBS-induced colitis in mice

Male WT and V1b−/− mice, weighing 20-25g were used. Animals received water and food ad libitum. Under general anaesthesia (Imalgène®/Rompun® 1/5 v/v, 0.05 ml/mouse, SC) colitis was induced by intra-colonic instillation of TNBS/ethanol solution (2,4,6-trinitrobenzene sulfonic acid, Sigma, 50 mg/kg, 50% ethanol, in 40μl) using a catheter introduced by rectal route at 4 cm from the anus. Animals were sacrificed 2 days after TNBS instillation. Three protocols were carried out. In a first series of experiments, SSR149415 was given daily from one day before to one day after TNBS intracolonic instillation. In a second set, SSR149415
was injected in a curative manner, *i.e.* from one day after colitis induction by TNBS. Finally, other animals were submitted to water deprivation for 24 hours, and colitis was induced the next day. Drugs (SSR149415 30 mg/kg p.o., doxantrazole 10 mg/kg i.p., or their respective vehicles) were administered in a preventive manner, *i.e.* from one day before to one day after colitis induction.

**DSS-induced colitis in mice**

Other WT and V1b⁻/⁻ mice mice were treated orally with Dextran Sodium Sulfate (4% w/v in drinking water) for 6 days and were sacrificed at day 7. One group of animals was treated with SSR149415 from day one prior to DSS administration to day 6 (preventive treatment). Another group was treated from day one after colitis induction (curative treatment).

**Genotyping**
Genotyping assay was developed and executed using Taq Qiagen Hot Start mix and the following primers for WT: V1361, 5' TCT GGC CAC AGG AGG CAA CCT 3'; V1900c, 5' ATC TCG TGG CAG ATG AGG CCA 3'; for KO animals: NEO S, 5' GAT GGA TTG CAC GCA GGT TCT 3'; NEO AS : 5' AGG TAG CCG GAT CAA GCG TAT 3'. PCR was performed using the following conditions: an initial step at 95°C for 15mn, then 34 cycles consisting of 45 sec at 94°C, 45 sec at 61°C and 45 sec at 72°C, and a final elongation step for 10mn at 72°C. Under these conditions, the common WT forward and WT reverse primers yield a 539 pb band for the WT allele, vs. a 378 pb band when utilizing the WT forward/Neo reverse primer for the targeted allele.

**DIG-labelled riboprobe generation**
The DIG labelled riboprobe was generated by in vitro transcription (Roche) of a PCR product generated with RNA Polymerase binding site extended ratAVPR1b specific primers (T3
linked sense primer 5'-AATTAACCCTCACTAAAGGGCTAACTCT-195 GAATTCTGAGCCTTC-3` and T7 linked antisense primer 5'-TAATACGACTC-196 ACTATAGGGCTGGAAGCGGTAGGTGATGTC-3`) or rat PECAM specific primers ((T3 linked sense primer 5'-AATTAACCCTCACTAAAGGGGTGGTAGAGC-ACAGTGGACAC 198 and T7 linked antisense primer 5'- TAATACGACTC-199 ACTATAGGGGAGAGCATTTCGCACACCTG) from a rat AVPR1b or rat PECAM cDNA clone as the substrate and PfuTurbo DNA-Polymerase (Stratagene, Cedar Creek, TX), respectively. After column-mediated purification (GE Healthcare) the DIG-labelled riboprobes were evaluated for integrity and quantity by gelelectrophorese (Invitrogen, Karlsruhe, Germany) and tested for their specificity and condition set up on transient transfected, ratAVPR1b overexpressing CHO (chinese hamster ovary cells) cells or control tissue.

Immunohistochemistry and in situ hybridization

Tissue sections (5 µm on SuperFrost/Plus) were generated (Microme HM340E), Hematoxylin & Eosin (H&E) stained, and utilized in Immunohistochemistry (IHC) or Digoxigenin-mediated RNA in situ Hybridisation (ISH) experiments. IHC experiments were performed using the Ventana Discovery system. Briefly, tissue sections were deparaffinized by heat, pretreated according to the need of the individual primary antibody applied and incubated with various primary antibodies, i.e. rabbit polyclonal anti-human AVPR1b antibody (LifeSpan LS-A3739, 10ng/µl with low heat (950-124)/protease (760-2020) combination treatment at 37°C for 1 hr) or with custom made, rabbit polyclonal anti-rat AVPR1b antibody directed against the epitope amino acid 308-319 of Acc# NP_05891 (Cambridge Research Biochemicals, Cleveland, UK). Secondary incubation was then performed with matching biotinylated goat anti-rabbit secondary
antibody (Vector, Wertheim-Bettingen, Germany) for 16 minutes. For visualisation, sections were incubated with the biotin-free, multimer technology detection kit UltraMap DAB, counterstained and mounted afterwards (Sakura, Staufen, Germany). High resolution images were taken at the Aperio Scanscope Digital slide scanner (20 x Objective).

ISH experiments were performed using Ventana Discovery system (Ventana, Illkirch, France). Briefly, tissue sections were deparaffinized by heat, 20 minutes pre-treated with protease and hybridized with Digoxigenin-labeled ISH riboprobe specific for rat AVPR1b (40 ng antisense (as) or sense (se) riboprobe matching at position 161-468 of Acc# NM_017205) or rat PECAM (25 ng, 347 nt) for 6 hr at 65°C. After washing 3 times for 10 minutes in 0.1 x SSC buffer at 75°C, sections were incubated with alkaline phosphatase-linked anti-DIG antibody (Roche, Mannheim, Germany) for 30 minutes. For visualization, sections were incubated with the BlueMap kit (NBT/BCIP mediated detection), counterstained with nuclear fast red and mounted afterwards. High resolution images were taken at the Aperio Scanscope Digital slide scanner.

Macroscopic and microscopic damage scores

Macroscopic colonic damage was assessed using a previously described method. Briefly, macroscopic criteria were based on the following (41, 43): presence of adhesions between the colon and other intra-abdominal organs (0=none, 1=mild, 2=major), consistency of colonic faecal material (as an indirect marker of diarrhoea) (0=formed, 1=loose, 2=liquid), thickening of the colonic wall, presence and extension of hyperemia and macroscopic mucosal damage (0= no damage; 1= hyperemia; 2= presence of an ulcer; 3= ulcer + inflammation; 4= two or more ulcers; 5= major damage (presence of necrosis < 2 cm); 6= very severe damage (presence of necrosis > 2 cm)).
Microscopic criteria for damage and inflammation were determined by light microscopy on haematoxylin-eosin stained tissue sections obtained from whole gut specimens taken from a region of the inflamed colon immediately adjacent to the gross macroscopic damage, and consisted to establish: loss of mucosal architecture, cellular infiltration, muscle thickening, presence of crypt abscess and goblet cell depletion (14).

Measurement of myeloperoxidase activity

MPO activity, a marker of polymorphonuclear neutrophil primary granules, was measured in colonic tissues, according to the method of Bradley et al. 1982 (4), slightly modified. After sacrifice, a sample of colon was suspended in potassium phosphate buffer (44 mM KH$_2$PO$_4$, 6 mM K$_2$HPO$_4$, 3H$_2$O, pH 6.0) and homogenized on ice using a Polytron. A cycle of freezing and thawing was repeated three times. Suspensions were then centrifuged at 6,000 g for 15 min at 4°C. Supernatants fractions were discarded and pellets were resuspended in hexadecyl trimethylammonium bromide buffer (HTAB 0.5%, w/v, in 50 mM potassium phosphate buffer, pH 6.0), a detergent inducing the release of MPO from neutrophile primary granules. These suspensions were sonicated on ice, and then centrifuged at 6,000 g for 15 min at 4°C. Pellets were discarded and supernatants were assayed spectrophotometrically for MPO activity. Supernatant was diluted in potassium phosphate buffer (pH 6.0) containing 0.167 mg/ml of O-dianisidine dihydrochloride and 0.0005% H$_2$O$_2$. Changes in absorbance at 450 nm were recorded every 30 s over 2 min. One unit of MPO was defined as the quantity of MPO degrading 1 µmol H$_2$O$_2$/min/ml at 25 °C. Finally, MPO activity expressed as Units of MPO activity/mg of wet tissue (U/mg).

Determination of TNF-α tissue levels

Samples of colon were removed from the area of gross injury, snap frozen and stored at —
80°C. On thawing, samples were weight, homogenized in 1 ml of protease inhibitors cocktail (aprotinin, leupeptin and pepstatin, 1 µg/ml), and centrifuged at 4 °C. A 100 µl aliquot of supernatant was then added to the EIA 96-well plate in duplicate and assayed using the manufacturer’s protocol (Rat TNF-α kit, Bender MedSystems, Vienna, Austria). Absorbance was read at 450 nm. TNF-α was expressed as pg/mg of wet tissue.

Measurement of colonic paracellular permeability

Immediately after sacrifice, portions of WT and V1b⁻/⁻ mouse proximal colon (exposed area, 0.3 cm²) were mounted into Ussing chamber (Physiological Instruments, San Diego, CA), each side containing 5 ml of Krebs buffer (118 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 25 mM NaHCO₃, 8.3 mM glucose, 2.5 mM CaCl₂), continuously gassed with 95% O₂/5% CO₂. Transepithelial electrical resistance was monitored throughout the experiment to assess tissue viability. After 20 minutes, fluorescein isothiocyanate-labeled 4-kD dextran was added at the mucosal side (2.2 mg/ml as final concentration). At the same time, Vasopressin (10⁻⁹ to 10⁻⁷ M), SSR149415 (10⁻⁶ M), or vehicle were added in the serosal compartment. Fluorescence was measured at the serosal side after 60 minutes. The role of mast cells was investigated in groups of mice pretreated with either doxantrazole or sodium cromoglycate. Doxantrazole (10 mg/kg in NaHCO₃ 5%, i.p. route) was injected 2 hours before sacrifice (12). Sodium cromoglycate (100 mg/kg) was dissolved in saline and injected by i.p. route 12h and 30 min before sacrifice (17).

Statistical analysis

Results are expressed as means ± standard error mean (S.E.M.). Statistical analysis was performed using analysis of variance (one-way or two-way, as appropriate, with the Bonferroni’s correction for multiple comparisons). A P value <0.05 was considered...
significant. Calculations were performed using GraphPad Prism™ (version 4.0, GraphPad Software Inc., San Diego, CA).
RESULTS

**V1b receptor is expressed by human and rat tissues**

V1b receptor was detected by immunohistochemistry in normal human colon, UC and CD cases. Positive labeling was found in enterocytes, both at the membrane and into the cytoplasm (Figure 1). Moreover, staining was seen in selected cell bodies in a subset of ganglia of the enteric nervous system (Figure 1). However, no difference in staining intensity and localization was observed between normal and inflammatory conditions (Figure 1).

Interestingly, rat tissues showed the same staining localization as shown by in situ hybridization (Figure 2A) and confirmed by immunohistochemistry (not shown). Seven days after the induction of colitis, an upregulation of the V1b receptor expression was observed in rat enterocytes, and in the areas of mucosal regeneration. Regarding the enteric nervous system, no staining difference was observed upon inflammatory conditions (Figure 2B).

**Effect of SSR149415 on experimental colitis in rats**

Body weight gain of non-inflamed controls was 25.5 ± 1.9 % at the end of the 8-day period of observation. After intrarectal DNBS, the body weight gain was significantly reduced with respect to non-inflamed controls (11.2 ± 2.9 %, p<0.001). Treatment with SSR149415 at the doses of 3, 10 and 30 mg/kg did not reduce this impairment in body weight gain (data not shown).

Macroscopic and microscopic damage scores were significantly (p<0.001) increased by intrarectal DNBS (Figures 3 &4). Oral SSR149415 at 3, 10 and 30 mg/kg significantly reduced macroscopic damage produced by DNBS (Figure 3A); the 3 doses induced a global similar effect. Likewise, microscopic damage scores and TNF-α tissue levels were also lowered by SSR149415 pretreatment (Figure 3B&C). Finally, animals receiving SSR149415 exhibited lower MPO levels at the dose of 10 mg/kg (5.5±1.23 U/mg vs. 18.3±2.9 U/mg with
DNBS, p<0.01, n=8), while the doses of 3 and 30 mg/kg decreased activity without reaching significance (p=0.0963 and p=0.0536, respectively, n=8).

**Experimental colitis in wild type and V1b−/− mice**

Two days after intrarectal administration of TNBS, severe macroscopic damages (Figure 5A) associated with high MPO activity (Figure 5B) and weight loss (Figure 5C) were observed in WT animals. By contrast, macroscopic damage scores and MPO levels were significantly lower in V1b−/− mice (366±82 U/mg of protein vs. 689±49, Figure 5, p<0.05). Weight loss was also less severe in V1b−/− mice (p<0.05). This strongly suggests, that vasopressin via V1b receptor activation is involved in the early phase of mucosal damage triggered by TNBS, since the absence of V1b receptors led to a markedly weaker inflammation. Preventive or curative treatment with SSR149415 (30 mg/kg po) significantly decreased macroscopic damage and MPO activity in WT animals, to levels similar to those of V1b−/− animals (393±49 U/mg of protein, Figure 5). No additive effect of SSR149415 and V1b deficiency mice were observed, which illustrates the antagonist specificity (Figure 5A&B). Regarding DSS-induced colitis, SSR 149415 was devoid of any preventive or curative effect (Supplementary data 1).

**Experimental colitis on water-deprived animals**

Water deprivation was performed to increase circulating vasopressin levels, according to Cristia et al (9). This resulted in a dramatic mortality in TNBS-treated animals (9 over 10 mice in the group). Administration of either doxantrazole, a mast cell stabilizer, or the V1b antagonist SSR 149415 prevented this elevated mortality and significantly reduced the colitis severity assessed by macroscopic damage scores and MPO activity (Figure 6). Interestingly, the effect of SSR149415 was more pronounced in water-deprived animals compared to mice with free access to water (Figure 6).
Effect of vasopressin on mouse colonic paracellular permeability

Vasopressin, added at the serosal side of the wild type mouse colonic wall, increased colonic permeability to FITC-dextran, used as a marker of the paracellular route. The dose-response revealed a bell-shaped curve, the maximal effect being reached at $10^{-8}$ M ($2.30 \pm 0.33$, $p<0.05$ vs control, Figure 7A). Basal permeability was unchanged in V1b$^{-/-}$ animals compared with controls ($0.91 \pm 0.09$ nmol/h/cm$^2$ vs. $0.82 \pm 0.09$, Figure 7B). The effect of vasopressin was absent in V1b$^{-/-}$ animals (NS vs. saline, Figure 7B), and was abolished when WT colonic preparations were pretreated with SSR149415 $10^{-6}$M ($0.79 \pm 0.13$ nmol/h/cm$^2$, $p<0.05$ vs vasopressin, Figure 7B).

Pretreating WT mice with doxantrazole or sodium cromoglycate assessed the involvement of mast cells. No change in permeability was observed in pretreated animals (Figure 7C), while vasopressin $10^{-8}$ M did not trigger any increase in colonic paracellular permeability in preparations from animals pretreated with either doxantrazole or cromoglycate (respectively $0.76 \pm 0.10$ nmol/h/cm$^2$ and $0.91 \pm 0.18$ nmol/h/cm$^2$, Figure 7C).

DISCUSSION

Our results identify vasopressin as a putative participant of gut inflammation and show that the V1b receptor is a key target in the inflammation process, as shown in TNBS-induced experimental colitis. So far, the role of V1b receptor was poorly documented in the colon, and only RT-PCR experiments reported mRNA expression in the rat small intestine (35). In the present work, we have shown that V1b receptor expression in normal and inflamed human and rat colon tissues shares a similar localization, i.e. enterocytes and in cells of the enteric nervous system, while no staining was observed above background in other areas such as
lamina propria. Moreover, in V1b-deficient mice, we observed a lower inflammatory response to TNBS and, in two models of hapten-induced experimental colitis, a reduction of gut inflammation under blockade of V1b receptors by SSR149415. In water-deprived animals, mast cell or V1b blockade ameliorated the dramatic mortality observed in TNBS-treated mice. Conversely, no improvement of inflammation was observed in DSS-induced colitis. Finally, Ussing chambers experiments revealed that vasopressin was able to increase colonic paracellular permeability of mouse tissue, this effect being dependent upon V1b receptor activation and mast cells. Thus, these findings report for the first time an original role for vasopressin in participating to inflammation, which can be, at least in part, linked to its ability to alter epithelial barrier.

Increased plasma levels of vasopressin have been described in various chronic inflammatory states in humans, such as cirrhosis (21), leukemia (23) and early phase of sepsis (25). In the Lewis rat, which displays elevated vasopressin plasma levels, an increased susceptibility to develop a colitis has been observed, which can be counteracted by immunoneutralization of vasopressin (33). By contrast, vasopressin-deficient Brattleboro rats are more resistant to gastrointestinal damage induced by endotoxins (42).

Vasopressin may influence inflammation through several sites of action. Indeed, V1 receptors have been described in peripheral blood mononuclear cells (2) and can trigger IFN-\(\gamma\) secretion by T-cells (40). In the experimental DNBS model of colitis in rats, oral SSR149415 (3, 10 and 30 mg/kg) prevented colon macroscopic and microscopic damages produced by DNBS for all the doses tested. In addition, we observed that in this model, SSR149415 is able to decrease the mucosal concentration of TNF-\(\alpha\) under inflammatory conditions. This is of peculiar relevance at the intestinal level since together with IFN-\(\gamma\), TNF-\(\alpha\) is a well known disruptor of enterocyte tight junctions (22). On the other hand, a recent work has shown that vasopressin, through V2 receptors, can reduce the immune response to an uropathogenic \textit{E.coli}. (8) In the
pulmonary tract, vasopressin is also able to decrease IL-6 release after a challenge with LPS (3). Thus, vasopressin may exert a dual role on inflammation: pro-inflammatory properties mediated by V1 receptors, and an immunomodulatory role triggered by V2 receptor activation. In the context of TNBS/DNBS-induced inflammatory colitis, we corroborate these findings, since (i) a V1b receptor antagonist, SSR149415, reduces the inflammatory parameters both in a curative and a preventive manner and, (ii) the colitis severity is weaker in V1b-deficient mice. Water deprivation is known to increase circulating vasopressin levels, and in this context we observed a strong mortality of animals in this group (90%), while treatment with the V1b antagonist abolished this lethality and ameliorated the colitis severity.

Conversely, we observed no improvement of inflammatory parameters on DSS-induced colitis. This may be due to the difference of colitis induction between DNBS/TNBS and DSS. Indeed, DNBS and TNBS act as an hapten that will stimulate the local immune cells (31). This is verified in our current experiments by TNF-α mucosal levels, that are lowered under SSR149415 treatment. However, it is supposed that sulphate moieties from DSS are responsible for its toxicity, through H₂S and HS⁻ production by enteric microbiota (34). In addition, colitis initiation does not require immune activation, since SCID mice develop a similar inflammation compared with controls (13).

According to V1b receptor localization, i.e. enterocytes and enteric ganglia, we can hypothesize that in our model, vasopressin may regulate inflammation by acting on epithelial cells and/or on the enteric nervous system. We have shown herein that vasopressin increases paracellular permeability of mouse colonic samples in Ussing chambers, this effect being inhibited by SSR149415, and absent in V1b-deficient animals. Moreover, we observed that preventive administration of SSR149415 also reduced colitis severity in mice submitted to water deprivation to increase their vasopressin blood levels. We can thus speculate that
SSR149415 limits the crossing of the hapten (DNBS or TNBS) to the internal milieu, which hampers its ability to stimulate the local immune system. This is also relevant with the absence of effect of the compound on DSS-induced colitis. It is well admitted that epithelial barrier breakdown is a key primary event in gut inflammation and IBD (47). Barrier integrity lies on several parameters and is continuously submitted to exogenous and endogenous stimuli. Of interest, one study has shown in a model of endotoxin-induced gastrointestinal damage that a peptide V1 antagonist reduced the severity of mucosal injury (42). Despite the presence of V1b receptor on enterocytes, our data suggest a new mechanism, that vasopressin exerts an indirect effect on colonic barrier function. Indeed, in colonic samples recovered from animals treated with mast cell stabilizers, doxantrazole and sodium cromoglycate, vasopressin was devoid of any effect, suggesting that the mast cell is also a putative target of vasopressin. Mast cells are immune cells playing a pivotal role in the regulation of barrier function. We have shown in an animal model of stress-induced impairment of epithelial barrier, an activation of mast cells leading to IFN-γ release by T-cells, which in turn induces gut leakiness (15). A recent study has also evidenced that activation of human mast cells by Corticotropin Releasing Hormone (CRH) triggered barrier defects (44). However, we did not observed any V1b receptor labeling in mast cells in our preparations, but in ganglia from the enteric nervous system. One feature of mast cells is their close relationship with enteric nerves. The activation of V1b receptors in enteric nerves may locally release mediators as CRH or Nerve Growth Factor (NGF) close to mast cells, leading to their degranulation. In addition, we have recently shown in an animal model of stress, a closer association between mast cells and enteric nerves, associated with an increased colonic permeability (1). One can hypothesize that activation of enteric nerves by vasopressin, through V1b receptors, may be responsible for epithelial barrier impairment in our experimental conditions. Of interest, we show that mast cell blockade by doxantrazole abolished the high mortality observed in water-
deprived, TNBS-treated animals, and decreased the colitis severity in TNBS-treated mice, either normally hydrated or submitted to water deprivation. Alternatively, a recent paper reported that the known $G_{q/11}$-coupled V1b receptors was also able to signal in transfected cells through a $G_s$ protein/cAMP pathway depending on the nature of the ligand as well as on its localization within specialized compartments of the plasma membrane. Thus, through cAMP involved in AQP2 recruitment and permeability, direct V1b receptor activation could modify intestinal barrier function (32). We noticed that vasopressin displayed a bell-shaped dose-response regarding permeability. This observation was not unexpected: indeed, some authors have reported similar dose-responses, either with vasopressin-(4-9) acting on V1a receptors (29), or $[\text{deamino-Cys}^1,\text{D-Arg}^8]$-vasopressin acting on V2 receptors (48).

A strong V1b receptor expression was denoted in the areas of mucosal regeneration, seven days after the induction of inflammation. Recently, Cristia et al. have shown that a prolonged vasopressin administration in rats stimulates the proliferation of the colonic myofibroblast layer, this effect involving both V1 and V2 receptors (9). Trophic effects of vasopressin have been documented through V1a receptors in various cell types (39),(38). Our present observation could denote a pro-proliferative effect of vasopressin, which might play a role in the resolution of inflammation in the late phase, by contrast with its early pro-inflammatory role.

To conclude, our data provide an original pro-inflammatory role for vasopressin in early stages of colitis, involving V1b receptors and mast cells. Despite further studies need to be addressed, our work clearly identifies V1b receptor antagonists as putative new therapeutic agents in gut inflammatory conditions.
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FIGURE LEGENDS

Figure 1. V1b receptor labeling by immunohistochemistry in human colonic biopsies from healthy subjects (A), Ulcerative colitis (B), and Crohn’s disease patients (C). The V1b receptor is present in enterocytes, and some ganglia of the enteric nervous system. No difference of expression is observed under normal and inflammatory conditions.

Figure 2. V1b receptor mRNA localization by in situ hybridization in control and DNBS-treated rats. Similarly to that observed in humans, V1b mRNA is present in enterocytes and ganglia from the enteric nervous system. Seven days after colitis induction, an intense labeling is observed within the areas of mucosal regeneration.

Figure 3. Macroscopic (A) and microscopic (B) scoring of colonic damage, and TNF-α levels measured in colonic tissue homogenates (C) induced by DNBS in rats, and reduction by pretreatment of animals by oral SSR149415 (3, 10 or 30 mg/kg). Data are expressed as mean±sem. n=8 rats per group, ***P < .001 vs. Saline; *P <0.05, **P <0.05, ###P <0.001 vs. DNBS.

Figure 4. Cross sections of rat distal colon treated with saline (A), DNBS (B), DNBS+SSR149415 3 (C), 10 (D) or 30 (E) mg/kg. DNBS induced a dramatic loss of mucosal architecture with goblet cell depletion and mucosal ulcerations, the granulocyte infiltrate extending throughout the mucosa and submucosa, also involving the muscularis propria, which appears thickened. Scale bar: 100 μm.

Figure 5. Macroscopic damages (A) and myeloperoxidase activity (B) determined in wild-type and V1b-deficient mice, treated or not with SSR149415 30 mg/kg. Panel C represents the limited weight loss in V1b<sup>−/−</sup> animals, compared with WT, submitted to TNBS-induced colitis. Data are expressed as mean±sem, n=10-12 mice per group. *p<0.05, **p<0.01, ***p<0.001 vs. WT/TNBS-treated group.

Figure 6. Effect of SSR149415 and doxantrazole pretreatment on colitis induced by TNBS in
mice submitted to water deprivation. Colitis was assessed by macroscopic damage scores (A) and MPO activity (B). Animals submitted to water deprivation and TNBS showed a mortality of 9 over 10 animals, thus the data were not analyzed. Doxantrazole or V1b antagonist preventive treatment prevented the mortality and ameliorated the signs of inflammation. Data are mean±sem, n=10 per group. *p<0.05, **p<0.01, ***p<0.001 vs. TNBS-treated group.

**Figure 7.** Measurement of colonic paracellular permeability to FITC-labeled dextran of wild-type and V1b-deficient mouse colonic explants mounted in Ussing chambers. Vasopressin (VP) 10^{-9} to 10^{-7} M was added in the serosal compartment of the chamber, and permeability was assessed after one hour (A). In some experiments, SSR 149415 10^{-6} M was added in the serosal compartment 10 minutes before vasopressin (B). Colonic paracellular permeability of tissues in response to vasopressin was also studied in wild-type animals pretreated with doxantrazole or sodium cromoglycate (C). Data are expressed as mean±sem, n=8-10 mice per group. *p<0.05 vs vehicle; "p<0.05 vs VP 10^{-8} M.

**Supplementary Figure 1.** Effect of preventive and curative effect of SSR149415 30 mg/kg on DSS-induced colitis in mice. Colitis severity was assessed by following animal weight (A), and measuring MPO activity (B) the day of sacrifice. Data are expressed as mean±sem, n=8-10 mice per group. **p<0.01, ***p<0.001 vs. control group.