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Ectopic expression of OX1R in ulcerative colitis mediates anti-inflammatory effect of orexin-A

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ABSTRACT

Orexins (orexin-A and orexin-B) are hypothalamic peptides that are produced by the same precursor and are involved in sleep/wake control, which is mediated by two G protein-coupled receptor subtypes, OX1R and OX2R. Ulcerative colitis (UC) is an inflammatory bowel disease, (IBD) which is characterized by long-lasting inflammation and ulcers that affect the colon and rectum mucosa and is known to be a significant risk factor for colon cancer development. Based on our recent studies showing that OX1R is aberrantly expressed in colon cancer, we wondered whether orexin-A could play a role in UC. Immunohistochemistry studies revealed that OX1R is highly expressed in the affected colonic epithelium of most UC patients, but not in the non-affected colonic mucosa. Injection of exogenous orexin-A specifically improved the inflammatory symptoms in the two colitis murine models. Conversely, injection of inactive orexin-A analog, OxB7–28 or OX1R specific antagonist SB-408124 did not have anti-inflammatory effect. Moreover, treatment with orexin-A in DSS-colitis induced $OX1R^{-/-}$ knockout mice did not have any protective effect. The orexin-A anti-inflammatory effect was due to the decreased expression of pro-inflammatory cytokines in immune cells and specifically in T-cells isolated from colonic mucosa. Moreover, orexin-A inhibited canonical NFκB activation in an immune cell line and in intestinal epithelial cell line. These results suggest that orexin-A might represent a promising alternative to current UC therapies.

1. Introduction

Ulcerative colitis (UC) and Crohn's disease are the two main forms of chronic inflammatory bowel diseases (IBD). UC is a chronic, relapsing-remitting and disabling disease that specifically affects the mucosa and superficial submucosa of the colon and rectum, with a distal to proximal gradient of inflammation [1]. UC is characterized by mucosal architectural changes, including early alterations of mucus-secreting goblet cells, crypt distortion, cryptitis, and typical crypt abscesses resulting from the transepithelial infiltration of immune cells during the acute phase of the disease [1]. In addition, these lesions can over time evolve towards dysplasia and cancer [2]. The exact etiology of UC remains unknown, and the course of the disease is variable. Multiple pathogenic factors are thought to be involved in UC, including environmental changes, susceptibility gene variants, dysbiosis, and dysregulated intestinal innate and adaptive immune responses [3]. Recently, the inflammasome pathway, regulatory RNAs, damage-associated molecular patterns (DAMPs) as well as ER stress in epithelial cells have all been associated with UC [3]. Currently, the treatment of UC remains limited and varies with the severity of the symptoms. The failure or intolerance to drug therapy, or the constant battle with the disease (acute severe colitis, perforation, uncontrollable bleeding, severe side effects of the medications and risk of cancer) are all indications for colectomy [4]. Thus, the identification of new therapeutic targets represents a major challenge in the treatment of UC.

Targeting G protein-coupled receptors (GPCRs), a large superfamily (800 members) of seven transmembrane cell surface receptors, has demonstrated significant potential for the treatment of chronic

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inflammatory diseases [5]. Most of those receptors are involved in inflammation and more specifically in colitis such as i) chemokine receptors including CCR5 [6], CCR6 [7]; ii) histamine receptor such as H4R [8]; cannabinoid receptor such as CB2 [9]; some orphan receptors [10] and; iii) neuropeptide receptors including VPAC/PACAP receptors [11].

Orexins (OxA and OxB) are hypothalamic neuropeptides that are both produced from the 131-residues prepro-orexin precursor [12]. These two peptides have been shown to be involved in multiple physiological processes, including the regulation of the sleep/wakefulness state, energy homeostasis, reward seeking, and drug addiction [13,14]. While the most important target of the orexins is the central nervous system, orexins also play a role in various peripheral organs such as the intestine, pancreas, adrenal glands, kidney, adipose tissue and reproductive tract [15], although their roles remain controversial [16]. Studies have demonstrated that OxA exerts neuroprotective effects, suggesting that it could affect the microglia, the resident macrophage-like cells of the brain [17]. In post-stroke cerebral neuroinflammation, treatment with OxA could reduce the infarct size through a microglial-mediated pathway inducing modulation of immune mediators [18]. Recent study reveals that OxA improved the survival of mice in which lipopolysaccharide (LPS) was intraperitoneally injected [19]. However, the role of orexins in neuroinflammation is still weakly documented and to date, the potential modulatory role of orexins in chronic inflammation, including IBD, is entirely unknown.

Orexins mediate their central and peripheral biological effects by binding to two GPCR subtypes, OX1R and OX2R [20], leading to the subsequent release of intracellular calcium transients *via* a Gq-dependent pathway [21]. Our group has recently reported that: 1) OX1R is highly expressed in colon, pancreas and prostate cancers, but is not detectable in normal tissue counterparts [22–24]; 2) OxA and OxB induce a massive apoptosis in cell lines derived from colon, pancreas and prostate cancers [22–24]; and 3) OxA and OxB display an anti-tumor effect in nude mice xenografted with these cell lines [22,24]. Considering our data in colon cancer and the potential role of orexins in neuroinflammation, we hypothesized that the OX1R/OxA pathway may have an important role in colonic inflammation in UC.

We provide here the first demonstration that OX1R is aberrantly expressed in the inflamed mucosa of UC patients, both in the epithelial and immune cells. This expression is associated with a strong specific immunomodulatory effect of OxA *via* OX1R and down-regulation of the pro-inflammatory cytokine production and secretion, as shown in two murine models recapitulating either the acute or chronic phase of UC.

2. Materials and methods

2.1. Patients and tissue collection

Nineteen patients (7 men and 12 women) with UC were selected from the records of the Departments of Pathology at the Beaujon Hospital, Clichy, France and the Centre Hospitalier Universitaire de Nantes, Nantes, France. Charts from patients were retrospectively reviewed for clinical and pathological data. The median age was 32 years (range 20–53). It should be noted that 10/19 patients have received a drug therapy whose 8 patients presented a drug resistance. The use of human material and retrospective study were approved by the Institutional Review Board (CEERB GHU Paris Nord No. IRB12-059 and 12-033).

2.2. Animal procedures

Wild-type (wt) female BALB/c mice (Harlan laboratories), 7 to 12 weeks of age (weight 18–22 g), or 8 weeks of age (weight 18–22 g) female wt C57BL/6 or female C57BL/6 IL10$^{-/-}$NOX1$^{-/-}$ or female C57BL/6 OX1R$^{-/-}$ mice were co-housed in filter-topped cages in a 12-h light/dark cycle with environmental enrichment. Food pellets (standard diet) and water were provided *ad libitum*. The animals were treated according to the guidelines of the Ministère de la Recherche et de la Technologie (Ministère de l'Enseignement Supérieur et de la Recherche), and experiments were approved by the Comité d'Ethique Paris Nord (No 121).

DSS-induced colitis was induced in wt BALB/c, wt C57BL/6 and C57BL/6 OX1R$^{-/-}$ mice by oral treatment with tap water containing 5% (w/v) of DSS (TdB Consultancy, Uppsala, Sweden) for wt BALB/c mice or 3.5% (w/v) of DSS for wt C57BL/6 and C57BL/6 OX1R$^{-/-}$ mice. DSS mice were treated by i.p. injection of 0.22 μmol/kg OxA or OxB7-28 inactive analog or SB-408124 antagonist two days before DSS induction, followed by daily i.p. injection of 0.22 μmol/kg OxA or OxB7–28 inactive analog or SB-408124 antagonist for 7 days in the absence or presence of the SB-408124 OX1R antagonist. C57BL/6 IL10$^{-/-}$NOX1$^{-/-}$ mice, 8 weeks of age, were treated with 2 i.p. injections of 0.22 μmol/kg OxA per week for 3 weeks. A group of IL10$^{-/-}$NOX1$^{-/-}$ mice were also treated by gavage every day with 53 mg/kg of 5-ASA or water (control mice) for 3 weeks. All control mice (wt BALB/c, wt C57BL/6, C57BL/6 IL10$^{-/-}$NOX1$^{-/-}$ and C57BL/6 OX1R$^{-/-}$) received the same volume of PBS by injection or oral gavage.

UC symptoms were monitored by measuring the colon length (DSS-induced colitis model), weight and DAI (Disease Activity Index) score, which was determined by the presence and severity of diarrhea, blood in the stool, inflammation, edema, and weight loss. The DAI scores ranged from 0 (no symptoms) to 4 (watery diarrhea, blood in the whole colon, heavy inflammation, ulcerations and edema). The animals were randomly assigned to each treatment group. Data processing did not exclude results from any of the animals.

2.3. Histopathological analyses

Colons from the two UC mouse models were collected and the length was measured after sacrifice. Sections (0.5 cm) of the distal, medium and proximal colon were cut, fixed in formalin and embedded in paraffin blocks (Shandon Cytoblock, Thermo Scientific, USA), then cut into 3 μm sections, which were then stained with hematoxylin and eosin and periodic acid–schiff. A histological score (HAI) was determined by scoring the damage of the crypt, inflammatory infiltrate, number of goblet cells, and integrity of epithelium were scored from 0 (normal) to 4 (severe) in a double blinded fashion by two pathologists. OX1R expression was assessed by IHC in human and mice. After dewaxing, the 3 μm paraffin sections were rehydrated, antigen retrieval was performed by pretreatment with high temperature at pH 9, and immunohistochemical procedures were carried out using an automated immunohistochemical apparatus according to the manufacturer's instructions (Bond-Max slide stainer, Menarini, Leica Microsystems). Briefly, after antigen retrieval, sections were incubated for 30 min with a polyclonal anti-OX1R antibody (Life Technology, PA5–33837, polyclonal rabbit, 1/100), rinsed, and then incubated with a biotinylated secondary rabbit anti-goat antibody (Vector BA-500, 1/400). Sections were rinsed and the reaction was developed according to the manufacturer's guidelines (streptavidin-peroxidase with an automate BOND, Leica Microsystems). Substitution of the primary antibody with PBS was used as a negative control. OX1R immunostaining scores were obtained by multiplying the intensity (negative, 0; weak, 1; moderate, 2; and strong, 3) by the percentage of stained epithelial cells (0 − 300) by high-power field (20 ×) and were performed by three investigators. Specificity of the anti-OX1R antibody was tested using recombinant HEK-OX1R cells expressing OX1R and the parental HEK cells. Moreover, addition of immunogenic peptide to the incubation completely abolished labeling by the anti-OX1R antibody.

2.4. Cytokine quantification

Colonic tissues from control, DSS (wt BALB/c, wt C57BL/6 and