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Inflammation-induced functional connectivity of Melanin-Concentrating Hormone and IL-10

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Abstract

Melanin-concentrating hormone (MCH) was identified in mammals as a hypothalamic neuropeptide regulating appetite and energy balance. However, similarly to most of the brain peptides, MCH is also produced in the gastrointestinal system and can act locally as an immunomodulator. We have previously reported high expression of MCH and its receptor MCHR1 in the affected mucosa of patients with inflammatory bowel disease. Furthermore, MCH deficiency in mice attenuated experimental colitis, pointing to MCH as a mediator of intestinal inflammation. In the present study, in order to gain further insights into the underlying mechanisms of such effects of MCH, we treated mice with established experimental colitis due to IL-10 deficiency with a MCHR1 antagonist (DABA-822). While treatment with the same drug was successful in attenuating TNBS-induced colitis in previous studies, it offered no benefit to the IL-10 knockout mouse model, suggesting that perhaps IL-10 is a downstream target of MCH. Indeed, in experiments focusing on monocytes, we found that treatment with MCH inhibited LPS-mediated IL-10 upregulation. Conversely, in the same cells, exogenous IL-10 prevented LPS-induced MCHR1 expression. Taken together, these findings indicate a functional cross-talk between MCH and IL-10 which prevents resolution of inflammation.

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Conflict of Interest

Leo R. Fitzpatrick has been the recipient of a Research Grant from, and Ofer Reizes has been a former employee of Procter and Gamble Pharmaceuticals that developed the MCHR1 antagonist DABA-822. The rest of the authors declare no conflict of interest.

Contributors

Ziogas designed and performed the in vivo studies, analyzed data and wrote the manuscript; Ziogas, Karagiannis, Geiger and Gras-Miralles performed and analyzed the in vitro studies and reviewed the manuscript. Fitzpatrick and Reizes contributed to the experimental design of the in vivo studies and reviewed the manuscript. Najarian did the histological scoring of colitis and reviewed the manuscript. Kokkotou had input in the experimental design, analyzed data and wrote the manuscript. The authors have read and approved the final article.

Keywords

melanin-concentrating hormone; MCHR1; antagonist; IL-10; colitis; DABA-822; macrophages; cytokines; intestinal inflammation; knock-out; deficiency; neuropeptides

1. Introduction

Melanin Concentrating Hormone (MCH) was discovered in mammals as a hypothalamic orexigenic neuropeptide and studies so far have addressed primarily its role in the regulation of appetite and energy balance [29]. It has been shown that MCH expression is upregulated in the hypothalamus of obese, leptin-deficient mice, and by fasting in wild-type mice [30]. Conversely, depending on the genetic background, mice lacking MCH are lean and hypophagic [35], while transgenic mice overexpressing MCH were found to be obese and insulin resistant [24]. However, accumulating evidence suggests that MCH also modulates inflammatory responses via activation of its receptor MCHR1 on various cell types [6, 22]. Indeed, in the human blood, low level MCHR1 mRNA expression was detected in all immune populations examined, including CD19+ B-cells, CD4+ T-cells, CD8+ T-cells CD14+ monocytes and granulocytes [37]. In rodents, MCHR1 transcripts were found in the bone marrow, spleen, thymus and lymph nodes [22]. Our own FACS analysis confirms expression of MCHR1 on CD4+ T-cells isolated from peripheral blood mononuclear cells (PBMCs) or colonic tissue [1]. Moreover, treatment of PBMCs with MCH induces intracellular calcium release, and an increase of intracellular cAMP levels, by coupling of MCHR1 to different G-proteins [22]. In activated PBMCs, MCH was found to inhibit cell proliferation and IL-2 production [5, 37]. Nevertheless, the functional significance of such immunomodulatory properties of MCH remains to be seen.

We have recently reported that MCH-deficient mice develop attenuated TNBS-induced experimental colitis [20]. We subsequently identified colonic epithelial cells as targets of MCH in the intestine. Specifically, MCHR1 expression in these cells is upregulated in patients with Inflammatory Bowel Disease (IBD) and in vitro in response to bacterial products (*C.difficile* toxin A) or IL-1beta [19, 20]. In turn, treatment of colonocytes with MCH stimulates expression of IL-8 and MIP1-beta [19, 20]. Potential sources of MCH in the gastrointestinal tract [11] include the enteric nervous system [20], enteroendocrine cells [11, 12], Th2 lymphocytes [32], and the endothelium [27]. In addition to colonocytes, cells of the myeloid lineage have been identified as major targets of MCH during intestinal inflammation. For instance, our studies indicate that human primary monocytes and RAW 264.7 mouse monocytic cells are positive by immunofluorescence for MCHR1, the expression of which is upregulated by treatment with LPS [17]. Importantly, in these cells MCH was found to promote migration and phagocytosis, both critical processes of host defense mechanisms, while the effects of MCH on cytokine production by monocytes have not been investigated.

In previous experiments, we used two models of mouse experimental colitis with different pathophysiology. The TNBS-induced colitis model is primarily a Th1-dependent process and shares features with Crohn's disease, while DSS-induced chronic colitis rather

resembles ulcerative colitis and incorporates features of both innate and adaptive immune responses that are at least in part microbiome-dependent [13]. In both cases, we found significant attenuation of established colitis in response to treatment with an anti-MCH antibody [20, 41].

Therefore, in the present study, to gain further insights into the mechanism(s) by which MCH modulates intestinal inflammation, we examined the effects of MCH blockade in mice with spontaneous chronic enterocolitis due to IL-10 deficiency [21]. IL-10 is secreted primarily by monocytes, dendritic cells and some T-cells, though B-cells and mast cells can also produce IL-10 [10, 33]. Its main effect in colitis is inhibition of Th1- and Th17-mediated immune responses, as well as induction of regulatory T-cells. The significance of the IL-10 pathway in IBD is underscored by recent genome-wide association studies that identified IL-10 as a susceptibility gene for ulcerative colitis. Most importantly, individuals homozygous for recessive loss-of-function mutations in any of the two IL-10 receptor genes, develop severe IBD early in their life [8]. IL-10 deficient mice were treated with the small molecule MCHR1 antagonist DABA-822. While administration of this compound does not modulate food intake or body weight in mice, presumably due to its lack of brain penetration, it has been shown to reduce the severity of TNBS-induced colitis [15]. We found that the beneficial effects of blocking MCH in experimental colitis were cancelled in the absence of IL-10, suggesting perhaps a cross-talk between MCH and IL-10. Indeed, using in vitro approaches we demonstrate that MCH inhibits LPS-induced upregulation of IL-10 secretion by monocytes and that IL-10 itself negatively regulates MCHR1 expression in the same cells.

2. Materials and Methods

2.1. Treatment of IL-10 deficient mice with a MCH antagonist

C.129P2(B6)-*Il10^{tm1Cgn}*/J mice and their controls were purchased from the Jackson Laboratory and subsequently bred in our facility. Male eight-week old IL-10 deficient mice and their wild-type littermates were used in the described experiments. It has been previously reported that treatment with piroxicam (Sigma), a nonsteroidal anti-inflammatory drug, can synchronize the induction of colitis in IL-10 deficient mice [2]. Piroxicam was mixed with powdered chow at a concentration of 80mg per 250g of food and given to wild-type (control) and IL-10 deficient mice for two weeks. Subsequently, mice were switched to normal rodent chow and received treatments with the MCHR1 peripheral antagonist DABA-822 (Aroz Technologies, Cincinnati, Ohio) or vehicle (n=8 mice per group). The medication was administered at 30mg/kg by oral gavage twice daily for an additional two weeks (n=8 mice per group) (Fig. 1). Dose selection and route of administration for DABA-822 was based on previous studies [7, 15].

Histological assessment of colitis was performed in paraffin embedded H&E stained transverse colonic sections by a Pathologist specializing in GI (RN) blinded to the experimental groups. Briefly, the following parameters were scored 0–3: crypt distortion and shortening; epithelial damage; and inflammatory infiltrates. Graphs represent the average scoring for all parameters (maximum score =3). Two fields from each colon section (distal, middle, proximal) were evaluated and averaged for each mouse.

All experimental procedures using animals have been approved by the Institutional Animal Care and Use Committee (IACUC) of Beth Israel Deaconess Medical Center.

2.2. LPS-treatments

Four month old MCH-KO mice and their wild-type littermates (n=4–5/group) were injected ip with 5ug of LPS (L2630, Sigma). Blood and tissues were harvested 16h later. Spleen was processed through a cell strainer (BD-Falcon) using 3ml of media. The cell suspension was centrifuged and the pellet was resuspended in 5ml erythrocyte lysis buffer. Two-million cells in complete RPMI media were cultured for 72 hours and the supernatant was collected and kept at –80°C for further analysis. IL-10 in serum and cell culture supernatants was measured by ELISA (eBioscience).

2.3. Cell culture and stimulations

RAW 264.7 mouse monocytic cells were purchased from ATCC. Cells were treated with 100ng/ml LPS from *Escherichia coli* serotype 0111:B4 (Sigma), 10^{–6}M MCH (Bachem), 10 ng/ml IL-10 (R&D Systems), 10ng/ml IL-1beta (R&D Systems), their various combinations or vehicle, and were harvested at 4 hours post-treatment for RNA analysis and at 72 hours for protein secretion in the tissue culture supernatant. Experiments using RAW cells were run in 5–6 replicas. IL-10 levels in culture supernatant were measured by ELISA (R&D systems). RNA was extracted using RNAqueous extraction kit (Ambion). Reverse transcription was performed using an I-Script cDNA synthesis kit (Bio-Rad Laboratories), followed by real-time PCR analysis of IL-10 or MCHR1 expression using Taqman pre-developed assays (Applied Biosystems). TATA-binding protein (TBP) gene expression served as a housekeeping control and results are expressed as arbitrary mRNA units (AU) (vehicle=100), unless indicated otherwise.

2.4. Statistical Analysis

Results are presented as group mean±SEM. Data were analyzed in Statview 2.0 Software (SAS Institute), using a non-parametric test (Mann-Whitney *U*-test), or repeated measures ANOVA for evaluating changes in body weight. A level of p<0.05 was considered statistically significant.

3. Results

3.1. Treatment of IL-10 deficient mice with a MCHR1 antagonist

DABA-822 is a small molecule MCHR1 antagonist with only peripheral effects due to its limited brain penetration [15]. Previous studies have demonstrated attenuation of TNBS-induced experimental colitis in mice treated with DABA-822 [7]. In the present study, IL-10^{–/–} mice received a two-week treatment with piroxicam to establish colitis, followed by treatment with DABA-822, or vehicle, for two additional weeks. Changes in mouse body weight due to induction of colitis were monitored overtime and were similar between the two treatments (Fig. 1). As expected, IL-10 deficient mice treated with piroxicam developed severe intestinal inflammation characterized in H&E stained histological sections by disruption of the epithelial architecture and mucosal erosions (Fig. 2A). However, treatment with DABA-822 failed to contain intestinal inflammation in the IL-10 deficient mice.

Indeed, histological scoring of the severity of colitis was indistinguishable between the two treatment groups (1.857 ± 0.465 vs 1.905 ± 0.575 , DABA-822 vs vehicle respectively; $p=0.84$; Fig. 2B).

Consistent with the histology, real-time PCR of analysis of colonic expression of IFN γ , TNF α , IL-1 β and IL-6 revealed no differences between DABA-822 and vehicle treated IL-10-deficient mice with colitis (1312 ± 447 vs 1197 ± 224 , $p=0.732$ for IFN γ ; 265 ± 18 vs 314 ± 51 $p=0.367$ for TNF α ; 945 ± 527 vs 1092 ± 365 $p=0.744$ for IL-1 β ; 296 ± 127 vs 314 ± 108 $p=0.889$ for IL-6; arbitrary mRNA units, DABA-822 vs vehicle treatment, respectively; Fig. 3). Cytokine expression in wild-type mice was set at 100.

Taken together, these results prompted us to further investigate the link between MCH and IL-10.

3.2. IL-10 regulation in MCH-deficient mice

To start delineating the interplay between MCH and IL-10, we first examined serum IL-10 levels in MCH-KO and their wild-type littermates at baseline (Fig. 4A, left panel) and at 16 hours post LPS-treatment (Fig. 4A, right panel) and we did not find any significant differences between the two genotypes under the conditions that we tested ($p=0.563$). However, we found higher IL-10 secretion in tissue culture supernatants of splenocytes isolated from LPS-treated MCH-KO mice compared to similarly treated wild-type mice ($p=0.0106$, Fig. 4B), suggesting perhaps an inhibition of IL-10 in the presence of MCH.

3.3. MCH modulates IL-10 expression

To further explore the above possibility, we examined potential direct effects of MCH on IL-10 expression. We and others have demonstrated the presence of functional MCHR1 in human monocytes and in the mouse RAW 264.7 monocytic cell line [17, 37]. Using RAW 264.7 cells, we found that treatment with MCH significantly inhibited LPS-stimulated expression of IL-10. Specifically, while MCH had not a significant impact on IL-10 levels at baseline, it strongly prevented the LPS-mediated IL-10 mRNA induction (1835 ± 236 vs 586 ± 119 arbitrary mRNA units, LPS vs LPS+MCH, $p=0.0143$; Fig. 5A), as well as protein secretion in the cell culture supernatants (387 ± 18 vs 101 ± 15 pg/ml, LPS vs LPS + IL-10, $p=0.0003$; Fig. 5B). Such findings confirm that in an inflammatory milieu, MCH negatively modulates IL-10 expression.

We subsequently examined the MCH-mediated modulation of additional cytokines which are upregulated in response to LPS (Fig. 6). We found that, under the conditions tested, treatment with MCH was found to inhibit expression of IL-6 (100 ± 14.2 vs. 21.1 ± 2.9 arbitrary mRNA units, LPS vs LPS+MCH, $p=0.0143$) and to a lesser degree of TNF α (100 ± 10.4 vs. 65.5 ± 5.8 arbitrary mRNA units, LPS vs LPS+MCH, $p=0.0679$), while expression of KC (100 ± 12.5 vs. 115.5 ± 35.1 arbitrary mRNA units, LPS vs LPS+MCH, $p=0.8065$) or TGF β (100 ± 16.1 vs. 78.0 ± 5.4 arbitrary mRNA units, LPS vs LPS+MCH, $p=0.2207$) remained unaltered. A profound upregulation of IL-1 β expression was also noted (100 ± 34.1 vs. 1673 ± 117 arbitrary mRNA units, LPS vs LPS+MCH, $p=0.0062$), which might provide further insights into the mechanisms by which MCH promotes

inflammation. Thus, it appears that the effects of MCH on LPS-induced cytokines are pathway specific.

3.4. IL-10 modulates MCHR1 expression

We have previously shown an upregulation of MCHR1 expression in colonocytes and monocytes by proinflammatory stimuli such as IL-1 β and LPS, respectively [17, 19, 20]. We thus speculated that anti-inflammatory signals could perhaps inhibit MCHR1 expression in the same cells. Indeed, we found that treatment of RAW 264.7 cells with IL-10 prevented the LPS-mediated induction of MCHR1 (192 ± 29 vs 57.6 ± 16 arbitrary mRNA units, LPS vs LPS+IL-10, $p=0.0197$, Fig. 7), while IL-10 alone had minimal effects on MCHR1 mRNA expression. These results, in conjunction with the effects of MCH on IL-10 expression, as described in Fig. 5 clearly indicate a cross-talk between IL-10 and MCH regulated pathways in the context of inflammation.

4. Discussion

During an inflammatory process, there is an immediate surge of proinflammatory cytokines followed by upregulation of the anti-inflammatory ones, for example IL-10. Failure of this balanced response, which under normal conditions leads to the resolution of inflammation results to chronic uncontrolled inflammation, as is the case in IBD [33]. In the present study we describe cancellation of the protective effects of MCH blockade, mediated by a small molecule MCHR1 antagonist, in IL-10 deficient mice with colitis. The same treatments were effective in attenuating TNBS-induced acute colitis [7] and chronic DSS-induced colitis [41] in wild-type mice, suggesting that IL-10 might represent a downstream target of MCH. To further delineate the interaction(s) between MCH and IL-10, we focused on in vitro studies using primarily RAW 264.7 mouse monocytes, based on the fact that monocytes/macrophages are a major source of IL-10 during intestinal inflammation [10]; that LPS is a potent stimulator of IL-10 production in RAW264.7 cells; and that treatment with LPS upregulates MCHR1 expression in these cells, as we have recently reported [17].

We found that in monocytes, MCH inhibits IL-10 upregulation associated with an inflammatory response. From a mechanistic point of view, this finding is consistent with the previously described stimulation of IL-10 expression in monocytes by α -MSH, the primary role of which is anti-inflammatory [4]. α -MSH, one of the products of the *pomc* gene, is a neuropeptide with appetite suppressing effects. Most importantly, α -MSH acts as a functional antagonist of MCH in the regulation of appetite, behavior and skin pigmentation [9, 18]. As it relates to intestinal inflammation, it has been reported that treatment of mice with α -MSH attenuated experimental colitis, while mice deficient for the α -MSH receptor MC1R developed aggravated DSS-induced colitis [25, 31]. Such findings further support the proinflammatory role of MCH, as described here and in our previous studies [19, 20]. Both of these peptides are highly conserved in evolution and appear to modulate energy balance as well as host defense responses, two of the most essential processes for the organism's survival [22].

Besides MCH, several neuropeptides of the brain-gut axis have been shown to modulate IL-10 expression not only by monocytes but also by T-cells. For instance, somatostatin and

calcitonin-gene related peptide upregulate IL-10 production by Th2 cells [23]; treatment with substance P or ghrelin in combination with LPS synergistically enhanced IL-10 secretion by monocytes [14, 38]; and urocortin II augmented IL-10 expression in *Listeria monocytogenes* - stimulated RAW 264.7 cells [34]. Adipokines, a class of cytokine-like regulators of energy balance and insulin sensitivity that are secreted mainly by adipocytes, have also been implicated in intestinal inflammation. Among them, Relm- α inhibited IL-10 release from LPS-activated macrophages [26]; and adiponectin upregulated the anti-inflammatory IL-10 and IL-1RA in monocytic cells [40]. However, only few of the above studies examined directly the impact of such effects on disease development in IL-10 deficient mice. For instance, while adiponectin or leptin deficiency offered no protection from colitis in these mice [28, 36], exogenous administration of GLP-2 [16] or a substance P antagonist [39] ameliorated spontaneous colitis in mice lacking IL-10, indicating a primarily IL-10 independent mode of action for these neuropeptides. In contrast, the beneficial effects of treatment with urocortin II in *Listeria monocytogenes* infection were cancelled in IL-10 deficient mice [34], as described here for blocking MCH.

Information about signals that modulate MCHR1 expression is scarce, with the exception of IL-1 β in colonocytes [20], LPS in monocytes [17], both positive regulators, and MCH itself in adipocytes, a negative regulator [3]. We describe here that IL-10 strongly inhibits LPS-induced upregulation of MCHR1 in monocytes, which in conjunction with the MCH effects on IL-10, as delineated in this work, establish a functional interplay between IL-10 and MCH. Of note, in analogy to MCHR1, IL-10 blocked IL-12 induced expression of the substance P receptor NK-1R in T-cells [39].

In summary, together with our previous reports, findings of the present study establish an immunomodulatory role for MCH in colitis, that targets both proinflammatory and anti-inflammatory pathways, though in opposite directions. Specifically, proinflammatory cytokines or microbial products, upregulate MCHR1 expression in macrophages and colonic epithelial cells [17, 19, 20], a process that is antagonized by anti-inflammatory signals like IL-10. MCH acting on these and perhaps additional immune cell types, stimulates proinflammatory cytokine production while in parallel inhibits the anti-inflammatory IL-10. As such, modulating the MCH effects might hold promise for the treatment of IBD and perhaps additional inflammatory and autoimmune conditions.

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The abbreviations used are

MCH	melanin concentrating hormone
MCHR1	MCH receptor1
IBD	inflammatory bowel disease

Ab	antibody
AU	arbitrary units
GI	Gastrointestinal
TNBS	2,4,6-trinitrobenzene sulfonic acid
DSS	dextran sulfate sodium

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Highlights

- In contrast to other models of experimental colitis, treatment with a MCHR1 antagonist did not attenuate disease in IL-10 deficient mice
- An underlying mechanism for this observation could be direct interactions between MCH and IL-10
- In vitro, MCH inhibits LPS-induced IL-10 expression in monocytes
- Treatment of monocytes with IL-10 prevents upregulation of MCH receptor 1
- MCH might promote intestinal inflammation via inhibition of anti-inflammatory pathways

Figure 1. Treatment of IL-10 deficient mice with colitis with a MCHR1 antagonist

IL-10 deficient mice and their wild-type littermates were exposed to piroxicam for 2 weeks, to accelerate colitis development. Following colitis establishment, mice were treated with the MCHR1 small molecule peripheral antagonist DABA-822 or vehicle for two weeks. Mouse body weight was monitored throughout the treatments. During the two weeks of treatment with piroxicam mice lost significant weight indicative of colitis development. In the recovery phase, upon cessation of piroxicam, mice started to regain weight slowly. However, no differences were observed in overtime changes in body weight between DABA-822 and vehicle treated mice lacking IL-10.

Figure 2. IL-10 deficient mice with colitis are unresponsive to treatment with a MCHR1 antagonist

IL-10^{-/-} mice received piroxicam treatments for a period of two weeks. Mice were then treated with the peripheral MCHR1 antagonist DABA-822 or vehicle for the ensuing two weeks.

A. Representative H&E stained histological sections of the mouse colon in a low magnification showing extensive tissue damage and immune cell infiltrates in IL-10 deficient mice with colitis, irrespectively of their DABA-822 or vehicle treatments.

B. The severity of colitis in the various experimental groups was scored (0–3) in a blinded fashion by a GI Pathologist (RN).
ns; not statistically significant

Figure 3. Effect of treatment with a MCHR1 antagonist on cytokine expression in IL-10 deficient mice with colitis

Mice were treated as in Fig 1 and at the end of the experiment (day 28) tissues were harvested for further analysis. Colonic mRNA expression of IFN γ , TNF α , IL-1 β and IL-6 was measured by TaqMan and results are presented as arbitrary mRNA units (AU). Wild-type mice without colitis serve as control (=100). While there was a robust cytokine upregulation in IL-10 deficient mice exposed to piroxicam compared to wild-type mice treated likewise, no differences in cytokine levels were apparent between IL-10 deficient mice with colitis treated with DABA-822 or vehicle

ns: not statistically significant

Figure 4. IL-10 expression in MCH-KO mice

- A. Serum IL-10 levels in MCH-KO and their wild-type littermates at baseline (left panel) and in response to LPS in vivo treatment for 16 hours (right panel).
- B. Spontaneous IL-10 secretion by mouse splenocytes cultured for 72 hours. Cells were isolated from the spleen of MCH-KO and WT mice treated with LPS for 16 hours.

* $p < 0.05$ between MCH-deficient and wild-type mice using a non-parametric test

Figure 5. MCH attenuates IL-10 induction in mouse monocytes

RAW 264.7 mouse monocytic cells were treated with MCH, LPS, their combination or vehicle.

(A) IL-10 mRNA expression was measured at 4 hours by quantitative PCR and B) IL-10 secretion in cell culture supernatants was measured at 72 hours by ELISA.

* $p < 0.05$; *** $p < 0.001$; LPS vs LPS+MCH treatments

Figure 6. Treatment with MCH modulates LPS-induced cytokine expression in monocytes

The effect of MCH treatment (4 hours) on the expression of various cytokines was examined by real-time RT-PCR in RAW 264.7 mouse monocytic cells in the presence or absence of LPS. For each cytokine, values obtained from LPS treated cultures have been arbitrarily set to 100 and represent arbitrary mRNA units.

* $p < 0.05$; ** $p < 0.01$; LPS vs. LPS+MCH

Figure 7. IL-10 modulates MCHR1 expression in monocytes

RAW 264.7 cells were treated with LPS, IL-10, their combination or vehicle for 4 hours and the MCHR1 mRNA expression was analyzed by quantitative PCR. Results are expressed as arbitrary mRNA units (AU).

* $p < 0.05$, LPS vs LPS+IL-10 treatments