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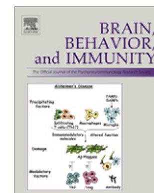

Short Communication

Repeated exposure to water immersion stress reduces the Muc2 gene level in the rat colon via two distinct mechanisms

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ABSTRACT

The mucin family plays a number of important roles in intestinal homeostasis. Among its members, the gel-forming Muc2, produced in goblet cells, is a major component of mucus and contributes to intestinal barrier integrity. Whereas psychological stress is known to impair intestinal barrier, the effects of chronic or repeated stress on mucin expression and goblet cell differentiation have not been well documented. The present study first examined the effects of different levels of exposure (3 days, 1 and 2 weeks) to water immersion stress on intestinal mucin gene expression in rats, and then explored the mechanisms underlying the stress-induced decrease in the colonic Muc2 level. Repeated water immersion stress for 1 and 2 weeks decreased colonic Muc2 gene levels to 40% of that of non-stressed animals, while exposure to stress for 3 days induced only a 25% decrease. The goblet cell numbers counts in the colons of the 1- and 2-week stress groups, but not the 3-day stress group, were decreased to 85% of that in non-stressed animals. Cdx2 expression, a transcriptional factor related to Muc2 synthesis in the goblet cells, was decreased in all stress groups, whereas Rath1 and Klf4 expressions, transcriptional factors related to goblet cell differentiation in Notch signaling were decreased in the 1-week stress group. Collectively, the repeated exposure to water immersion stress decreases Muc2 synthesis in the goblet cells via decreased Cdx2 expression and subsequently reduces the goblet cell number via Notch signaling suppression.

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1. Introduction

Psychological stress is important risk factors for the onset and development of intestinal diseases (Collins, 2001). One of the major causes of stress-induced intestinal diseases is impaired intestinal barrier function (Soderholm and Perdue, 2001). The impairment of barrier integrity followed by permeation of undesired substances, such as undigested food, microbes, and microbial toxins, from the lumen, can induce excessive activation of the mucosal immune system and sustained inflammation. Previous studies have demonstrated that various stresses, particularly acute stresses, impair intestinal barrier integrity and increase intestinal permeability (Matsuo et al., 2009; Saunders et al., 1994). However, the effect of chronic stress on intestinal barrier integrity has not been well documented. As many diseases are caused by chronic or repeated rather than a single exposure to stress, it is also important to understand the effect of exposure to different levels of stresses on intestinal function.

One of the most important roles of the intestine is to provide a physical barrier against undesired substances mentioned above (Turner, 2009). Barrier integrity is coordinated by a number of barrier components, including the mucus layer. The major component of the mucus is secreted mucins, large glycoproteins with highly polymeric protein backbone structures (Johansson et al., 2011). Upto 20 mucin genes have been identified and broadly classified into two types, secretory and membrane-bound. In both the small and large intestines, Muc2 is the major secretary mucin synthesized in goblet cells. It is known that Muc2 expression in the goblet cells is regulated by certain transcription factors including Cdx2 (Mesquita et al., 2003) and GATA4 (van der Sluis et al., 2004). Studies have suggested that reductions in Muc2 synthesis and/or goblet cell number resulting in a barrier defect may be involved in the onset or development of intestinal diseases. For example, patients with ulcerative colitis often shows depletion of recognizable goblet cells in the colonic epithelium (Theodossi et al., 1994), and Muc2-deficient mice show an exacerbation of dextran sulfate sodium-induced colitis (Van der Sluis et al., 2006). On the other hand, membrane-bound mucins, such as Muc1, 3, 4, 14 and 17, are expressed in the intestinal goblet cells and absorptive cells. The membrane-bound mucins form glycocalyx on the apical membrane and seem to provide a barrier with similar properties to those of the mucus layer (McGuckin et al., 2011), although apart from the gel-forming Muc2 their contribution to intestinal barrier integrity remains unclear.

The intestinal epithelial cells consists of five main cell types; absorptive enterocytes, goblet cells, Paneth cells, Tuft cells, and enteroendocrine cells. In the colon, pluripotent stem cells at the

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lower crypt region differentiate into mature cell lineages during migration toward the luminal surface region. The differentiation of epithelial cells is regulated by complex epithelial-epithelial and epithelial-mesenchymal interactions. Among these, the Notch pathway plays a crucial role in the cell fate decision (Vooijs et al., 2011). When Notch signaling is activated, the downstream molecule, Hes1, promotes an absorptive cell fate over a secretory cell fate. In contrast, when Notch signaling is suppressed, Hath1 (also called Math1 in mice and Rath1 in rats) promote a secretory cell fate, and Krüppel-like factor 4 (Klf4) is then required for the specification of goblet cells. However, the effects of stresses on epithelial cell fate and Notch signaling remain unclear.

The present study first investigated the impact of different levels of exposure to water immersion stress on intestinal mucin gene expression. Repeated water immersion stress is an easy, reproducible, and widely used model to study the pathophysiologic aspects of the chronic stress response. Thereafter, we examined the mechanisms underlying the stress-induced reduction of Muc2 level with a particular emphasis on goblet cell differentiation and Muc2 transcriptional regulation.

2. Materials and methods

2.1. Animals and stress exposure

Male Wistar rats (6 weeks old, $n = 28$; SLC Japan, Hamamatsu, Japan) were housed in individual cages in a room with controlled temperature (22 ± 2 °C), relative humidity (40–60%) and lighting (light 0800–2000 h) throughout the study. The rats had free access to the AIN-93G formula diet and tap water. After an acclimation period, rats were divided into four groups using the randomized block design based on body weight; the control (without stress), 3-day stress, 1-week stress, and 2-week stress groups. In the stress session, the animals were placed in a wired stainless steel cage (dimensions, 11 length × 25 width × 19 height (cm), Natsume Seisakusho Co. Ltd., Tokyo, Japan), which was divided into 10 compartments, and immersed for 3 h to the level of the xiphoid process in a water bath maintained at 25 °C. The animals were subjected to this stress session starting at 1:00PM once a day for 3 days, 1, or 2 weeks. The control animals were not subjected to any stress sessions, but were handled at 1:00 PM for a few seconds and spent for 3 h in their own cages placed in the room where the stress exposure was performed. To avoid the acute influence of the last stress session and to evaluate the consequences of chronic stress, the animals were killed 24 h after the last stress session. The small intestines (mid-part) and colon were removed for real-time PCR and histochemical analysis as described below. The animal study was approved by the Hiroshima University Animal Committee, and the rats were maintained in accordance with the Hiroshima University guidelines for the care and use of laboratory animals.

2.2. Real-time PCR

The gene expression levels of mucins (Muc1-4), Rath1, Klf4, Cdx2, and GATA4 in the rat intestines were quantified by real-time PCR. The total RNA from rat intestinal tissues was isolated using TRI reagent (Sigma, St. Louis, MO, USA), and reverse-transcribed using a ReverTra Ace® qPCR RT kit (TOYOBO, Tokyo, Japan) according to the manufacturer's instructions. Quantitative real-time PCR was performed using a Step One system (Life technologies, Inc., Grand Island, NY, USA) and a KAPA SYBR FAST qPCR kit (KAPA BIOSYSTEMS, Cape Town, South Africa). The primer sequences used for PCR are shown in Supplementary Table S1. Data were analyzed by the ΔΔCt method and presented as fold changes in gene expression after normalization against the internal control GAPDH gene expression level.

2.3. Histology and immunohistochemistry

The mid-portion of rat colon tissues was embedded in OCT compound (Sakura Finetek Japan, Tokyo, Japan) after fixation with 4.0% paraformaldehyde in phosphate-buffered saline and frozen sections (8 μm in thickness) were prepared on glass slides. The sections were stained with periodic acid-Schiff and counter-stained with hematoxylin and the number of goblet cells was determined at the light microscopic level (DMI600B, Leica Microsystems, Tokyo, Japan). Separate sections were blocked in 5% normal goat serum for 0.5 h and incubated for 16 h with rabbit polyclonal anti-Muc2 (Santa Cruz Biotechnology, CA, USA), followed by incubation for 1 h with goat AlexaFluor 488-conjugated anti-rabbit IgG (Life technologies) and rhodamine-conjugated phalloidine (Cytoskeleton Inc, CO, USA). The fluorescence was visualized using a Leica FW4000 fluorescence microscope (Leica Microsystems) and the fluorescence intensity of Muc2 was quantified using Image J software. For estimations of goblet cell numbers and Muc2 signal intensities, two sections were prepared from all rats and ten complete crypts per section were selected. The means of 20 values measured per a full crypt were considered as the individual results.

2.4. Statistical analysis

All values are expressed as means with their SEM. Statistical analyses were performed by 1-way ANOVA followed by Duncan's multiple range test. A difference with a P value <0.05 was considered significant. Statistical analyses were performed using the general linear models procedure of the Statistical Analysis Systems program (version 6.07; SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Effects of repeated stress exposure on intestinal Muc gene expression

Muc2 and Muc3 gene expression in the colon was influenced by repeated water immersion stress (Fig. 1A–D). Muc2 gene expression was gradually decreased during the first week and remained at a reduced level throughout week 2, with the Muc2 expression levels in the 1- and 2-week stress groups approximately 40% of that in the control group. The 3-day stress group also showed a modest decrease in Muc2 expression, although the decrease was not significant. Muc3 expression in the colon decreased during the first 1 week under repeated stress, but a normalization of Muc3 level was observed after 2-week stress exposure. No significant differences in Muc1 and Muc4 gene expression were observed among the groups, although Muc4 expression tended to decrease after stress exposure for 1 and 2 weeks. Muc1, 2, 3, or 4 genes expression levels in the small intestine were not influenced by repeated stress (data not shown). As the gel-forming mucin, Muc2, has a crucial role in intestinal barrier function, we focused on the underlying mechanisms for the repeated stress-induced decrease in Muc2 gene level in the colon.

3.2. Effects of repeated stress exposure on goblet cell number and Muc2 protein level

As the Muc2 gene level in the colon, but not small intestine, was decreased by stress, goblet cells in the rat colon were visualized using Periodic acid-Schiff staining method and counted at the microscopic level (Fig. 1E). Goblet cell numbers in a crypt of the

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