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Rapid communication

Intestinal expression of ACE2 in mice with high-fat diet–induced obesity and neonates exposed to maternal high-fat diet

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ABSTRACT

Objective: The 2019 novel coronavirus disease (COVID-19) is threatening global health and is especially pronounced in patients with chronic metabolic syndromes. Meanwhile, a significant proportion of patients present with digestive symptoms since angiotensin-converting enzyme 2 (ACE2), which is the receptor for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is highly expressed in the intestine. The aim of this study was to evaluate the effects of a high-fat diet (HFD) and a maternal HFD on the intestinal ACE2 levels in adults and neonates.

Methods: We examined intestinal ACE2 protein levels in mice with diet-induced obesity (DIO) and neonatal mice exposed to a maternal HFD. We also investigated Ace2 mRNA expression in intestinal macrophages.

Results: Intestinal ACE2 protein levels were increased in DIO mice but decreased in offspring exposed to a maternal HFD compared with chow-fed controls. Ace2 mRNA expression in intestinal macrophages was detected and downregulated in DIO mice. Additionally, higher intestinal ACE2 protein levels were observed in neonates than in adult mice.

Conclusions: The influence of an HFD on intestinal ACE2 protein levels is opposite in adults and neonates. Macrophages might also be involved in SARS-CoV-2 intestinal infection. These findings provide some clues for the outcomes of patients with COVID-19 with metabolic syndromes.

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Introduction

The 2019 novel coronavirus disease (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, is prevalent worldwide [1]. In patients with COVID-19 infection, gastrointestinal (GI) reactions are commonly reported, and SARS-CoV-2 RNA has been detected in feces and GI tissues [2,3], indicating the existence of GI infections and fecal–oral transmission. Accumulating evidence indicates that individuals with metabolic diseases experience more severe outcomes from COVID-19 [4,5]. Angiotensin-converting enzyme 2 (ACE2) is the cellular receptor for SARS-CoV-2 [6] and, according to the Human Protein Atlas (HPA) database, it is highly expressed in the GI tract [7]. Exploring the factors affecting the expression of intestinal ACE2 may predict the outcomes of intestinal infection with SARS-CoV-2. It has been reported that ACE2 levels in adipose tissue are upregulated in obese/diabetic states [8,9]. It is thus far unclear whether metabolic disorders may alter the expression of ACE2 in the intestine. We undertook this study to investigate the alteration of intestinal ACE2 expression in mice fed short- and long-term high-fat diets (HFDs) as well as pups exposed to maternal HFDs.

Recent studies have reported that SARS-CoV-2 can directly attack immune cells. In the lungs, ACE2-positive alveolar macrophages can be infected by SARS-CoV-2 [10]. Intestinal macrophages also play an important role in gut homeostasis; however, most intestinal studies in COVID-19 focus on epithelial cells, and it is unclear whether intestinal macrophages express ACE2 and contribute to GI infection. Here, we also sought to determine whether...
intestinal macrophages may express ACE2 and whether it is altered in diet-induced obesity (DIO).

Materials and methods

Animals

C57BL/6 mice were obtained from the Animal Core Facility of Nanjing Medical University and group housed under a 12-h light/dark cycle at 23°C with ad libitum access to food and water. For the HFD study, male mice were kept on a normal chow diet (XietongShengwu, 1010013) until 8 wk of age, and then fed either a chow diet or an HFD (58% HFD with sucrose, Research Diets D12331) for 12 and 48 wk. For the maternal HFD study, 8-wk-old female mice were exposed to either a normal chow diet or an HFD for 12 wk. A normal chow diet-fed male mouse was added to each cage for breeding and they remained in the cages either with chow diet or HFD. Resulting male offspring were sacrificed at postnatal day 12 (PD12). All the animal experiments were approved by the Committee on Animal Care of Nanjing Medical University, and complied with National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Isolation of primary microglia and intestinal macrophages

Intestinal macrophages and microglia were isolated as described by us and others with minor modifications [11,12]. For intestinal macrophages, mice were sacrificed, and the intestine was carefully removed, cut longitudinally, and cleaned in Hank's balanced salt solution (HBSS). Tissues were cut into pieces and washed twice in RPMI-1640 medium (Gibco, Waltham, MA, USA) supplemented with 5% fetal bovine serum (Gibco) and 5 mM EDTA at 37°C and shaken for 20 min to remove epithelial cells. Tissue were then digested in Accutase solution (Millipore, CA, USA) for 40 min, and passed through a 70-μm filter. For microglia, brains were homogenized in RPMI-1640 medium. Resuspended brain and gut lysates were layered on a 30%/70% (v/v) Percoll gradient and centrifuged without a brake at 500 g for 30 min. The mononuclear cells were collected from the gradient interface. After 30 min of incubation, the non-adherent dead cells and lymphocytes were washed away with HBSS. Attached live cells were harvested for Ace2 expression analysis and immunofluorescent staining.

Western blotting

The ileum and colon were dissected and rinsed in phosphate-buffered saline to remove luminal content. Tissues were lysed in radioimmunoprecipitation buffer (Beyotime, Los Angeles, CA, USA) supplemented with cocktail protease inhibitors (Thermo Fisher Scientific, Waltham, MA, USA). 30 μg proteins were separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel, and transferred onto nitrocellulose membranes (Millipore). The membranes were blocked with 5% milk (Phygene, FuZhou, CN) for 1 h at room temperature, then incubated with the following primary antibodies overnight at 4°C: anti-ACE2 (1:1000; R&D Systems, Minneapolis, MN, USA), anti-GAPDH (1:5000; Affinity Biosciences, Cincinnati, OH, USA), and anti β-actin antibody (1:500; Santa Cruz, Dallas, TX, USA).

Real-time quantitative PCR and agarose gel electrophoresis

Total RNA was extracted from primary cells or gut tissues using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and subjected to reverse transcription with HiScript-II-Q RT SuperMix for quantitative polymerase chain reaction (qPCR; Vazyme, Nanjing, China). Real-time qPCR was carried out using SYBR Green Master

Fig. 1. ACE2 expression is increased in the ileum of mice fed on HFD. (A) Body weight of mice fed an HFD for 12- and 48-wk. (B) Basal blood glucose level is elevated in mice fed an HFD for 48-wk. (C,D) qPCR shows relative Ace2 mRNA levels are much higher in the ileum than the colon but do not differ between chow- or HFD-fed animals at both time points. (E,F) Western blot using ACE2 antibody indicates that ACE2 protein levels are increased in the ileum of mice fed HFD for 12- and 48-wk compared with chow-fed mice. ACE2 protein levels in the colon did not differ between HFD- and chow-fed mice. (G,H) Quantification of Western blot intensities in (E,F) estimating the amount of ACE2 protein levels in the ileum. (I) Significantly higher ACE2 level in the ileum of mice fed an HFD for 48-wk by immunohistochemical analysis. (J) The surface ACE2-ir area percentage in each villi quantified by imaged J. Data are presented as means ± SD. "P < 0.05, "P < 0.001, "P < 0.01, vs controls. ACE, angiotensin-converting enzyme; ACE-ir, angiotensin-converting enzyme immunoreactive; DI, diet induced; HFD, high-fat diet.
of mice were analyzed for relative mRNA expression and ACE2 protein levels. qPCR shows that Ace2 mRNA expressions are much higher in the ileum than colon as literature reported, however, there is no difference between chow-fed and HFD fed groups at either time points (Fig. 1CD). Protein levels of ACE2 were analyzed by Western blot. Consistent with the HPA database and qPCR results, we observed that ACE2 is highly expressed in the ileum and is expressed at low levels in the colon, suggesting that the small intestine could be more prone to infection by COVID-19. Notably, ACE2 protein levels were much higher in the ileum of the HFD groups at both 12 and 48 wk (Fig. 1E–H). Compared with 12-wk HFD feeding, ACE2 protein levels in the ileum were not further elevated by 48-wk HFD feeding. In the colon, ACE2 protein levels did not differ between the chow and HFD groups. The higher surface protein level of ACE2 in the ileum of mice fed a 48-wk HFD was confirmed by immunohistochemistry (Fig. 1I,J). These results show that ACE2 is highly expressed in the ileum and upregulated in DIO mice but is not further increased by age or prolonged HFD feeding.

**Ace2 mRNA expression is downregulated in intestinal macrophages of mice fed a HFD**

It has been reported that alveolar macrophages and Kupfer cells express high levels of Ace2 and therefore could be targeted by SARS-CoV-2 [13]. Considering that little attention has been given to intestinal macrophages, we then sought to determine whether intestinal macrophages may express high levels of Ace2. Considering that little attention has been given to intestinal macrophages, we then sought to determine whether intestinal macrophages may express high levels of Ace2. Considering that little attention has been given to intestinal macrophages, we then sought to determine whether intestinal macrophages may express high levels of Ace2. Considering that little attention has been given to intestinal macrophages, we then sought to determine whether intestinal macrophages may express high levels of Ace2. Considering that little attention has been given to intestinal macrophages, we then sought to determine whether intestinal macrophages may express high levels of Ace2. Considering that little attention has been given to intestinal macrophages, we then sought to determine whether intestinal macrophages may express high levels of Ace2.
We next checked Ace2 mRNA expression in intestinal macrophages isolated from mice fed an HFD or chow diet. Unexpectedly, the Ace2 mRNA level was significantly decreased in intestinal macrophages of mice fed an HFD for 48 wk (Fig. 2D), which was opposite to the increased Ace2 expression in the whole tissue of the ileum. These results suggest that intestinal macrophages may take part in the pathologic process of COVID-19, but the potential mechanisms need further study.

**ACE2 protein is highly expressed in the neonatal intestine and is decreased by maternal HFD feeding**

Considering that an increasing number of women have metabolic syndromes in their child-bearing years and that there are some cases of SARS-CoV-2 infection in newborns, we next evaluated the effects of maternal HFD on neonatal intestinal Ace2 expression. Surprisingly, on PD12, in the ileum of the male offspring exposed to maternal HFD, Ace2 mRNA levels and protein levels were both significantly decreased compared with that in the maternal chow-fed group (Fig. 3A–C), which was opposite to the results in the adult DIO mice (Fig. 1E–H). Immunohistochemistry also confirmed the decreased ACE2 surface protein levels in the intestinal epithelium of neonatal mice exposed to maternal HFD (Fig. 3D,E). Additionally, we compared intestinal ACE2 protein levels at different ages in chow-fed mice. The highest expression of intestinal ACE2 protein was detected in neonates compared with 20- and 56-wk-old mice (Fig. 3F,G). Taken together, these results suggest that neonates with high levels of intestinal ACE2 have a high risk for inducing SARS-CoV-2 tropism, although maternal HFD may not be a predisposing factor for COVID-19 in neonates.

**Discussion**

In this study, we reported that chronic HFD feeding increased intestinal ACE2 protein expression, which may partly contribute to the higher susceptibility of individuals with obesity to COVID-19. ACE2 was reported to be expressed in alveolar macrophages in the lung and CD169-positive macrophages in the spleen and lymph nodes, strengthening the evidence that SARS-CoV-2 can directly
It is of great significance to understand the maternal factors affecting the susceptibility of newborns to COVID-19 to prevent more newborns from being infected. In contrast to the results in adult DIO mice, the ACE2 protein levels were significantly decreased in the ileum of offspring exposed to maternal HFD. Thus, maternal HFD may not be a promoting factor for COVID-19 infection in the neonatal intestine and may even reduce susceptibility. However, contrary to recent studies in humans stating that intestinal Ace2 mRNA expression is increased with age [17,18] and the lower disease severity in children, we detected the highest protein levels of intestinal ACE2 in neonates compared with young (20wk-old) and old (56-wk-old) adult mice. Ortiz et al. also reported that ACE2 proteins are expressed on the cell surface of lung cells and are higher in young children [19]. These conflict conclusions may be partly due to the inconsistency between mRNA and protein results of ACE2. We found that Ace2 mRNA results are not always the same as the Western blot or immunohistochemical results. This discrepancy is more obvious in adult intestine in our study, indicating that in adult intestine, ACE2 protein turnover rate might be slower. Thus, for ACE2, both protein and mRNA levels should be assessed before making conclusions.

This study provided some clues for COVID-19 susceptibility in individuals with metabolic diseases; however, due to biosafety requirements, intestinal Ace2 expression was analyzed in mice without SARS-CoV-2 infection. Whether patients with obesity exhibit more common GI manifestations and higher intestinal viral loads requires additional clinical information. Meanwhile, the role of Ace2 in intestinal macrophages is still poorly understood. Further investigations may shed some light on these points.

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