Activation of autophagy in rats with plateau stress-induced intestinal failure

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Abstract: Hypobaric hypoxia may damage the intestinal mucosa, which may induce multiple organ dysfunction. However, little work has been done regarding whether high altitude hypoxia is associated with failure of the intestinal mucosal barrier. The aim of this study was to investigate the change of the autophagy after the intestinal failure in rats acutely exposed to plateau stress. Fifty Wistar rats were randomly divided into five groups: the plain group, plateau for 6 h, 12 h, 24 h, and 48 h (n = 10 in each group). The acute exposure to plateau was established at a simulated altitude of 4767 meters (m) in a decompression chamber. Intestinal injury was verified by light microscopy. The autophagosomes in the intestinal epithelial cells were observed by transmission electron microscopy (TEM). The protein expression of Beclin1 and LC3B in the intestinal epithelial cells were analyzed by immunohistochemistry. Compared with the plain group, acute exposure to plateau led to a time-dependent damage of the intestinal epithelium. The autophagosome was observed after the intestinal failure following acute exposure to high altitude for 6 h. The expression of Beclin1 and LC3B protein in the rats exposed to acute plateau for 6 h, 12 h, 24 h and 48 h were significantly higher than those in the plain group. The expression of autophagy also showed a significant increase in rats with intestinal failure following acute exposure to plateau stress.

Keywords: High altitude, hypoxia, intestinal failure, autophagy, Beclin1, LC3B

Introduction

The major features of high altitude areas (altitude ≥ 3000 m) are low pressure, low oxygen, low temperature, high sunshine exposure and changeable climate [1]. People from plain acutely stressed to plateau often develop intestinal-related diseases, and the main symptoms include abdominal distention, loss of appetite, abdominal pain, and diarrhea. In severe cases, peptic ulcers and gastrointestinal bleeding may occur [2]. Currently, acute altitude illnesses such as high altitude induced cerebral edema and pulmonary edema have become an area of intensive research. Nevertheless, little work has been done on the pathogenesis and prevention of high altitude-related intestinal disorders.

Intestines are not only the vital organ that is essential for food digestion and absorption of nutrients, but they also play important role in immune reaction, endocrine and mucosal barrier functions. Intestinal mucosal injury causes intestinal barrier dysfunction leading to deterioration of the primary diseases and even multiple organ failure (MOF) and systemic inflammatory response syndrome (SIRS). In patients with acute exposure to high altitude stress, damage to intestines often occurs and the low oxygen may play an important role in the process of intestinal injury.

Autophagy is a tightly regulated pathway involving the lysosomal degradation of cytoplasmic organelles or cytosolic components. This pathway can be stimulated by multiple forms of cellular stress, including hypoxia, reactive oxygen species, DNA damage, protein aggregates, and organelle damage. The degradation products are reused to maintain cellular metabolic turnover and homeostasis [3]. Many studies have
confirmed that autophagy is closely associated with hypoxia in tumor, and it probably has a preventive effect against cancer initiation [4]. However, little work has been done regarding whether autophagy is associated with the intestinal failure resulted from acute exposure to plateau stress.

We have successfully established the rat model of acute plateau stress by exposing the rats to a hypobaric hypoxic condition of an altitude of 4767 m in a decompression chamber. Autophagy activation was observed in the gastrointestinal tract in rats with intestinal failure caused by acute plateau stress. This model has provided a theoretical foundation for further studying the role of autophagy in intestinal epithelial cell damage.

Materials and methods

Reagents and experiment apparatus

The anti-Beclin1 antibody and the anti-LC3B antibody were obtained from Abcam (Santa Cruz USA). The streptavidin-peroxidase immu-
nohistochemical staining kit (SP Kit) and DAB kit (25×) were obtained from Bioss (Beijing, China). The Computer Image Analysis System (Olympus, Tokyo Japan) was used to analyze the immuneostaining results. The transmission electron microscopy (TEM, JEM-1230) (Nagano Japan) was used to evaluate the ultrastructural cellular features. The decompression chamber (FLYDWC50-II) (Guizhou, China).

Animals and sampling
Fifty 6-week old healthy adult male Wistar rats (average body weight 260 ± 20 g) were purchased from the Experimental Animal Center of Lanzhou University (altitude 1500 m). The rats were acclimatized at temperature of 23°C and humidity of 50% for one week before being put into decompression chamber (simulated altitude of 4767 m). The plain group rats were sacrificed and the rats in the acute plateau group were sacrificed after 6 h, 12 h, 24 h and 48 h, respectively. The rats were anaesthetized using chloral hydrate (0.3 ml/100 g body weight). The ileum tissue was isolated and washed in buffered saline. A 1-cm piece of tissue was fixed in 10% neutral formalin for subsequent immunohistochemistry and hematoyxin & eosin (H&E) staining. Another 1-cm piece of ileum tissue was fixed in 2.5% glutaraldehyde for TEM as described below.

Figure 2. Morphological features of autophagosomes in the intestinal epithelial cells. In the rats of the plain group, the intestinal epithelial cell structure was almost normal with a slightly reduced number of lysosomes and no autophagosomes (A). In contrast, in the rats exposed to acute plateau stress for 24 h, there was an increased number of lysosomes in the intestinal epithelial cells, and presence of double membrane structure characteristic of autophagosomes (B). Magnifications: (A), ×4000; (B), ×10,000.

Light microscopy
After being fixed in 10% neutral formalin for 24 hours, the ileum tissues were embedded into paraffin blocks. Sections of 4 µm thickness were cut and standard H&E staining performed. The overall histological changes were examined under the light microscopy.

Transmission electron microscopy
The ileum tissue were fixed in 2.5% glutaraldehyde at temperature of 4°C for 12 hours, followed by fixing in 1% osmic acid and dehydration in graded ethanol. The samples were then treated in propylene oxide, embedded in ethoxyline resin, and the ultra thin sections were cut. The sections were double stained with uranyl acetate and lead citrate before being observed under the TEM.

Immunohistochemistry
A two-step immunohistochemistry method was performed. Paraffin sections were dewaxed and incubated with 3% to block the endogenous peroxidase activity. Pepsin K was applied following the incubation with anti-Beclin1 antibody and anti-LC3B antibody (1:500) at 4°C overnight. After incubation with the secondary antibody (IgG/Bio), DAB was used to develop
color. The slides were then dehydrated in graded alcohol and sealed. The images were acquired under the light microscopy and the expression level of Beclin1 and LC3B were analyzed by quantitative image processing software (Image-Pro Plus 6.0 analysis software, Media Cybernetics, Inc., Washington, USA). The mean optical density (MOD) was used to express the level of protein expression.

Statistical analysis

All data was processed using SPSS 18.0 and expressed as the mean ± standard deviation (SD). The measurements were analyzed by Student’s t test. A P value of < 0.05 was defined as statistical significance.

Results

Morphological changes of the intestinal tissues

In rats of the plain group, the intestinal villi were in good order and the mucosal epithelium is intact (Figure 1A). On the contrary, in the rats exposed to acute plateau stress, the villi appeared disorganized and with epithelium mucosae exfoliated accompanying infiltration of chronic inflammatory cells in the lamina propria (Figure 1B-E). And with the exposure time prolonged, the more serious damage can be observed, such as loss of goblet cells (Figure 1C) and separation of mucosal epithelium and lamina propria (Figure 1C-E). In rats exposed to

Figure 3. LC3B expression in intestinal epithelial cells. LC3B was present in cytoplasm of intestinal epithelial cells. There was almost no LC3B expression in the blank group (A) but high in groups exposed to plateau stress. Furthermore, a time dependent increase in the expression was visible in rats exposed to plateau stress for 6 h (B), 12 h (C), and 24 h (D) for 6 h (B), 12 h (C), and 24 h (D) except for 48 h (E). Magnifications: ×200.
plateau stress for 24 h and 48 h, the villi’ number was even reduced and capillary congestion can be clearly seen (Figure 1D-E).

**Morphology of autophagosome**

In the plain group, no obvious morphological changes were observed in the intestinal epithelial mucosa except for a reduced number of lysosomes. No autophagosomes were observed (Figure 2A). In contrast, in rats with acute plateau stress exposure, a marked swelling in the mitochondria in intestinal epithelial cells was present, together with an increased number of lysosomes and the presence of double membrane structure resembling autophagosomes (Figure 2B). The autophagosomes were observed as early as 6 h after the rats being exposed to the acute plateau stress. Formation of autophagosome peaked at 24 h and remains obvious in rats at 48 h being exposed to plateau stress condition (Figure 2B).

**Expression of Beclin1 and LC3B in intestinal mucosa**

LC3B was present in cytoplasm of intestinal epithelial cells when Beclin1 in nucleus. Unlike the blank group in which LC3B expression was almost undetectable (Figure 3A), a time dependent increase in the expression of LC3B was visible in the intestinal epithelial cells in rats exposed to plateau stress (Figure 3B-E) and there was statistical significance (Table 1). But it is interesting that in rats exposed to plateau stress for 48 h, there was a reduced expression of LC3B (Figure 3E). And the same situation also occurred in Beclin1 expression (Table 1).

**Discussion**

Acute plateau stress can use sympathetic activation and increased splanchnic vascular tone. Hypoxia has been suggested as a major factor leading to ischemia of the intestinal mucosa, enhanced vascular permeability, and impaired tight junction of the intestinal epithelia. All these may result in epithelial necrosis and impaired intestinal barrier [5]. Sprague-Dawley rats exposed to hypobaric hypoxia at a simulated altitude of 7000 m for 72 h could lead to exfoliation of intestinal villi, inflammatory cell infiltration, edema of lamina propria associated with red blood cell effusion and translocation of bacteria to major organs [6]. Moreover, the prebiotics treatment significantly alleviated changes in TNF-α and IL-10 induced by high altitude hypoxic stress [7]. These data suggest that high-altitude hypoxia could cause severe intestinal mucosal injury and increase bacterial and endotoxin translocation.

In our study, by exposing the rats to a hypobaric hypoxic condition (simulated an altitude of 4767 m in a decompression chamber, we have found that acute plateau stress could damage the intestinal epithelial cells, and the extent of mucosal damage correlates with the increased length of plateau stress. The gut is not only the target organ of systemic inflammatory response, but it can also be the initiator of systemic inflammatory response [8]. Intestinal mucosal barrier function has become an important indicator to determine the prognosis of critically ill patients. It is thus necessary to study the mechanisms of intestinal mucosa barrier damage after in major stressful conditions.

In recent years, studies have found that autophagy plays an important role in a variety of biological activities. For example, autophagy can accelerate cellular metabolism and is involved in the energy recycling from the decomposition products during ischemia and hypoxia. Increased autophagy promotes cell survival under ischemia and hypoxia [9]. It is known that hypoxia can cause autophagic activity, but whether or not autophagy plays any role in the intestinal mucosal injury after acute plateau stress remains unclear. In our study, we have found that the rats exposed to plateau stress exhibit an increased number of intestinal autophagosome than the rats in the plain group. We speculate that autophagy may help remove the damaged cells and long-lived pro-

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**Table 1. Expression of Beclin1 and LC3B in each group (n=10, x ± s)**

<table>
<thead>
<tr>
<th>Group</th>
<th>Beclin1</th>
<th>LC3B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plain group</td>
<td>0.142 ± 0.005</td>
<td>0.150 ± 0.007</td>
</tr>
<tr>
<td>Acute plateau stress, 6 h</td>
<td>0.161 ± 0.017</td>
<td>0.171 ± 0.015</td>
</tr>
<tr>
<td>Acute plateau stress, 12 h</td>
<td>0.179 ± 0.018</td>
<td>0.190 ± 0.017</td>
</tr>
<tr>
<td>Acute plateau stress, 24 h</td>
<td>0.195 ± 0.015</td>
<td>0.207 ± 0.016</td>
</tr>
<tr>
<td>Acute plateau stress, 48 h</td>
<td>0.185 ± 0.020</td>
<td>0.193 ± 0.019</td>
</tr>
</tbody>
</table>

*: P < 0.01, compared with control group; #: P < 0.01, compared with control group.
teins in the damaged intestinal epithelium, and therefore plays an important role in repairing the damage cells.

Increased autophagy in rats exposed to plateau stress was verified using two markers Beclin1 and LC3B. Beclin1 is a homologue of Atg6 which is essential for autophagy in yeast. Beclin1 is highly conserved in eukaryotes and is a key regulators for the process of autophagy [10]. The microtubule-associated protein 1 light chain 3 (LC3), a homologue of Atg8 also essential for autophagy in yeast, is associated to the autophagosome membranes after processing [11]. Our data clearly demonstrated an increased mucosal expression of Beclin1 and LC3B in rats acutely exposed to plateau stress. Indeed, dysregulated autophagy has been shown in various colonic diseases including colon cancer, ulcerative colitis, and Crohn’s disease [9].

In summary, our data showed that plateau stress can induce intestinal mucosal damage, and subsequent activation of autophagy. With the successful establishment of the current experimental model, our future work will focus on how autophagy is involved in the regulation of mucosal damage. The generated data would be very useful in designing appropriate approaches for the prevention of high altitude induced intestinal function failure.

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Disclosure of conflict of interest

None.

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References