Effect of low-dose ethanol on NLRP3 inflammasome in diabetes-induced lung injury

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Abstract: To observe the changes in NLR family pyrin domain containing 3 (NLRP3) inflammasome in a rat model of diabetes-induced lung injury, and investigate the effect of low-dose ethanol on the production of NLRP3 inflammasome. The type I diabetic mellitus (DM) rat model was established, and the rats were divided into four groups: normal control group (CON group), low-dose ethanol group (EtOH group), diabetes group (DM group) and DM+EtOH group. The rats were fed for 6 and 12 weeks, respectively. The ratio of lung wet weight/body weight (lung/body coefficient) was calculated, and the changes of pulmonary morphology and fibrosis were observed by HE and Masson staining. The changes in pulmonary ultra-structure were examined by electron microscopy. The expressions of mitochondrial acetaldehyde dehydrogenase 2 (ALDH2) and NLRP3 inflammasome key factors, NLRP3, ASC and caspase-1 proteins were detected by western blot. Compared with the CON group, the lung/body coefficient was increased (P<0.05), lung fibrosis occurred, ALDH2 protein expression was decreased, and NLRP3, ASC and caspase-1 protein expressions were increased in the DM rats (P<0.05). Compared with the DM group, the lung/body coefficient and fibrosis degree were decreased, ALDH2 protein expression was increased (P<0.05), and NLRP3, ASC and caspase-1 protein expressions were decreased in the DM+EtOH group (P<0.05). Hence, low-dose ethanol increased ALDH2 protein expression and alleviated diabetes-induced lung injury by inhibiting the production of NLRP3 inflammasome.

Key words: diabetic rat, low-dose ethanol, lung injury, mitochondrial acetaldehyde dehydrogenase 2, NLRP3 inflammasome

Introduction

Diabetes mellitus (DM) is a common chronic metabolic disease, and its morbidity is closely linked to its complications. Schuyler et al. [1] first proposed that the lung is one of the major target organs of DM, but the mechanisms of diabetes-induced lung injury remain unclear. DM is closely related to chronic inflammation [2]. Zhang et al. reported that alleviating inflammation could reduce lung injury in diabetic rats [3]. As the core of the inflammatory response, the NLR family pyrin domain containing 3 (NLRP3) inflammasome is increasingly considered to be a driver of DM complications, and is an important effector in innate immunity. NLRP3 protein, adaptor protein apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) and caspase-1 precursor (pro-caspase-1) are the core protein components of NLRP3 inflammasome. NLRP3 inflammasome...
recruits the downstream adaptor protein ASC and the effector protein caspase-1 to assemble NLRP3 inflammasome. Subsequently, caspase-1 promotes the secretion of inflammatory factors including interleukin-18 (IL-18) and IL-1β, and exerts an inflammatory reaction [4].

Activation of NLRP3 inflammasome is known to be associated with the pathogenesis of various auto-inflammatory, autoimmune, and chronic inflammatory and metabolic diseases [4–6], but its involvement in diabetes-induced lung injury is less reported. Mitochondrial aldehyde dehydrogenase 2 (ALDH2) decomposes acetaldehyde and its metabolites (which induce cell damage) into non-toxic carbon dioxide and water [7, 8]. We and others have previously reported that low-dose ethanol activated ALDH2 activity and enhanced its antioxidant role, as well as reduced the intracellular and extracellular inflammatory responses and participated in organ protection [9–11]. However, there are few reports on the role of low-dose ethanol in NLRP3 inflammasome production in diabetes-induced lung injury.

Therefore, in this study, we first observed whether the over-production of NLRP3 inflammasome in lung tissue occurred in a DM rat model, and subsequently analyzed whether activation of ALDH2 with low-dose ethanol can attenuate the lung inflammatory response. The findings provide a theoretical basis for clinical prevention and treatment of diabetes-induced lung injury.

Materials and Methods

Animals

Male Sprague-Dawley (SD) rats [Specific pathogen Free (SPF) grade, weighing 180–220 g] were purchased from the Animal Center of Bengbu Medical College, Anhui, China [Reg. No. SCXK (su2009-001)]. All rats were fed with basic diet (6% fat, 23% protein and 64% carbohydrate) and distilled water, and maintained under the standard laboratory conditions at 23 ± 3°C room temperature with 12 h light/dark cycles.

Chemicals and antibodies

Streptozocin (STZ) was purchased from Sigma Aldrich (Saint Louis, MO, USA). Ethanol (EtOH) was obtained from Bengbu New Chemical Reagent Factory, China. The antibodies ALDH2 (Cat No. ab196494), caspase-1 (Cat No. 2225) and ASC (Cat No. ab155970) were obtained from Abcam (Cambridge, MA, USA). Antibody NLRP3 (Cat No. NBP-12446) was purchased from NOVUS (Centennial, CO, USA), and antibody GAPDH (Cat No. Abs118936a) was acquired from Absin Bioscience Inc. (Shanghai, China).

Establishment of diabetes rat model and experimental protocol

After one week of acclimation to laboratory conditions, the rats were fasted overnight for 12 h. The next day, the rats were intraperitoneally injected with a single rapid dose of 55 mg/kg STZ, which was freshly dissolved in 0.1 mol/L sodium citrate buffer, pH 4.5, to induce the DM model. The rats in the control group were injected with the same volume of sodium citrate buffer alone. After 72 h, the fasting blood glucose (FBG) level was measured. When FBG was >16.7 mmol/L and sustained for one week, the DM model was successfully established. All experimental rats were randomly divided into four groups (n=12): control (CON), low-dose ethanol (EtOH), diabetes (DM) and DM+EtOH groups. After successful modeling, the rats in the EtOH and DM+EtOH groups were fed with 2.5% ethanol for one week, and subsequently fed with 5% ethanol for six weeks (n=6/group) and 12 weeks (n=6/group). The rats in the other groups received regular drinking water [10].

Determination of fasting blood glucose, lung wet weight and body weight

All rats were sacrificed at the end of the sixth and twelfth week. After FBG and body weight (g) were measured, 1% pentobarbital sodium (30 mg/kg) was intraperitoneally injected, the whole lung weight was obtained, and the ratio of lung wet weight (mg) / body weight (g) (lung/body coefficient) was calculated.

Measurement and scoring of rat pulmonary morphology and fibrosis by Hematoxylin-eosin (HE) and Masson staining

The left lower lung tissue (0.1 g) of each rat was fixed in 10% neutral formalin, embedded with paraffin, the lung tissues were cut into 4 µm thick sections, and observed under the microscope. Three sections were randomly selected for each rat. The changes in pulmonary morphology were observed by hematoxylin-eosin (HE) staining, and the lung histological changes were assessed according to the morphological changes: alveolar and interstitial edema, alveolar and interstitial hemorrhage, inflammatory infiltration, alveolar hyperemia, atelectasis or excessive alveolar expansion. The diffuse alveolar damage (DAD) score was evaluated and blindly graded by applying an arbitrary grading scale ranging from Grade 0 to 3: Grade 0, no change or very slight change; Grade 1, slight change; Grade 2, moderate change; Grade 3, heavy change. The total lung injury score of each
visual field was calculated [12].

The fibrosis degree was observed by Masson staining, and the Ashcroft score was used to evaluate the pulmonary fibrosis: Grade 0, normal lung; Grade 1, minimal fibrous thickening of alveolar or bronchiolar walls; Grade 3, moderate thickening of walls without obvious lung structural damage; Grade 5, increased fibrous with definite lung structural damage and formation of fibrous bands or small fibrous masses; Grade 7, severe distortion of architecture and large fibrous area; Grade 8, total fibrous obliteration of the field. The mean score, which reflected the severity degree of pulmonary fibrosis in each visual field, was assessed [13].

Observation of the pulmonary ultra-structural changes under transmission electron microscope (TEM)

In the 12-weeks group, the lung tissue (1 mm³ size) was immersed in 2.5% glutaraldehyde and fixed at 4°C for 24 h. Then, the pulmonary ultra-structural changes, especially the changes of lamellar bodies and mitochondria, were observed under TEM. For quantification of the lamellar bodies and mitochondria, the images were analyzed by point-counting method and expressed as the percent volumes of each organelle in the cytoplasm of pulmonary tissue slice [14, 15].

Measurement of ALDH2, NLRP3, ASC and caspase-1 protein expressions by Western blot

The lung tissue (0.1 g) was homogenized in the mixture of radioimmunoprecipitation assay lysate and PMSF (0.1 mM) for 40 min on ice. The mixture was centrifuged at 15,000 g for 15 min at 4°C, the supernatant was collected, and the concentration of protein was measured by bicinchoninic acid assay. Equal quantities of protein lysates (60 µg) were electrophoresed on 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. After blocking with 5% nonfat milk for 90 min at room temperature, the membrane was probed overnight at 4°C with primary antibodies against ALDH2 (1:3,500), NLRP3 (1:500), ASC (1:300) and caspase-1 (1:1,000). After washing, the membranes were incubated with the corresponding secondary antibodies (1:6,000) at 37°C in a water bath for 40 min, and centrifuged at 100 rpm for 20 min at room temperature. Finally, the membrane was visualized using the enhanced chemiluminescence method. GAPDH (1:3,000) was used as a loading control. The autoradiographs were scanned using the ChemiDoc XRS gel image system and analyzed with the Image Lab software (version 3.0; Bio-Rad Laboratories Inc., Hercules, CA, USA). The density value of each band was expressed as arbitrary units.

Statistical analysis

All data were analyzed by GraphPad Prism 6.0 software. The experimental data were expressed as mean ± SEM. The statistical analysis was performed by one-way ANOVA and comparison between groups was performed by Newman-Keuls test. A P-value <0.05 indicates statistical significance.

Results

Changes in fasting blood glucose (FBG), body weight and the lung/body coefficient

Compared with the CON group, the FBG and lung/body coefficient in the DM group were increased, and body weight was decreased at 6 and 12 weeks. With the prolongation of DM course, the coefficient was further increased, indicating that DM induced lung swelling. Compared with the CON group, there was no significant difference in the EtOH group (Table 1).

Compared with the DM group, there were no changes in FBG, but the lung/body coefficient was decreased, and the body weight was increased in the DM+EtoH group, suggesting that low-dose ethanol could reduce lung swelling under high glucose condition (Table 1).

Morphological changes in rat lung tissue

The HE staining results in the DM group at sixth week showed that the lung structure was destroyed, alveolar atelectasis or excessive expansion occurred, the alveolar wall was thickened, with infiltration of inflammatory cells. These changes worsened at the twelfth week. In the DM+EtoH group, the alveolar atelectasis and expansion was alleviated, the inflammatory infiltration was

| Table 1. Changes of fasting blood glucose, body weight and lung/body coefficient in different groups (mean ± SEM, n=6) |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Group          | FBG (6W)        | FBG (12W)       | BW (6W)         | BW (12W)        | Lung/body coefficient (6W) |
|                |                 |                 |                 |                 | Lung/body coefficient (12W) |
| CON            | 5.87 ± 1.14     | 5.75 ± 1.02     | 406.3 ± 38.2    | 464.8 ± 30.5    | 3.70 ± 0.05       |
| EtoH           | 5.66 ± 0.98     | 6.02 ± 1.06     | 397.5 ± 31.3    | 470.2 ± 32.6    | 3.80 ± 0.02       |
| DM             | 25.31 ± 3.99**  | 28.43 ± 4.07**  | 171.6 ± 23.7**  | 273.3 ± 34.0**  | 5.45 ± 0.03**     |
| DM+EtoH        | 21.20 ± 3.10    | 24.65 ± 2.88    | 216.8 ± 34.4**  | 252.4 ± 22.8**  | 4.50 ± 0.01**     |

FBG: fasting blood glucose; BW: body weight. **P<0.01 vs. CON; ^^P<0.01 vs. DM; ##P<0.01 vs. 6W.
lowered, and the DAD scores were decreased as compared to the DM group (Fig. 1).

**Fibrosis changes in rat lung tissue**

The Masson staining results showed pulmonary fibrosis and increased Ashcroft score in the DM group in contrast to the Con group. The Ashcroft score was decreased in the DM+EtoH group in contrast to the DM group (Fig. 2).

**Transmission electron microscopy (TEM) observation of the rat lung tissue**

At 12 weeks, the TEM results showed that the lamellar bodies in the type II lung epithelial cells contained concentric or parallel layers, the mitochondria had intact structure, and the cristae was clear in the control and EtoH groups. Compared with the CON rats, most lamellar bodies were vacuolated, the parallel layers had disappeared, most of the mitochondria were swollen, vacuolated and ruptured, and the cristae had almost disappeared in the DM group. In the DM+EtoH group, some lamellar bodies were partly vacuolated, and the mitochondria were swollen but not ruptured, and the percent volumes of lamellar bodies and mitochondria were decreased in contrast to DM rat. These results suggested that the lung ultrastructure injuries were ameliorated with EtoH treatments (Fig. 3).

**Changes in ALDH2 protein expression in the rat lung tissue**

The western blot results showed at sixth and twelfth week that ALDH2 protein expression was significantly increased in the EtoH group and decreased in the DM group compared with the Con group. ALDH2 protein expression in the DM+EtoH group was higher than that in the DM group (Fig. 4).

**Changes in NLRP3 inflammasome-related protein expressions in rat lung tissue**

Compared with the CON group, there were no differences in NLRP3, caspase-1 and ASC protein expressions at sixth and twelfth week in the EtoH group, while
NLRP3, caspase-1 and ASC protein expressions were increased in the DM group. Compared with the DM group, the expressions of NLRP3, caspase-1 and ASC proteins were decreased in the DM+EtoH group (Fig. 5).

Discussion

With the aging of the global population and socio-economic development, DM has become a major chronic disease that seriously endangers human health. Controlling diabetes-related complications has become a global health problem [16]. This study found pulmonary inflammation and fibrosis in DM rats, and the degree of injury was more serious in 12-week DM rats than in 6-week DM rats. When the DM rats were treated with low-dose ethanol for 6 and 12 weeks, the lung injury was alleviated with the up-regulation of aLDH2 protein. These results suggested that low-dose ethanol can inhibit high glucose-induced pulmonary inflammation and fibrosis by increasing ALDH2 protein expression.

Pulmonary fibrosis is a terminal stage of lung disease characterized by proliferation of fibroblasts, extracellular matrix accumulation, inflammatory injury and destruction of tissue structure [17]. Pulmonary fibrosis is closely related to the activation of NLRP3 inflammasome in aged mice model [18]. Chronic inflammation may be a trigger for DM [2]. Hyperglycemia worsened the inflammatory state by activating NLRP3 inflammasome in gingival tissue [19]. In untreated type II DM patients, the expression and activity of NLRP3 inflammasome were enhanced in monocytes. The NLRP3 inflammasome was significantly increased in the early stage of carotid atherosclerosis and in type II DM patients [20–22]. These studies suggested that NLRP3 inflammasome plays a key role in the development of DM. NLRP3 inflammasome is known to participate in transforming growth factor β1, bleomycin and lipopolysaccharide-induced pulmonary inflammation and fibrosis [23–27], further indicating that pulmonary fibrosis and NLRP3 inflammasome are closely related. Chen et al. observed pulmonary fibrosis accompanied by the increase of NLRP3 in a rat DM model at 12 weeks [28].
In this study, we observed destruction of lung structure and pulmonary fibrosis, accompanied by increase in key components of lung NLRP3 inflammasome, NLRP3, ASC and caspase-1 protein expressions both at sixth and twelfth week in a rat DM model, suggesting that NLRP3 inflammasome was involved in diabetes-induced lung injury not only at the beginning of diabetes but also throughout the disease course, and inflammation may be...
an important cause of pulmonary fibrosis.

Aldehyde dehydrogenase 2 (ALDH2) is a mitochondrial enzyme, which metabolizes ethanol and toxic aldehydes such as 4-hydroxy-2-nonenal [29, 30]. ALDH2 plays an antioxidant role to resist various injury-induced ROS formation. Our previous studies had shown that low-dose ethanol could stimulate ALDH2 expression in rat lung tissue [31], the cardiomyocyte injury was alleviated when ALDH2 activity was increased [10, 32], and ALDH2 deficiency worsened ethanol-induced liver inflammation and fibrosis in mice [33]. These reports verified that ALDH2 is important in different organ injuries, and the mechanisms are related to attenuation of ROS release and inflammation. In this study, we observed that the low-dose ethanol-fed DM rats had less lung injury than the DM rats. Moreover, NLRP3 inflammasome expression was decreased and fibrosis degree was alleviated with the increase of ALDH2 protein expression by low-dose ethanol. These results suggested that activation of ALDH2 could play a protective role against diabetes-induced pulmonary fibrosis by decreasing NLRP3 inflammasome production. In this study, the diabetes rats were treated only with low-dose ethanol, but not with specific ALDH2 agonist. Hence, we could not exclude the other roles of low-dose ethanol, such as improving insulin sensitivity [34]. Therefore, it is necessary to continue the intensive investigation of the mechanisms related to the protection by low-dose ethanol.

In summary, the results indicated that pulmonary fibrosis may be associated with the increase of NLRP3 inflammasome in a diabetes rat model. Low-dose ethanol can reduce NLRP3 inflammasome expression and alleviate lung injury through activation of ALDH2 protein expression. This study provides a theoretical foundation for further exploration of diabetic pulmonary fibrosis therapeutics.

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