Environmental Research 167 (2018) 1–6

Contents lists available at ScienceDirect

Environmental Research

journal homepage: www.elsevier.com/locate/envres

The effects of atmospheric hydrogen sulfide on peripheral blood lymphocytes of chickens: Perspectives on inflammation, oxidative stress and energy metabolism☆

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ARTICLE INFO

Keywords:
Hydrogen sulfide
Inflammatory
Lymphocytes
Chicken

ABSTRACT

Excessive hydrogen sulfide (H_2S) affects poultry health. Exposure to air pollution induces inflammation, oxidative stress, energy metabolism dysfunction and adverse health effects. However, few detailed studies have been conducted on the molecular mechanisms of H_2S-induced injury in poultry. To understand how H_2S drives its adverse effects on chickens, twenty-four 14-day-old chickens were randomly divided into two groups. The chickens in the control group were raised in a separate chamber without H_2S, and the chickens in the treatment group were exposed to 30 ppm H_2S. After 14 days of exposure, peripheral blood samples were taken and the lymphocytes were extracted to detect inflammation, oxidative stress and energy metabolism in broilers. Overall, an increase in the inflammatory response was detected in the peripheral blood lymphocytes following H_2S exposure compared to the control group, and the expression levels of the heat shock proteins (HSPs) and the transcription factors nuclear factor κB (NF-κB), cyclooxygenase 2 (COX-2) and inducible nitric oxide synthase (iNOS) were up-regulated in the H_2S group, which further suggested that H_2S induced an inflammatory response via the NF-κB pathway. Because of the activation of NF-κB, which is a major regulator of oxidative stress, we also observed that reactive oxygen species (ROS) production was elevated under H_2S exposure. In addition, we presumed that energy metabolism might be damaged due to the increased ROS production, and we found that H_2S down-regulated the expression levels of energy metabolism-related genes, which indicated the occurrence of energy metabolism dysfunction. Altogether, this study suggests that exposure to excessive atmospheric H_2S induces an inflammatory response, oxidative stress and energy metabolism dysfunction, providing a reference for comparative medicine.

1. Introduction

With the development of the poultry industry, hydrogen sulfide (H_2S), as a main contaminant in the air, is causing increasing problems to the health of broilers. H_2S in chicken houses is mainly produced by the chicken excreta in the decomposition process of corruption. If the protein in the feed is high and the digestive system is in disorder, a large amount of H_2S is excreted through the intestine. In large, enclosed-layer chicken houses, H_2S increases significantly if there are broken eggs. Many studies show that H_2S has a wide range of toxic effects on various organ systems, including the nervous system (Solnyshkova et al., 2004), respiratory system (Lim et al., 2016), cardiovascular system (Haouzi et al., 2015) and digestive system (Atteneramos et al., 2007). There are also a few report studying the effects of H_2S on the immune system. Rogers et al. found that a low level of H_2S significantly impaired the antibacterial system of the rat, consequently compromising the immune responses (Rogers and Ferin, 1981). Robinson et al. found that H_2S reduced the percent of alveolar macrophages (Robinson, 1982). Moreover, leukocytes counts were decreased in rats exposed to 400 ppm of H_2S, and the exudation of neutrophils was observed (Lopez et al., 1987). H_2S also induces an inflammatory response in rat colonocytes both in vivo and in vitro (Beaumont et al., 2016).

The injury mechanisms of toxic and harmful gases are manifold. Among the features of the immune system, cytokines offer important information in assessing immune function, and they are also important indicators to evaluate the immune response (Li et al., 2017). Many

☆ All of the authors have read and approved the paper and it has not been published previously nor is it being considered by any other peer-reviewed journal.

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https://doi.org/10.1016/j.envres.2018.06.051
Received 26 March 2018; Received in revised form 11 June 2018; Accepted 26 June 2018

environmental gases [chlorine (Cl_2), ammonia (NH_3) and sulfur dioxide (SO_2)] alter the immune system by humoral and cellular immunity if the gas concentrations reach certain levels and, thus, change the cytokine expression levels (Jonasson et al., 2013; Li et al., 2014; Yi et al., 2016). Oxidative stress is one of the main foci of environmental toxicology research. Reactive oxygen species (ROS) is a normal metabolite in animals that is regulated by a variety of antioxidant defense mechanisms (enzymes and antioxidants). In recent years, it has been shown that antioxidant levels are closely related to the inflammatory response (Pan et al., 2017). NF-κB is an oxidative stress sensitive transcription factor that is involved in inflammation, innate immunity, cell differentiation and apoptosis, and ROS is an important molecule that activates NF-κB. The activation of NF-κB induces the expression of TNF-α, IL-1β, IL-6 and other inflammatory cytokines, causing inflammation. In addition, it has been reported that exposure to excessive harmful environmental gases could weaken antioxidant ability (Kim et al., 2015). Additionally, HSPs are synthesized under stress and have a strong and powerful resistance to stress; they regulate autophagy and antioxidation; and they participate in the biological function of organism immunity. HSP70 activates the NF-κB signaling pathway, such that the cells synthesize and release various pro-inflammatory factors, including TNF-α, IL-1β and IL-6, chemotactic activating dendritic cells and T cells, thereby regulating immune function (Chen et al., 2017). In addition, HSPs also can protect against antioxidant enzyme activity and enhance the ability to fight free radicals (Zhao et al., 2017). Energy metabolism dysfunction is another focus of environmental toxicology research because it is linked inextricably with immune function. Cheng et al. found that blocking metabolic pathways with metformin diminished cytokine production and increased mortality in systemic fungal infection, and the immunometabolic defects were partially restored by therapy with recombinant IFN-γ (Cheng et al., 2016). Moreover, an attenuation of inflammation in the intestinal mucosa was found through immunostaining, and the expression levels of energy metabolism-related genes were down-regulated (Päivärinta et al., 2016). Additionally, heavy metal cadmium markedly down-regulated the expression of energy metabolism-related genes in chicken spleens, and the mRNA levels of TNF-α and IL-1β were up-regulated while IL-2 and IFN-γ were down-regulated (Zhe et al., 2017).

Lymphocytes, as the first line of defense of the immune system, are sensitive to environmental challenges, especially environmental gases (Forget et al., 2016; Wei et al., 2015). Nevertheless, at the present time, only a few studies have addressed the initial effects of H_2S on chickens, and these were mainly focused on meat quality (Ahmed et al., 2014; Wang et al., 2011). However, the effects of H_2S on peripheral blood lymphocytes in chickens have not been reported systematically, and the mechanism of H_2S-induced injury needs to be revealed. In this experiment, we treated broilers in experimental conditions, detected the effects of H_2S on the mRNA and protein expression levels of inflammatory cytokines, HSPs and energy metabolism-related genes, and evaluated the level of ROS in chicken peripheral blood lymphocytes.

2. Materials and methods

2.1. Preparation of the animals

All the procedures used in this experiment were approved by the Institutional Animal Care and Use Committee of Northeast Agricultural University. Twenty-four 1-day-old Ross 308 male broilers (Weiwei Co. Ltd.,Harbin, China) were housed in individual wire-bottom cages in an environmentally controlled room under standard brooding practices. Then, the broilers were transferred to environmentally controlled exposure chambers. The concentrated H_2S was delivered in a whole-body animal exposure chamber from days 15 to 28. Each exposure chamber was a 4500 × 3000 × 2500 mm (length × width × height) sealed unit, sectioned for housing 12 birds per chamber (n = 10 per group, the remaining two chickens in each group were on standby for any unexpected condition). The broilers in the treatment group were exposed to 30 ppm H_2S during the experimental period. The broilers in the control group were raised in a separate chamber without H_2S for the same period, and the concentration of ambient H_2S was kept below 2 ppm. The concentration of H_2S in both chambers was monitored with a LumaSense Photoacoustic Field Gas-Monitor Innova-1412 (Santa Clara, CA, USA) during the entire experiment. All the broilers were given ad libitum access to water and basal diets during the entire experiment, and the ingredients and chemical composition of the basal diets are shown in Table S1. The temperature and airflow were controlled during the exposure to ensure adequate ventilation, to minimize the buildup of animal-generated contaminants (dander, NH_3, CO_2) and to avoid thermal stress. The chambers were maintained at 18–26 °C, and the chickens were kept at under a 16-h light/8-h dark cycle. At day 28, all the birds were subjected to peripheral blood sampling for further experiments.

2.2. Preparation of the peripheral blood lymphocytes

Peripheral blood was collected into a 10 mL injector with 1 mL of a heparin sodium (7500 U/mL) (Sigma, USA, Cat# H3149). Fifteen milliliters of lymphocyte separation medium (TBD sciences Cat# LTS1077) was added to a 50 mL sterile centrifuge tube, and 10 mL of peripheral blood was transferred slowly on the lymphoprep. The lymphocytes were collected after centrifugation (2000 × *g*) for 25 min at room temperature. The mixed lymphocytes were then washed 3 times with phosphate-buffered saline (PBS). Subsequently, the lymphocytes were divided into three parts; one part was used to detect ROS production, and the remaining two parts were added to 1 mL of Trizol and to 1 mL of PBS, respectively, and were then stored at − 80 °C for qRT-PCR and western blot, respectively.

2.3. RNA isolation and qRT-PCR

The gene expression levels were evaluated by qRT-PCR. The primers for the detection of the targets mRNA are shown in Table 1. The total RNA was isolated from the cell samples using the Trizol reagent (Invitrogen, USA, Cat# 15596018) according to Yang et al. (Yang et al., 2018). The mRNA relative levels were calculated according to the $2^{-\triangle\triangle Ct}$ method. β-actin served as the endogenous controls for normalization.

2.4. Protein extraction and western blot analysis

The protein samples were separated by 8% and 12% SDS-PAGE and were transferred to PVDF membranes (Merck Millipore, USA, Cat# ISEQ. 00010, LOT# R6PA4145H). The membranes were blocked with 5% skim milk for 3 h at 37 °C and were incubated for 14 h at 4 °C, with the following diluted primary antibodies: COX-2, NF-κB, iNOS, HSP27, HSP40, HSP60, HSP70, HSP90, NADPH oxidase 2 (NOX2), avian uncoupling protein (avUCP), hexokinase 2 (HK2), pyruvate dehydrogenase complex X (PDHX), succinate dehydrogenase B (SDHB) and pyruvate kinase (PK); the dilutions of antibodies are shown in Table S2. After washing three times for 15 min each with PBST, the membranes were incubated for 2 h at 37 °C with peroxidase-conjugated secondary antibodies against rabbit IgG (Santa Cruz Biotechnology, Argentina, Cat# sc-2357, RRID: AB_628497). After washing three times for 15 min each, the bound antibodies were visualized by chemiluminescence using the ECL-plus reagent (GE Healthcare, Buckinghamshire, UK). The β-actin content was analyzed as the loading control using a rabbit polyclonal antibody.

2.5. Determination of ROS generation in the peripheral blood lymphocytes

For determining ROS generation, the peripheral blood lymphocytes were collected after treatment. The levels of ROS were measured using