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RESEARCH ARTICLE

Inflammation-Induced Adaptive Transitions in the Metabolomic Profile of Mouse Lung and Plasma

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SUMMARY

Airway inflammation is an important feature of chronic airway diseases like asthma and chronic obstructive pulmonary disease. In the present study, we aimed to evaluate the changes in major metabolites in the mouse airways in a model of experimental airway inflammation. For this purpose lipopolysaccharide (LPS) was intranasally applied to mice and fourty eight hours later their lungs and plasma were isolated in order to perform metabolomic analysis. Metabolomics is a very powerful technique using combined gas chromatography with mass spectrometry (GC-MS) aimed to identify and quantify as much as metabolites in a biological system. The metabolomic profile of LPS-applied and control mice were performed on the lung homogenates and plasma samples. GC-MS based metabolomics profile allowed to identify 87 and 244 metabolites in lung and plasma samples, respectively. The multivariate analysis results showed that a significant difference between LPS and control groups were obtained in lung and plasma samples. As a result of analyses, pyruvic acid, glyceric acid, adenosine, nonanoic acid and monomethylphosphate were increased, dehydroascorbic acid, theorine, 3-hydroxyaspartic acid, palmitic acid, palmitoleic acid, valine, succinic acid, lactic acid, tetradecanoic acid, ethanolamine, and N-methylglutamic acid were decreased in the lung homogenates, and urea, linoleic acid and L-alanine were decreased in the plasma samples isolated from LPS-group of mice. These results indicate that the purine and energy metabolisms are affected by airway inflammation.

Key Words: *Metabolomic, GC-MS, Profiling, Mouse lung, Lipopolysaccharide, Inflammation*

Fare Akciğeri ve Plazmasında İnflamasyonla İndüklenen Adaptif Değişiklikler

ÖZ

Solunum yolu inflamasyonu astım ve kronik obstrüktif akciğer hastalığı gibi bir çok kronik solunum yolu hastalığının önemli bir komponentidir. Bu çalışmanın amacı farede oluşturulan deneysel solunum yolu inflamasyonu modelinde solunum yollarındaki majör metabolitlerdeki değişimi incelemektir. Bu amaçla farelere intranazal olarak lipopolisakkarit (LPS) uygulanmış ve kırksekiz saat sonra metabolomik analiz için akciğerleri ve plazmaları alınmıştır. Metabolomik, kütle spektrometresi ile gaz kromatografisini (GC-MS) kombine eden ve biyolojik sistemdeki metabolitleri tanımlamak ve ölçmek için kullanılan çok güçlü bir tekniktir. LPS uygulanan ve kontrol grubu farelerinin metabolomik profili akciğer dokusu ve plazma numuneleri üzerinde gerçekleştirilmiştir. GC-MS bazlı metabolomik profili, sırasıyla akciğer ve plazma örneklerinde 87 ve 244 metaboliti tanımlamaya olanak sağlamıştır. Multivariate analiz sonuçları, farelerin akciğer ve plazma örneklerinde LPS ve kontrol grubu arasında anlamlı bir fark olduğunu göstermiştir. Analizler sonucunda, LPS ile indüklenen solunum yolu inflamasyonu modelinde akciğer dokusunda pirüvik asit, gliserik asit, adenosin, nonanoik asit ve monometilfosfat'ın arttığı; dehidroaskorbik asit, teorin, 3-hidroksyaspartik asit, palmitik asit, palmitoleik asit, valin, süksinik asit, laktik asit, tetradekanoik asit, etanolamin ve N-metilglutamik asit'in azaldığını, plazma örneklerinde üre, linoleik asit ve L-alanin düzeylerinin azaldığı gösterilmiştir. Bu sonuçlar, pürin ve enerji metabolizmasının hava yolu inflamasyonunda etkilendiğini göstermektedir.

Anahtar Kelimeler: *Metabolomik, GC-MS, Profilleme, Fare akciğeri, Lipopolisakkarit, İnflamasyon*

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INTRODUCTION

Airway inflammation is an important component of various airway diseases such as asthma and chronic obstructive pulmonary disease (COPD) (Sallmann et al., 2011). The inflammation of the airways also leads to bronchoconstriction which is responsible for the symptoms such as cough and shortness of breath. Therefore suppressing the inflammation is a key therapeutic strategy in these diseases.

The bacterial endotoxin, lipopolysaccharide (LPS) is a commonly used substance for inducing experimental inflammation in the airways (Bathoorn et al., 2008). LPS binds to toll-like receptor-4 (TLR4) and activates pro-inflammatory pathways (Hoshino et al., 1999) and Th2 cells of the adaptive immune system and subsequent release of cytokines, such as IL-6, TNFα, and IL-1β. When applied intranasally, LPS leads to neutrophil accumulation and, peribronchial and paranchymal inflammation of the lungs representing a suitable tool for experimental airway inflammation (Kaya-Yasar et al., 2017). Furthermore, LPS application also produces airway hyperreactivity which is a direct indicator for bronchial hyperresponsiveness (Kaya-Yasar et al., 2017; Starkhammar et al., 2012).

The recent developments in technologies allow comprehensive screening of the genome, transcriptome, proteome, and metabolome. Detailed knowledge of genomic, proteomic and metabolomic processes converged in the integrated "omics" approach clamps a massive potential for understanding mechanism of diseases, for their early diagnostics, choosing personalized therapeutic strategy and assessing its effectives. In metabolomics, the purpose is to identify and quantify as much as metabolites in a living organism (Nicholson et al., 1999). Metabolomics is very sensitive to a variety of external stimuli, drug exposures, genetic modifications, and disease pathways (Fiehn, 2002). Therefore metabolomics can help identifying the phenotype/pathophysiology of diseases and finding new therapeutic targets.

In the present study, we aimed to evaluate the changes in the major metabolites in airway inflammation. For this purpose, we performed a metabolomic analysis in the lungs and plasma samples isolated from control and LPS-applied mice. Analytes in a metabolomic sample comprise a highly complex mixture. Combined gas chromatography with mass spectrometry (GC-MS) is one of the most powerful techniques and commonly used in metabolomics studies. Metabolomic profiling of lung homogenate and plasma samples were performed using GC-MS and difference in metabolomic profile was evaluated between control and LPS-applied mice.

MATERIALS AND METHODS

14-16 weeks old CD1 mice were used in the present study. The procedures on animals were approved by Hacettepe University Animal Research Ethics Committee (No:2017/12-6).

In vivo experimental airway inflammation

LPS was used in order to induce experimental airway inflammation in mice. For this purpose, LPS was intranasally (i.n.) applied (60 µl; 0,1 mg/ml in PBS) to mice. The control group received vehicle (60 µl PBS) by the same route. 48 hours after LPS/vehicle application, mice were sacrificed by cervical dislocation and the chest was exposed by a midline incision on the thorax. Blood was isolated by cardiac puncture and the lungs were removed afterwards. Isolated lungs were immediately frozen by liquid nitrogen and stored at -80 until the analysis. Blood samples were centrifuged at 1000 g and the plasma was isolated for further analysis.

Tissue and plasma sample preparation for metabolomic analysis

Methods used for sample preparation and GC-MS instrumental conditions have been adopted from our previous studies (Nemutlu et al., 2015; Trushina et al., 2012). Briefly, the lung tissue samples were powdered using a mortar cooled down using liquid nitrogen and 50 µg of fine powder was transferred into an ependorf tube and extracted using 900 µL methanol:water (8:1, v/v) mixture containing 5 µg internal standard (IS), myristic-d27 acid, at ambient temperature. For plasma samples, 20 µL plasma were transferred into an ependorf tube and extracted using same solution used for tissue. Followed by extraction, tissue and plasma samples were spin down and 200 µL of supernatant was transferred into an eppendorf tube, and then completely dried in a vacuum concentrator. Subsequently, the tubes were methoximated and derivatized using N-methyl- N-trimethylsilyltrifluoroacetamide with 1% trimethylchlorosilane (MSTFA + 1% TMCS, Pierce) and then analyzed using GC-MS system (Shimadzu GC-MS-QP-2010 Ultra).

Metabolomic analysis

GC-MS based metabolomic analysis was performed as described in our previous paper (Nemutlu et al., 2015; Trushina et al., 2012). The data deconvolution was conducted using AMDIS and then SpectConnect was used to create a data matrix of metabolite peaks. The data matrix obtained from

GC-MS metabolomic analyses was transferred to an Excel work file and normalized to sum of area. Any metabolite traits having more than 50% of the values missing were excluded from the data matrix. Missing values in the data matrix were filled with the half value of the smallest concentration in the metabolite group. The final data matrix was import into the SIMCA-P+ (v13.0, Umetrics, Sweden) program for multivariate analysis. The principal component analysis (PCA) was performed to reveal the homogeneity of the data, any groupings, outliers, and trends, while partial least squares differentiation analysis (PLS-DA) used class discrimination, simplify interpretation, and find potential biomarkers. The variable importance in projection (VIP) values estimated to distinguish the most important metabolites for the stratifications of the groups. The Student's unpaired *t*-test was used to statistically compare changes in mean expression per metabolite between the groups. The differences were considered statistically significant at $P<0.05$. All data are expressed as mean ± standard error.

RESULTS

A GC-MS based metabolomics study has been performed for the metabolomic profiling of the mouse lungs and plasma samples in a model of experimental airway inflammation. GC-MS based metabolomics profile allowed to identify 87 and 244 metabolites in lung and plasma samples, respectively. The data from GC-MS analysis is transformed into meaningful data through multivariate analysis interpreted with PCA and PLS-DA methods. Initially, the data were examined with PCA scatter plot and two of the lung tissue samples were found outlier and excluded from study (data not shown). Then PLS-DA were performed for lung and plasma samples. The goodness and robustness of the models were evaluated using R^2 (the fraction of variance explained by a component) and Q^2 (the fraction of the total variation predicted by a component) values, respectively. These values for lung samples were R^2: 0.998 and Q^2: 0.628. The higher values (0.5 >) indicate the validity of the methods and that the model was stable and good to fitness and prediction. But, these values were found 0.902 for R^2 and 0.154 for Q^2 in plasma model, respectively. The lower Q^2 value (0.5 <) indicates that the model prediction was not good.

Lung metabolomic profile: Local response to lung inflammation

The PLS-DA scores plots shown in Figure 1 demonstrate that LPS application caused changes in the metabolomics profile. In order to deeply investigate the metabolomics profile differences between the control and LPS groups, PLS-DA score plot was drawn (Figure 1A). As seen in the figure, the two groups presented a completely different metabolomics profile from each other (Figure 1A). A VIP graph was also utilized in order to identify the metabolites affecting differentiation in the PLS-DA score plot (Figure 1B). As a result of analyses, pyruvic acid, glyceric acid, adenosine, nonanoic acid and monomethylphosphate were significantly increased while dehydroascorbic acid, theorine, 3-hydroxyaspartic acid, palmitic acid, palmitoleic acid, valine, succinic acid, lactic acid, tetradecanoic acid, ethanolamine, and N-methylglutamic acid were decreased in the lung homogenates in LPS-induced experimental airway inflammation model (Figure 2).

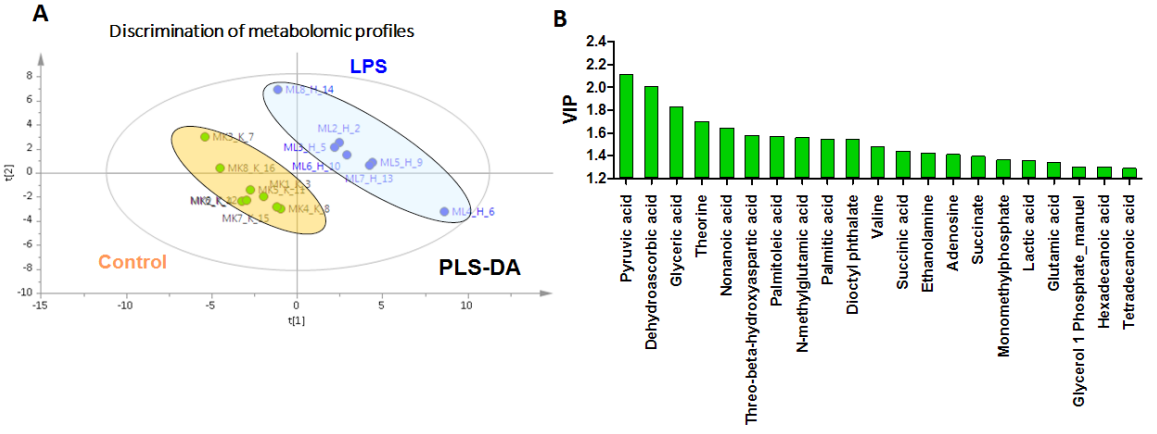

Figure 1. Comparison of lung metabolic profiling of control and LPS groups.

A) PLS-DA score plot shows clear separation between groups;

B) VIP charts of metabolites that are effective in separating the groups.

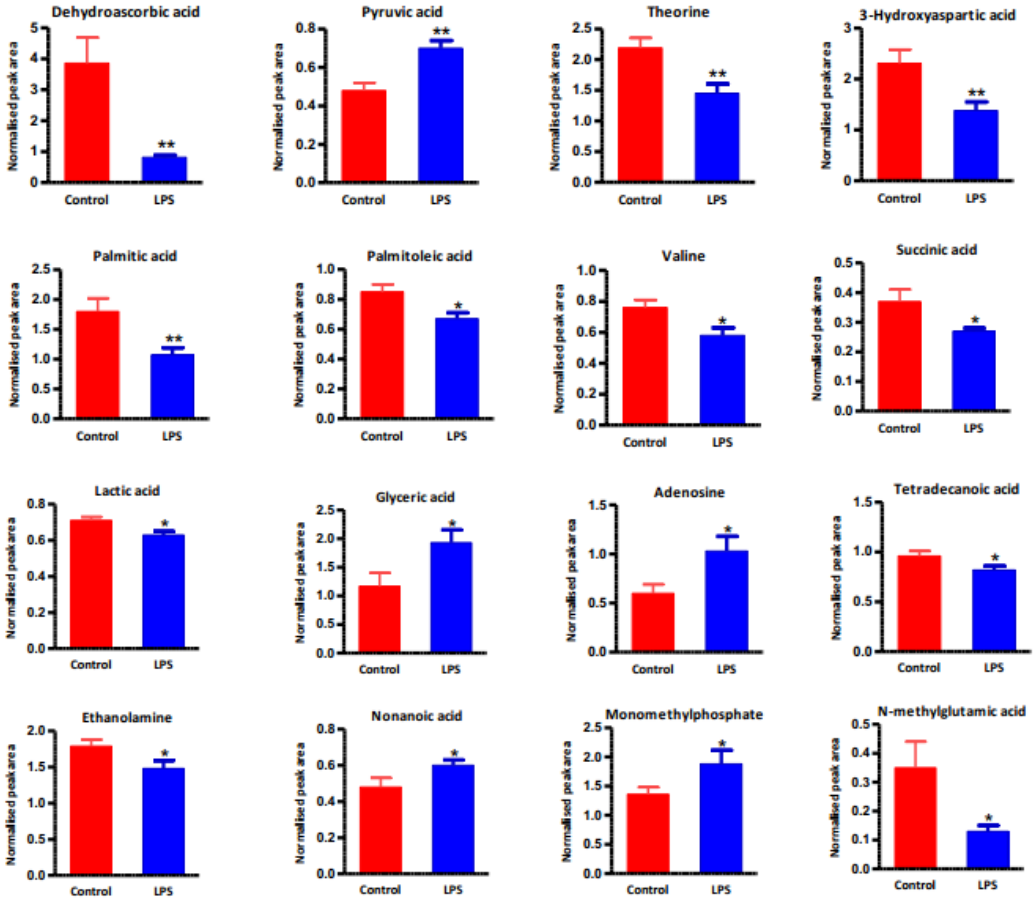

Figure 2. Metabolite levels in lung samples from control and LPS groups (* p <0.05 and ** p <0.01)

Plasma metabolomic profile: Systemic response to lung inflammation

The systemic effect of the airway inflammation was investigated using plasma samples. The difference between control and LPS groups were investigated with PLS-DA score plot and VIP graph (Figure 3). The metabolites; urea, linoleic acid and L-alanine were found to be significantly decreased in LPS-induced experimental airway inflammation (Figure 4).

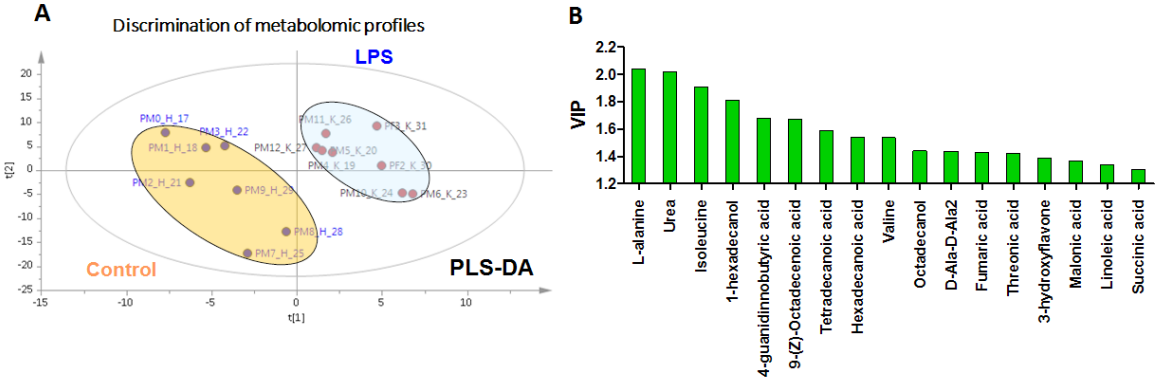

Figure 3. Comparison of plasma metabolic profiling of control and LPS groups.
A) PLS-DA score plot shows clear separation between groups;
B) VIP charts of metabolites that are effective in separating the groups.

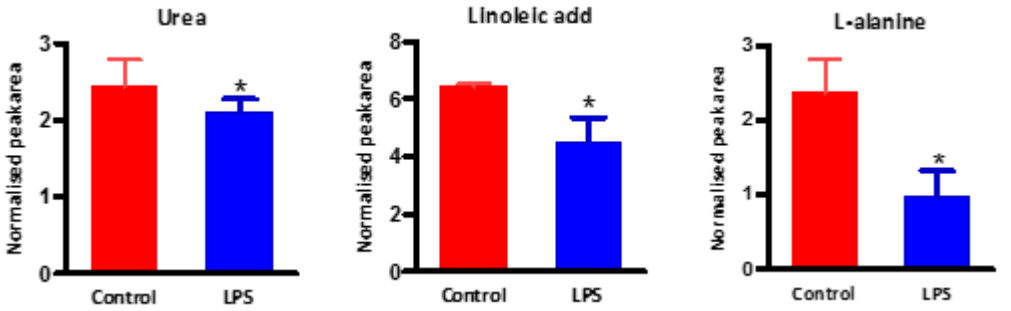

Figure 4. Metabolite levels in plasma samples from control and LPS (* $p < 0.05$)

DISCUSSION

Local application of LPS into the airways leads to airway inflammation which represents several features of airway diseases such as asthma and COPD. In most of the chronic airway diseases, inflammation leads to significant metabolic changes that are important for the disease progression (Angelis et al., 2014). The metabolomics approach helps comprehensive evaluation of the metabolic pathways and thus can help to understand the pathophysiology of diseases, identify new therapeutic targets and also diagnosing some airway diseases like asthma (Checkley et al., 2016).

The lung homogenate and plasma samples of LPS treated mice were compared to that of control group in order to identify the difference in their metabolite profiles. Fourteen metabolites (adenosine, dehydroascorbic acid, ethanolamin, glyceric acid, lactic acid, nonanoic acid, palmitic acid, palmitoleic acid, pyruvic acid, succinic acid, tetradecanoic acid, theorine, 3-hydroxyaspartic acid, valine, monomethylphosphate, and N-methylglutamic acid) have been found to be significantly altered between control and LPS groups. Among these metabolites pyruvic acid, glyceric acid, adenosine, nonanoic acid and monomethylphosphate were found to be significantly increased while dehydroascorbic acid, theorine, 3-hydroxyaspartic acid, palmitic acid, palmitoleic acid, valine, succinic acid, lactic acid, tetradecanoic acid, ethanolamine, and N-methylglutamic acid were decreased in the lung homogenates in LPS-induced experimental airway inflammation. On the other hand in the plasma samples urea, linoleic acid and L-alanine were found to be significantly decreased in the LPS group.

The purine nucleotide adenosine is an important mediator involved in asthma. Inhalation of adenosine has been shown to induce bronchoconstriction in asthmatic patients but not in healthy subjects (Cushley et al., 1983). Adenosine levels in plasma and bronchoalveolar lavage fluid of asthmatic patients have also been shown to be increased after allergen exposure (Driver et al., 1993; Mann et al., 1986). Factors such as trauma, cellular stress, hypoxia, ischemia or inflammation, rapidly lead to an increase in adenosine levels due to ATP degradation (Fredholm, 2007). Studies suggest that adenosine accumulation due to inflammation may be an important signal for initiating and/or sustaining chronic inflammation as well (Cicala and Ialenti, 2013). Therefore, adenosine can contribute to both disease development and function as a part of the inflammatory response.

In humans, asthmatics have increased levels of peptides related to glutamate cycle, as well as markers of inflammation like nicotinamide, adenosine monophosphate and arachidonate (Comhair et al., 2015). In addition, purine nucleosides, especially adenosine, are released too under stress conditions like inflammation, and they are revealed to be involved in many allergic conditions, mainly, the pathogenesis of asthma and the subsequent chronic obstructive pulmonary diseases (COPD) (Zhan-Guo Gao, 2017). While the concentration of extracellular adenosine is about 300 nM in normal cells, its concentrations rapidly exceed 1 μM in cells undergoing damage (Min et al., 2016). Adenosine accumulation is limited by its catabolism to inosine by adenosine deaminase (ADA) and then inosine is finally degraded to the stable end-product uric acid (Wilson et al., 2009). It is also possible that a further increase of adenosine levels may occur from metabolically active or stressed cells as a result of the reduced activity of ADA or adenosine kinase (AK), an enzyme that normally phosphorylates adenosine to AMP (Caruso et al., 2013). On the other hand, mice deficient of ADA, develop chronic pulmonary inflammation and airway obstruction (Blackburn et al., 2000). Thus, failure of the pathway leading to extracellular adenosine generation increases vulnerability to acute injury, while an excessive extracellular adenosine accumulation, such as in ADA deficient mice, causes chronic injury. Ultimately, moderate and short

lasting increased levels of extracellular adenosine may function as a natural endogenous protective pathway, while strongly high and long lasting extracellular adenosine tissue levels may contribute to the extent of the inflammatory tissue damage (Hasko et al., 2008).

It is well known that cells with low metabolic demands use oxidative phosphorylation to synthesize adenosine triphosphate (ATP), but under anaerobic or stress conditions, this energy is produced by glycolysis, which involves the conversion of glucose to lactate in order to synthesize ATP. However, cells with high metabolic demands, such as activated T cells, synthesize ATP through glycolysis during rapid proliferation, even under aerobic conditions (Kim et al., 2018), which results in characteristic shifts in the metabolic profile of the cell (Fiehn, 2002). LPS-induced lung inflammation and pro-inflammatory cytokines such as TNF-α and IFN-γ induce endoplasmic reticulum stress, which helps the cell to handle stress conditions (Kim et al., 2015). Decreased succinic acid and increased pyruvic acid levels in LPS mice indicate shifting energy metabolism from oxidative phosphorylation to glycolysis. On the other hand, the threonine, valine, 3-hydroxyaspartic acid and N-methylglutamic acid levels were decreased in LPS group indicating the changing in amino acid metabolism. Threonine is an essential amino acid necessary for intestinal mucosal protein synthesis, especially mucin, and for intestinal integrity, immune barrier function, and oxidative status (Liu et al., 2017). Suliman et al. were found that inflammation contributes to low amino acid concentrations in patients (Suliman et al., 2005). However, the mechanism for the decreased amino acid concentrations in LPS group's lungs is not clear. Evidence suggests that inflammation may lead to increased loss of nitrogen in the urine, increased amino acid oxidation, and increased metabolic demands of amino acids or may shifting in energy metabolism from carbohydrate metabolism to amino acid metabolism and leading increased rate of muscle and protein breakdown in the lungs of LPS group of mice.

The dehydroascorbic acid, an oxidized form of ascorbic acid, increased in LPS group due to oxidative stress by reactive oxygen species at high rates (Wilson, 2002). Moreover, both vitamin C and dehydroascorbic acid play a crucial role in sheltering lipids against oxidative damage (Astley, 2003). Therefore, the decreased level of dehydroascorbic acid may also affect the decrease in the level of fatty acids of palmitic acid, palmitoleic acid, tetradecanoic acid and ethanolamine in LPS group except for nonanoic acid which has been increased by LPS application. The increased level of fatty acid may lead to an increase of end product glycerol and its oxidized form, glyceric acid, in LPS group of mice.

Even if they are not considered as a diagnostic parameter by clinicians yet, measurements of metabolites are being gradually important. One reason that the limited sensitivity of current instrumentation and the little abundance of biomarkers in biological resources has provided another obstacle to the discovery and validation of new possible biomarkers (Rossi et al., 2014). Moreover using single test for diagnosis of diseases may result in many false positives (Hewitt, 2008). Therefore by providing an overall view on the metabolite changes in experimental models, metabolomic studies may lead to identify new biomarkers and therapeutic targets for inflammatory airway diseases.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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