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Research paper

Long-term allergen exposure induces adipose tissue inflammation and circulatory system injury

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ABSTRACT

The purpose of this study was to study whether allergen exposure can induce inflammation and lower the anti-inflammation levels in serum and in adipose tissues, and further develop cardiovascular injury. Our data showed that heart rate was significantly higher in the OVA-challenged mice compared to control mice. Moreover, there were higher expressions of pro-inflammation genes in the OVA-challenged mice in adipose tissues, and the expressions of anti-inflammation genes were lower. The levels of inflammation mediators were associated in serum and adipose tissues. The level of circulatory injury lactate dehydrogenase was significantly associated with the levels of E-selectin, resistin and adiponectin in the serum. The hematoxylin and eosin and immunohistochemistry stains indicated the OVA-challenged mice had higher levels of inflammation. In summary, the current study demonstrated allergen exposure can cause cardiovascular injury, and inflammatory mediators in adipose tissues play an important role in the pathogenesis of cardiovascular injury.

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1. Introduction

Cardiovascular disease (CVD) constitutes a group of metabolic diseases prevalent in all countries. According to statistical data, more than 30% of the population in the USA has cardiovascular diseases [1], and CVD is third among the top ten leading causes of death. In Taiwan, about 10% of the entire population die from CVD each year, and CVD is second among the top ten leading causes of death [2]. Therefore, understanding the risk factors and mechanisms for cardiovascular disease is crucial to its prevention and the development of therapeutic strategies.

Up to now, many studies indicated the development of cardiovascular diseases is primarily due to lifestyle factors and exposure to environmental pollutants [3–9]. Inflammation plays an important role in CVD. Previous studies have indicated obesity and other factors can affect the levels of interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α) in the blood, causing endothelial cell damage and produce adhesive molecules, such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and E-selectin. Adhesive molecules can attract LDL, white blood cells and platelets into locations that result in blood vessel damage and can cause fiber caps, influencing the flow of blood and disrupting heart function [10,11]. Moreover, IL-6 and

TNF- α can also produce C-reactive protein (CRP) and exacerbate the pathogenesis of CVD [12–14]. Therefore, IL-6, TNF- α , VCAM-1, ICAM-1, E-selectin, and C-reactive protein (CRP) are also important predictive markers for CVD.

People who are obese have higher amounts of subcutaneous and visceral adipose tissue, which induces changes in the levels of resistin, adiponectin, IL-6, TNF- α and interleukin-1 β (IL-1 β) and is related to the pathogenesis of cardiovascular diseases [15,16]. Resistin also damages endothelial cells and produces the adhesive molecules, VCAM-1, ICAM-1 and E-selectin. Therefore, many studies found higher levels of resistin in patients with CVD compared to the healthy population [15,17–22]. Adiponectin is also an important mediator of CVD. Adiponectin can reduce the risk of CVD through: (1) increases in the level of NO in blood vessels, (2) anti-oxidant activities, (3) down-regulation of endothelial cellular adhesive molecules, and (4) protection of the endothelium from apoptosis [23]. Epidemiological studies indicated patients with CVD have lower levels of adiponectin than healthy individuals [24–26], and animal studies also indicated lower levels of biomarkers with CVD in mice injected with adiponectin [27,28]. Taken together, adipokines from adipose tissue also significantly influence CVD.

We recently discovered acute exposure to allergens lowers adiponectin levels in serum and adipose tissue and concurrently induces adipose tissue inflammation [29]. This study hypothesizes allergen exposure can affect cardiovascular injury biomarkers

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through the action of adipose tissue inflammation and allergen exposure is one of the risk factors in cardiovascular injury. We tested the hypotheses by analyzing the levels and relationships of inflammatory mediators, adipokines and the biomarkers of cardiovascular injury in serum and adipose tissue and complete Hematoxylin and eosin (HE) and immunohistochemistry (IHC) staining of adipose tissue.

2. Materials and methods

2.1. Animals and animal care

Eight-week-old male C57BL/6 mice were obtained from the National Cheng-Kung University Laboratory Animal Center. The mice were grouped and kept in a constant lighting cycle (12-h light/dark), temperature, ($23 \pm 2^\circ\text{C}$) and relative humidity (50–60%) with free access to food and water. All study protocols were approved by the Animal Care and Use Committee of National Cheng-Kung University.

2.2. Allergen sensitization and challenge

In the first stage, the mice were sensitized with four intraperitoneal injections of 10 μg Ovalbumin (OVA, Grade IV, Sigma-Aldrich, St Louis, MO, USA) bound to 1 mg aluminum hydroxide (Sigma-Aldrich, St. Louis, MO, USA) in 200 μl of Phosphate Buffered Saline (PBS, pH7.4) on days 1, 8, 15 and 23. The control mice were sensitized with PBS (200 μl). On day 30, in the second stage, the mice were intranasally challenged with 50 μg of OVA in 25 μl of PBS once a day, five days per week for 12 weeks. Equal volumes of PBS were used in parallel experiments as a control. Twenty-four hours after the last intranasal challenge, the mice were weighed (AE240, Mettler-Toledo, Switzerland) and sacrificed with citosol (Kyorin Pharmaceuticals, Tokyo, Japan), and the blood, bronchoalveolar lavage fluid (BALF) and adipose tissue were collected.

2.3. HR, SPB and DBP measurements

Heart rate (HR), systolic blood pressure (SBP) and diastolic blood pressure (DBP) were continuously taken for five days before sacrifice using a blood pressure analysis system (BP-2000 Series II, Visitech systems, United States of America) in accordance with manufacturer protocols.

2.4. Cell populations in bronchoalveolar lavage fluid (BALF)

In this study, BALF samples were drawn, and the number and population of the cells were determined to examine whether allergen exposure successfully elicits airway inflammation. Two tubes of BALF were collected in order, and each included 3 repeated, 30-s-long drawings using 1 ml of saline. The BALF was centrifuged at 4°C and 2000 rpm for 10 min, and the pellets were resuspended in 1 ml of RPMI 1640 medium supplemented with 10% fetal bovine serum. Twenty microliters of cell suspension were drawn for total cell counts using a hemocytometer (Marienfeld, Germany) and a microscope (BH-2, Olympus, Tokyo, Japan). The rest of the suspension was cytocentrifuged (Thermo Electron Corporation, Waltham, MA, USA) to transfer cells onto a microscopic slide, which was subsequently stained with Diff-quick (Sysmex Corporation, Tokyo, Japan). The macrophages, eosinophils, and neutrophils were differentiated by the stain, and the cell numbers in each population were counted under a microscope (CX31RBSF, Olympus, Tokyo, Japan).

2.5. Blood cardiovascular injury indexes, adiponectin, resistin, and immune markers

We measured the levels of lactate dehydrogenase (LDH), creatinine phosphokinase (CPK) and creatine kinase muscle-brain (CK-MB) with an FUJIFILM DRI-CHEM analyzer (FUJIFILM DRI-CHEM +4000i, Tokyo, Japan) using an analysis slide LDH, CK-MB and CPK (FUJIFILM, Tokyo, Japan) according to manufacturer protocols, respectively. Enzyme-linked immunosorbent assays (ELISA) were performed to determine the serum levels of adiponectin, resistin, VCAM-1, ICAM-1, E-selectin, IL-6, TNF- α (R&D System, Minneapolis, MN, USA) and total immunoglobulin E (IgE) (BD Biosciences, San Diego, CA, USA).

2.6. Inflammatory marker expression in adipose tissue

In this study, brown (interscapular) and white adipose tissues (inguinal and gonadal) were harvested and conserved in RNAlater (Protech, Taipei, Taiwan) at 4°C for 24 h before storage at -20°C . For RNA extraction, 1 ml of RNA Plus Extraction Reagent (Invitrogen, Carlsbad, CA, USA) was mixed with the adipose tissue, and the mixture was homogenized using a BeadBeater (BioSpec Products, Inc., Bartlesville, OK, USA). The extracted sample was further treated with an RNA pure kit (Geneaid, New Taipei, Taiwan) to increase the purity. The mRNA expressions of plasminogen activator inhibitor-1 (PAI-1), IL-6, TNF- α , IL-1 β (pro-inflammatory M1 macrophage), interleukin-10 (IL-10), macrophage galactose N-acetyl-galactosamine-specific lectin 1 and 2 (Mgl1, and Mgl2) (anti-inflammatory M2 macrophage) in the adipose tissues were determined using real-time quantitative RT-PCR. Reverse transcription was performed using the SuperScript III First-Strand Synthesis Super-Mix (Invitrogen, Carlsbad, CA, USA). Quantitative real-time RT-PCR was performed with the Fast SYBR Green Master Mix (Applied Biosystems, Foster, California, USA) and the SYBR Green system (Applied Biosystems, StepOne Plus Real-Time PCR SYSTEM Thermal Cycler, Model 4376592, Foster, CA, USA).

The data were expressed as relative gene expression to the endogenous reference, 18S rRNA, using the $\Delta\Delta\text{Ct}$ method [30]. For comparison between the OVA- and PBS-treated groups, the level of hypoxanthine guanine phosphoribosyl transferase (HPRT) expression was used as the internal control. Primer sets were as follows: PAI-1 forward 5'-TGA TGG CTC AGA GCA ACA AG-3' and reverse 5'-GCC AGG GTT GCA CTA AAC AT-3'; IL-6 forward 5'-GAT GCT ACC AAA CTG GAT ATA ATC-3' and reverse 5'-GGT CCT TAG CCA CTC CTT CTG TG-3'; TNF- α forward 5'-ATG AGC ACA GAA AGC ATG-3' and reverse 5'-TCA CAG AGC AAT GAC TCC-3'; IL-1 β forward 5'-GCA GCT ATG GCA ACT GTT CCT-3' and reverse 5'-TCA TAT GGG TCC GAC AGC ACG-3'; IL-10 forward 5'-CAG AGC CAC ATG CTC CTA GA-3' and reverse 5'-TGT CCA GCT GGT CCT TTG TT-3'; Mgl1 forward 5'-TGA GAA AGG CTT TAA GAA CTG GG-3' and reverse 5'-GAC CAC CTG TAG TGA TGT GGG-3'; Mgl2 forward 5'-TTA GCC AAT GTG CTT AGC TGG-3' and reverse 5'-GGC CTC CAA TTC TTG AAA CCT-3'; and HPRT forward 5'-CTC ATG GAC TGA TTA TGG ACA GGA-3' and reverse 5'-GCA GGT CAG CAA AGA ACT TAT AGC-3'.

2.7. Hematoxylin and eosin (HE) and immunohistochemistry (IHC) staining

In this study, the degree of inflammation in adipose tissues was determined using the HE staining method. Adipose tissues were fixed in 10% neutral formalin (Macron chemicals, Charlotte, NC, USA) for 24 h. The tissues were embedded with paraffin using a paraffin embedding mechanism (LIPSHAW) and sliced to 5 μm using a paraffin slicing mechanism (LIPSHAW). The histological

sections were stained with Harris hematoxylin counterstained with eosin. In addition, F4/80 antibody was used to identify the adipose tissue macrophage (ATM). The above experimental processes were performed by the Department of Pathology, National Cheng Kung University Hospital. Finally, light microscope (CX31RBSF, Olympus, Tokyo, Japan) was applied to evaluate the inflammation state, and the F4/80 cells were further counted and expressed as the number per 100 cells.

2.8. Statistical analysis

We used a Student's *t*-test to analyze the differences between the PBS- and OVA-treated mice for the biomarkers in the BALF, serum, and adipose tissue. The relationship was examined using a Pearson correlation. SAS 9.2 was used to analyze the data. The statistical significance was set at $p < 0.05$.

3. Results

3.1. BALF cell and IgE level in serum

In this study, the number of cell-specifics in the BALF and the total IgE level in the serum were examined to understand whether OVA exposure effectively causes immune responses in the airway. Table 1 shows OVA exposure caused a significant increment in the number of total cells, macrophages, neutrophils and eosinophils in the BALF as compared to the PBS-treated group ($p < 0.05$). In addition, OVA exposure also induced significant increases in the level of total IgE in serum in the OVA-challenged mice as compared to the PBS-treated mice ($p < 0.05$). These data demonstrated OVA exposure induced hyper-reactivity of the immune system in the airways in the mice.

3.2. HR, SBP, and DBP

We measured the values of HR, SBP and DBP to understand whether exposure to allergens affects cardiovascular functions. Table 2 shows the differences in SBP and DBP between OVA challenged and PBS challenged mice, but these differences are not significant. The level of HR is significantly higher in the mice that were OVA challenged as compared to the PBS-challenged mice ($p < 0.05$).

3.3. LDH, CPK and CK-MB in serum

LDH, CPK and CK-MB are often used to test the cardiac structure. In this study, the levels of the markers were also analyzed to assess whether exposure to allergens affects cardiac function. Fig. 1 shows a higher LDH level ($p < 0.05$) in the OVA-challenged mice as compared to the PBS-challenged mice, and also higher expressions of CPK and CK-MB without significant differences. In this study, the ratio of CK-MB and CPK was further calculated. The ratios of the PBS-challenged mice and OVA-challenged mice were 0.30 and 0.32, respectively. There were no significant differences in the study mice.

Table 1
Different cell counts in BALF (n = 4–6/group).

Cell composition	PBS	OVA
Total cells (10^5 /ml)	0.73 ± 0.40	775.55 ± 108.26*
Macrophages (10^5 /ml)	0.58 ± 0.24	30.31 ± 3.56*
Eosinophils (10^5 /ml)	0.04 ± 0.05	7.58 ± 0.89*
Neutrophils (10^5 /ml)	0.11 ± 0.14	730.08 ± 103.28*
Total IgE (μg/ml) ^a	1.95 ± 0.94	4.86 ± 0.27*

* $p < 0.05$ when compared with PBS.

^a Total IgE in serum.

Table 2

Blood pressure and heart rates in the different mouse groups (n = 4–6/group).

Parameter	PBS	OVA
SBP (mmHg)	113.55 ± 9.90	111.00 ± 7.25
DBP (mmHg)	65.74 ± 13.87	68.03 ± 13.28
HR (bpm)	483.67 ± 52.36	512.16 ± 84.28*

SBP: Systolic blood pressure, DBP: Diastolic blood pressure.

HR: Heart rate.

* $p < 0.05$ when compared with PBS.

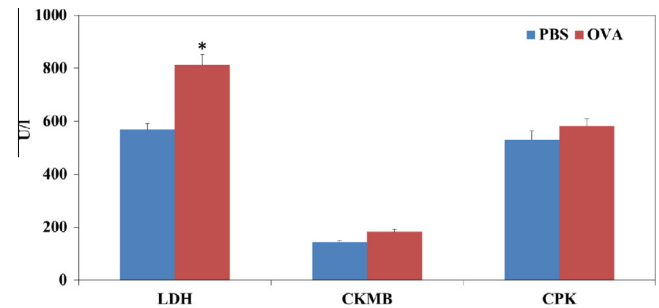


Fig. 1. Levels of markers in the damage of myocardium in serum. (* $p < 0.05$ when compared with PBS) (n = 4–6/group.) The levels of LDH, CPK and CK-MB were measured by FUJIFILM DRI-CHEM analyzer using an analysis slide LDH, CK-MB and CPK. LDH: lactate dehydrogenase; CPK: creatinine phosphokinase; CK-MB: creatine kinase muscle-brain.

3.4. Inflammatory markers and adipokines in serum

We hypothesized inflammatory responses may contribute to OVA-induced cardiovascular injury. Therefore, we investigated the serum levels of IL-6 and TNF- α , and ICAM-1, VCAM-1 and E-selectin expressed on the vascular endothelium, as well as resistin and adiponectin expressed by adipose tissues. Fig. 2A shows the OVA-challenged mice expressed higher levels of IL-6 as compared to the PBS-challenged mice ($p < 0.05$). The OVA-treated mice had significantly higher E-selectin level than the PBS-treated mice ($p < 0.05$), while no significant difference was found in the level of ICAM-1 and VCAM-1 (Fig. 2B). On the other hand, resistin increased by nearly twofold ($p < 0.05$), while adiponectin significantly decreased ($p < 0.05$) in the OVA-challenged mice (Fig. 2C).

3.5. Relationship between inflammatory markers, adipokines and cardiovascular injury

We assumed inflammation plays an important role in the pathogenesis of cardiovascular injury, therefore analyzed whether LDH is associated with the levels of IL-6, E-selectin, adiponectin and resistin. The analysis results are shown in Fig. 3. It can be seen LDH is positively associated with resistin and E-selectin ($p < 0.05$) and adiponectin also has a significant negative correlation with LDH ($p < 0.05$). Together, allergen exposure significantly affects the expression of adipokines and endothelial molecules, which affect the level of LDH.

3.6. Adipose tissue weight and inflammation

The levels of adipokines and E-selectin appeared to be influenced by OVA exposure, so we wondered if the characteristics of adipose tissue were affected concurrently. We harvested adipose tissues and analyzed the tissue weight and the expression profile of pro-inflammatory (macrophage M1) and anti-inflammatory (macrophage M2) markers using qPCR. As shown in Table 3, the weight proportions of both inguinal and gonadal adipose tissues

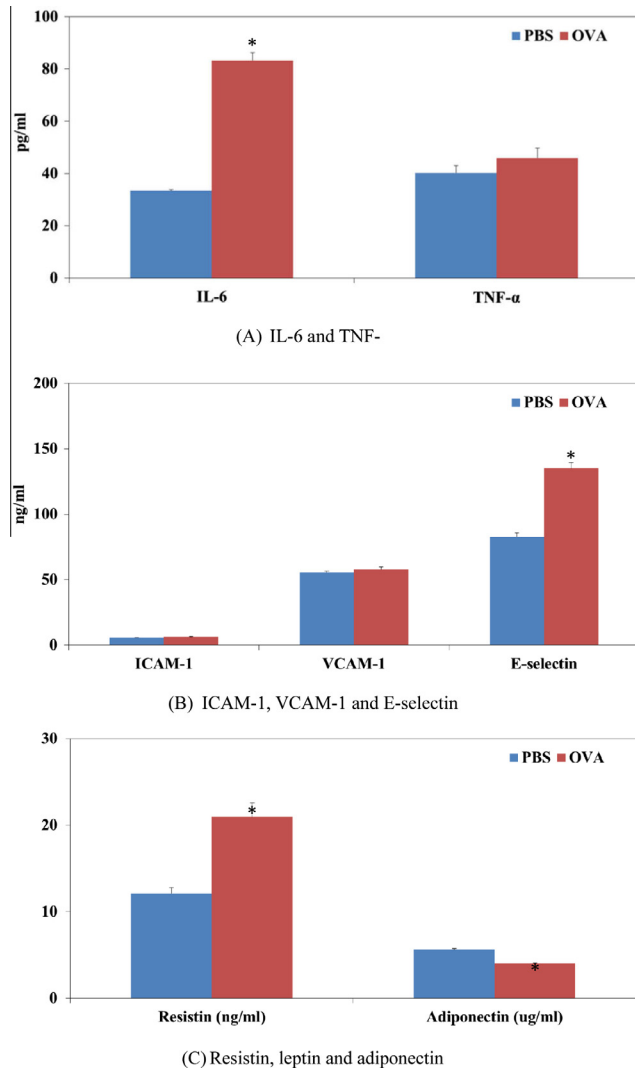


Fig. 2. Serum levels of inflammatory biomarkers. (A) IL-6 and TNF- α , (B) ICAM-1, VCAM-1 and E-selectin and (C) resistin and adiponectin (B) ($p < 0.05$ as compared with PBS) ($n = 4-6$ /group). The levels of IL-6, TNF- α , ICAM-1, VCAM-1, E-selectin, resistin and adiponectin were analyzed using ELISA. VCAM-1: vascular cell adhesion molecule-1; ICAM-1: intercellular adhesion molecule-1.

were substantially lower in the OVA-challenged mice as compared to the control mice ($p < 0.05$). On the other hand, the brown adipose tissue (BAT) weight did not show a detectable difference. In respect to gene expression in the gonadal adipose tissue, we found the expressions of TNF- α and IL-1 β mRNA were incremental ($p < 0.05$) (Fig. 4A) as well as decreased IL-10 and Mgl2 in the OVA-challenged mice ($p < 0.05$) (Fig. 4B). In the case of the inguinal adipose tissue, we detected higher expressions of IL-6 and IL-1 β mRNA ($p < 0.05$) (Fig. 5A), and likewise, lowered IL-10 and Mgl2 in the OVA-challenged mice ($p < 0.05$) (Fig. 5B). HE and IHC staining also indicated obvious inflammation expression in the adipose tissues of the OVA-challenged mice as compared to the PBS-challenged mice (Figs. 6 and 7). In summary, Table 3 and Figs. 4–7 demonstrate OVA allergen exposure induces a reduction in white adipose tissue weight, increases pro-inflammatory gene expression, and negatively regulates the expression of anti-inflammatory genes.

In this study, the relationships between the levels of adipokines and E-selectin in serum and the expressions of pro- and anti-inflammatory genes in adipose tissues were further analyzed to understand whether inflammatory markers in adipose tissue affect

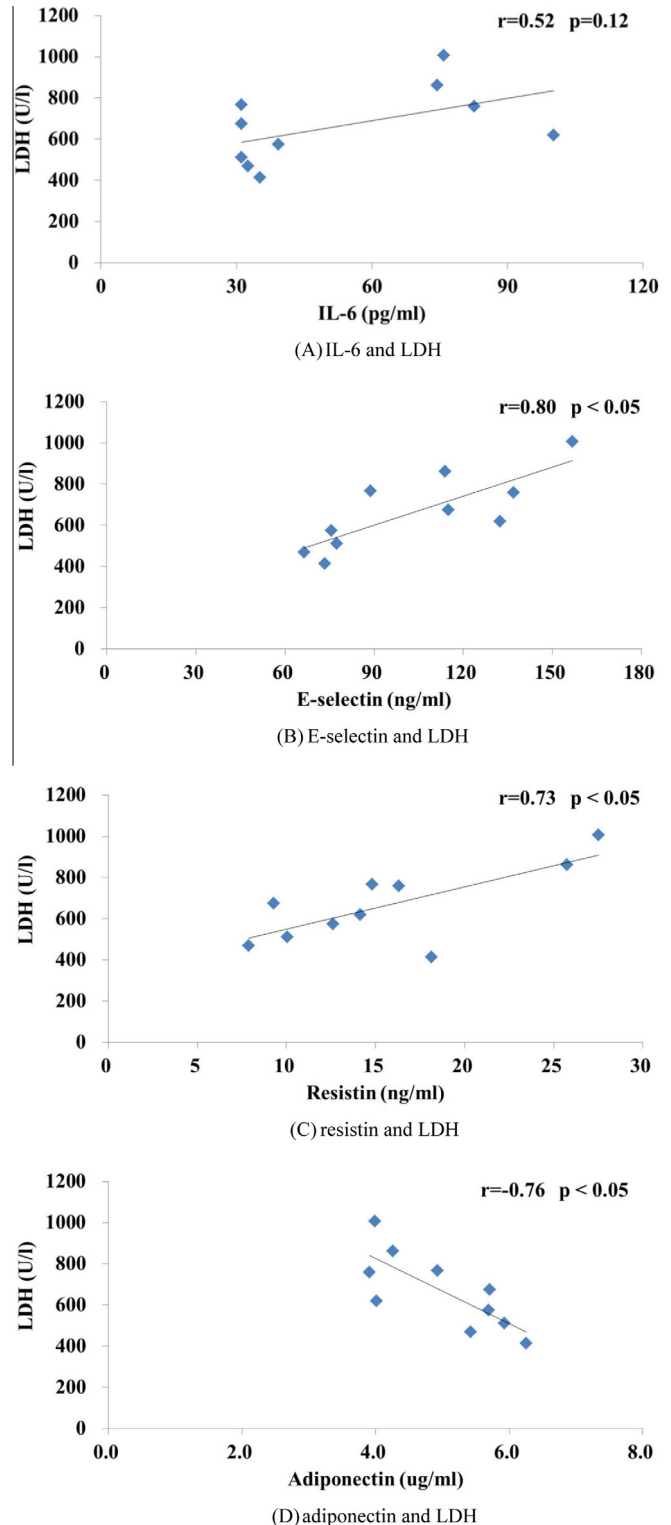


Fig. 3. Correlation of LDH with IL-6 (A), E-selectin (B), resistin (C) and adiponectin (D) (statistical significance was at $p < 0.05$). LDH: lactate dehydrogenase.

the distribution of adipokines and E-selectin in serum. Table 4 shows significant associations occurring in gonadal adipose tissue between adiponectin and IL-1 β ($p < 0.05$), E-selectin and IL-1 β ($p < 0.05$), and E-selectin and TNF- α ($p < 0.05$). In the inguinal adipose tissue, we found resistin was associated with IL-6 and IL-1 β ($p < 0.05$); E-selectin was associated with IL-1 β ($p < 0.05$), while adiponectin was negatively correlated with IL-6 and IL-1 β

Table 3

Body weight and weight percentage of adipose tissue in the different mouse groups (n = 4–6/group).

Category	PBS	OVA
Body weight (g)	23.2 ± 1.6	19.3 ± 0.9*
BAT (%) ^a	0.31 ± 0.08	0.30 ± 0.04
Inguinal (%) ^a	0.32 ± 0.07	0.09 ± 0.04*
Gonadal (%) ^a	1.37 ± 0.29	0.09 ± 0.03*

^a (Adipose tissue weight / body weight of mouse) × 100.

* p < 0.05 when compared with PBS.

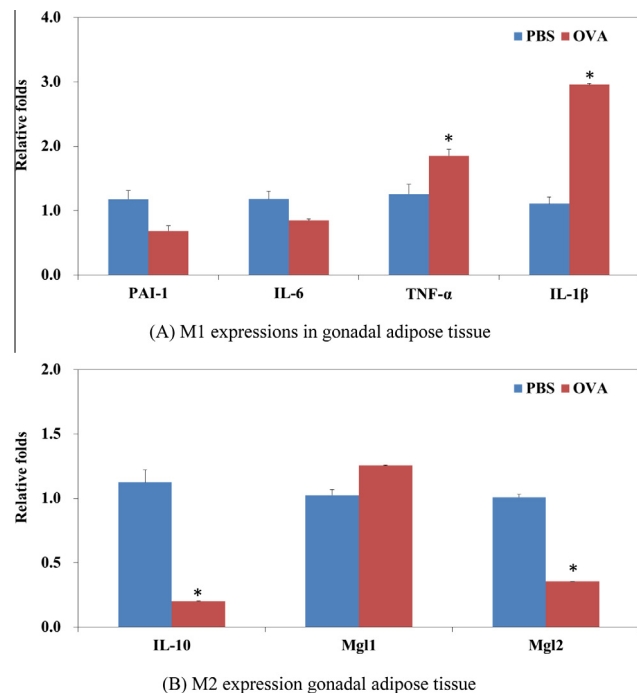


Fig. 4. M1/M2 gene expression in gonadal adipose tissue. (A) M1 and (B) M2 ($p < 0.05$ when compared with PBS) (n = 4–6/group). The RNA of PAI-1, IL-6, TNF-α, IL-1β, IL-10, Mgl1 and Mgl2 can be extracted using RNA Extraction Reagent. The mRNA expressions were analyzed using real-time PCR (SYBR Green). PAI-1: plasminogen activator inhibitor-1; Mgl1 and Mgl2: macrophage galactose N-acetyl-galactosamine-specific lectin 1 and 2. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

($p < 0.05$). Taken together, these results demonstrated that OVA exposure-induced adipose tissue inflammation has a considerable influence on the serum levels of both adipokines and E-selectin.

4. Discussion

This is the first study demonstrating allergen exposure is linked to circulatory system inflammation, and the results suggest adipose tissue inflammation as a possible mediator. Allergen exposure induces adipose tissue inflammation, and the inflammatory gene expression changes correlate with inflammatory markers E-selectin and adipokines in circulation. According to our experimental model, we propose allergen exposure may be one risk factor for cardiovascular injury.

We validated allergen exposure significantly induced hyper-reactivity of the immune system in the airway of OVA-challenged mice. As immune cell counts in BALF and the serum levels of total IgE are common indications of airway inflammation [31–34], we observed a substantially increased number of immune cells in BALF and elevated IgE levels in the blood of the mice under observation (Table 1). Moreover, we found the predominant cell

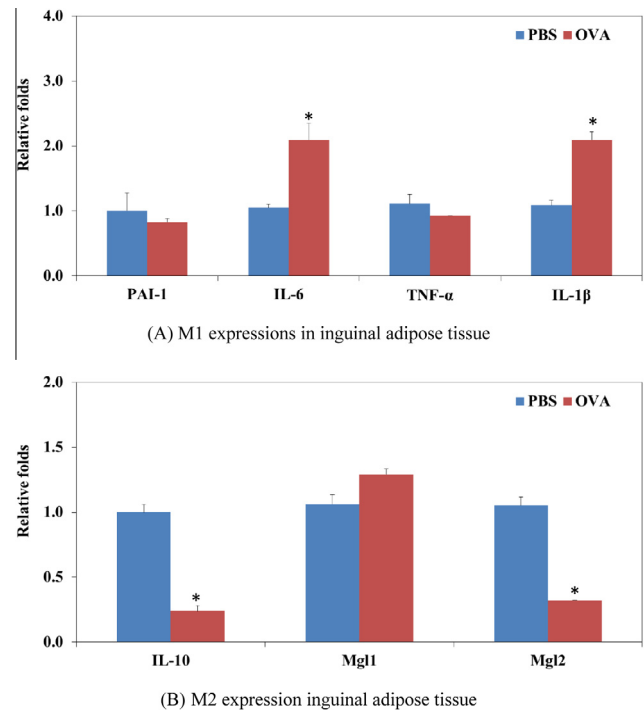


Fig. 5. M1/M2 gene expression in inguinal adipose tissue. (A) M1 and (B) M2 ($p < 0.05$ when compared with PBS) (n = 4–6/group). The RNA of PAI-1, IL-6, TNF-α, IL-1β, IL-10, Mgl1 and Mgl2 can be extracted using RNA Extraction Reagent. The mRNA expressions were analyzed using real-time PCR (SYBR Green). PAI-1: plasminogen activator inhibitor-1; Mgl1 and Mgl2: macrophage galactose N-acetyl-galactosamine-specific lectin 1 and 2. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

type in the BALF was neutrophils, which is an important biomarker for severe asthma [35,36]. Therefore, our results suggest long-term allergen exposure successfully induces airway inflammation and could also cause serious airway inflammation.

Previous studies found exposure to the OVA allergen can lower the level of adiponectin as an anti-inflammatory in both serum and adipose tissues [29], and a previous study indicated lower adiponectin concentrations can induce inflammation, which contributes to the pathogenesis of CVD [23]. This study further investigated the relationship between allergen exposure and cardiovascular injury, and assessed the role of adipose tissues in the association between cardiovascular injury and allergen exposure. Table 2 and Fig. 1 show there are higher concentrations of heart rate and serum LDH levels in mice that are OVA-challenged as compared to mice that are PBS-challenged, respectively. These results imply exposure to the OVA allergen can influence cardiovascular function. However, previous studies showed LDH is also secreted as a result of skeletal muscle damage [37,38], and some studies also indicated LDH is not an appropriate prediction marker for cardiovascular injury [39,40]. Moreover, our data show no differences between the OVA mice and PBS mice in regard to CPK, CKMB and the ratio of CKMB/CPK. However, our study found higher levels of IL-6, resistin and E-selectin in the OVA-challenged mice as compared to the PBS-challenged mice, and the OVA mice had lower levels of adiponectin (Fig. 2). Fig. 3 also shows LDH is associated with E-selectin, resistin and adiponectin.

Many studies demonstrated adipose tissue inflammation can influence the levels of resistin, adiponectin, IL-6 and TNF-α in the blood vessels and is involved in the development of CVD [11,15,41]. IL-6 and TNF-α can cause vascular damage and produce the adhesive molecules VCAM-1, ICAM-1 and E-selectin, which attract LDL, white blood cells and platelets into the blood vessels

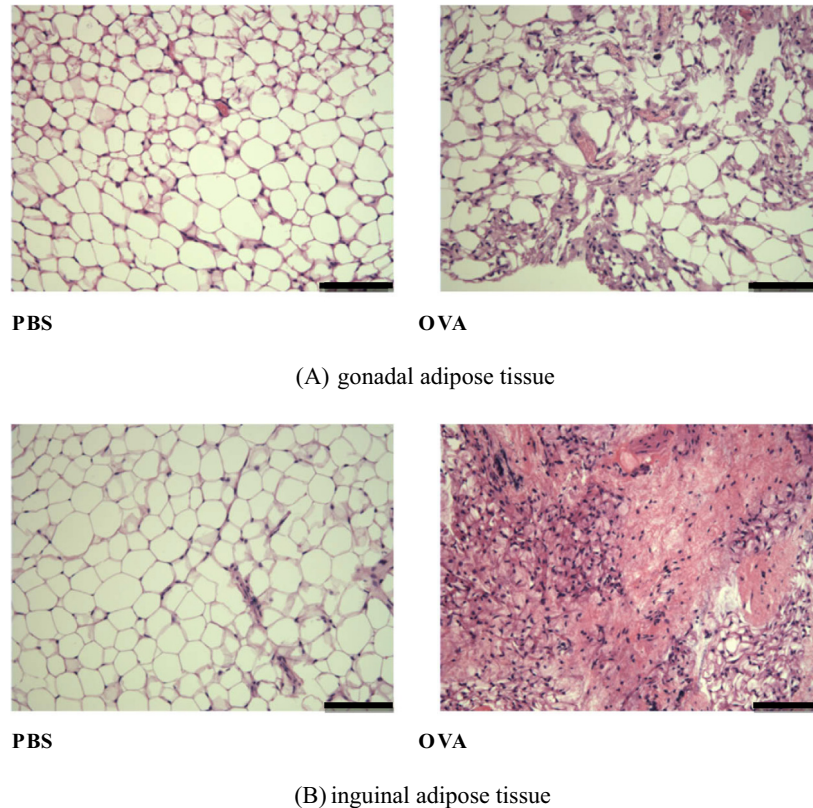


Fig. 6. Adipose tissue HE photo from PBS and OVA-challenged mice (A) gonadal adipose tissue and (B) inguinal adipose tissue (scale bar: 100 μ m). Adipose tissues were fixed in 10% neutral formalin, and then the tissues were embedded with paraffin and sliced to 5 μ m. The histological sections were stained with Harris hematoxylin counterstained with eosin.

and cause fiber caps and also influence heart function and blood flow [10,42]. Both adiponectin and resistin also influence the production of adhesive molecules. Using cell experimentation, Kawamami and his colleagues found resistin can induce VCAM-1 and ICAM-1, but there were lower levels of ICAM-1 and VCAM-1 with increasing adiponectin levels [43]. Both adiponectin and resistin can affect the function of the left ventricular mass (LVM) and also LV fractional shortening (LVFS) [44]. Moreover, adiponectin has an anti-inflammatory function, and adipose tissue inflammation lowers the levels of adiponectin in both blood and adipose tissue [45]; thus, there is not enough ability to reduce oxidation damage and inflammation [23]. Lower levels of adiponectin in patients with CVD compared to the healthy population have been found [25,46]. Previous studies also indicated adiponectin can lower the level of LDH and also cause myocardial ischemia-reperfusion injuries [47]. Additionally, the heart rate can serve as a biomarker of cardiovascular injury. de Chantemele and colleagues found higher heart rate variability in mice fed a high-fat diet as compared to mice fed a normal diet [48]. Epidemiological studies indicated a higher risk of CVD in people who have higher heart rates, regardless of gender [49]. Kolloch and co-workers discovered a higher risk of death associated with increased heart rate among subjects with CVD [50]. Therefore, heart rate is often a biomarker for predicting CVD. Our data show heart rate variability was higher in the OVA mice than it was in the PBS mice. To summarize, there were significant different of inflammatory mediators, adipokines, and adhesive molecules in serum between mice with OVA-challenge and PBS-challenge, and the level of LDH was associated with the inflammatory mediators. These results imply allergens could induce inflammation is related to diseases of the circulatory system.

Adipose tissue inflammation has been found to be characterized by induction of inflammatory M1 polarity in adipose tissue macrophages [51,52]. In the current study, we examined the expression of pro-inflammatory M1- and anti-inflammatory M2-related genes in dissected adipose tissues, and, as shown in Figs. 4 and 5, M1 genes were up-regulated while M2 genes were down-regulated in the OVA-challenged mice. We also found the weight of adipose tissues in the OVA-exposed mice was substantially reduced (Table 3), which agreed with a previous finding indicating adipose tissue weight was reduced due to an environmental stimulus [14]. Moreover, there were more inflammatory expressions of adipose tissues in the OVA-challenged mice compared to those that were PBS-challenged, suggesting there is obvious inflammation in allergen-exposed mice (Fig. 6). We also found OVA-challenged mice had higher expressions of F4/80 in adipose tissues than those that were PBS-challenged, demonstrating obvious ATM accumulation in the OVA challenged mice (Fig. 7). Most strikingly, our correlation analysis revealed a link between gene expression changes in adipose tissue and circulating inflammatory biomarkers (Table 4). Obesity is definitely one risk factor leading to inducement of inflammatory reactions in adipose tissue. However, obesity was not the central concern; rather, this study aimed at investigating whether exposure to the OVA allergen would induce inflammation in adipose tissue. The lower body weight of the OVA-challenged mice may be attributable to the fact allergen exposure may lead to inflammation of adipose tissues, which play a key role in energy homeostasis, therefore results in decreased food intake [53]. These combined results should strongly imply the induction of OVA allergen exposure in mice may result in adipose tissue inflammation and in turn, metabolic system alterations. Moreover, we found that there were higher expressions of M1 markers in adipose tissues,

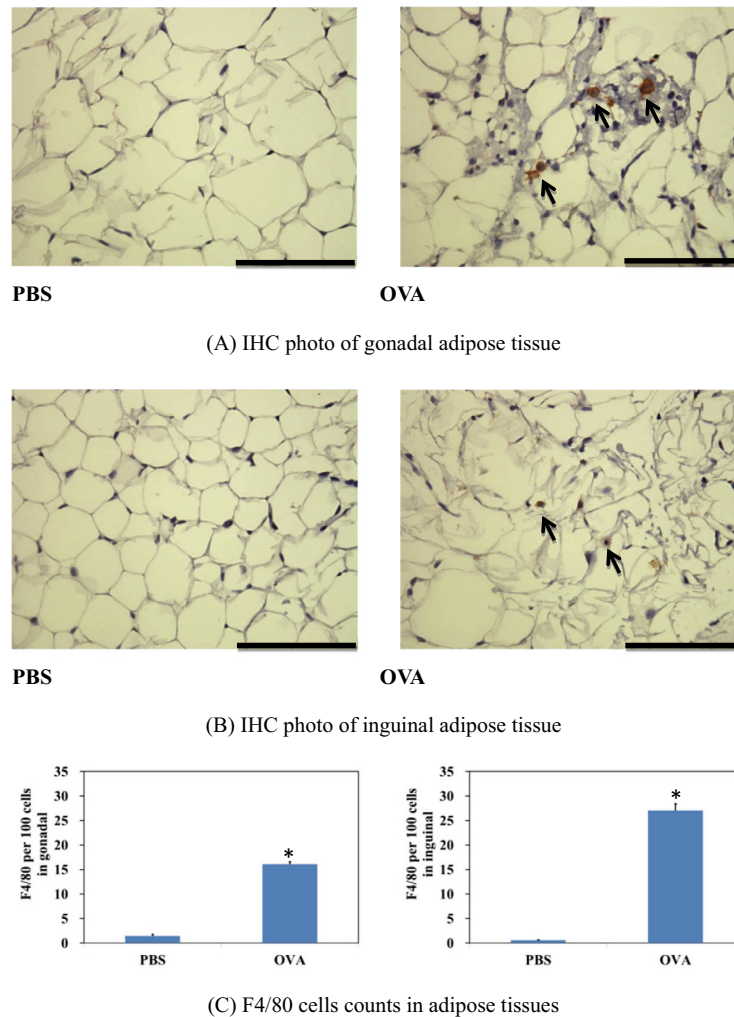


Fig. 7. Results of IHC with F4/80 antibody in adipose tissue. (A) Gonadal adipose tissue, (B) inguinal adipose tissue and (C) F4/80 cells number per 100 cells. Arrows indicate F4/80 (scale bar: 100 μ m). F4/80 antibody was used to identify the adipose tissue macrophage. Light microscope was applied to count the numbers (per 100 cells).

Table 4

Correlation between circulating resistin, adiponectin and E-selectin levels and adipose tissue inflammation and anti-inflammation gene expressions (*p*-value).

Adipose tissue expression	mRNA	Circulation		
		Resistin (ng/ml)	Adiponectin (μ g/ml)	E-selectin (ng/ml)
Gonadal adipose tissue	PAI-1	−0.27 (0.45)	0.45 (0.19)	−0.28 (0.44)
	IL-6	−0.29 (0.42)	0.08 (0.84)	−0.22 (0.53)
	TNF- α	0.43 (0.22)	−0.37 (0.30)	0.69 (<0.05)*
	IL-1 β	0.62 (0.06)	−0.98 (<0.001)*	0.77 (<0.05)*
Inguinal adipose tissue	PAI-1	0.23 (0.55)	0.44 (0.24)	−0.31 (0.41)
	IL-6	0.86 (<0.05)*	−0.63 (<0.05)*	0.60 (0.07)
	TNF- α	−0.37 (0.29)	0.60 (0.07)	0.03 (0.94)
	IL-1 β	0.77 (<0.05)*	−0.76 (<0.05)*	0.78 (<0.05)*

* Statistical significance at *p* < 0.05 when compared with PBS.

and M2 levels were lower in the OVA-challenged mice than they were in PBS-challenged mice (Figs. 4 and 5). Inflammatory mediator levels were also higher in the OVA-challenged mice (Fig. 2). There were also significant associations between inflammation and anti-inflammation levels in both blood and adipose tissues (Table 4). These results imply OVA allergen exposure can cause adipose tissue inflammation and also change the levels of adipokines in adipose tissues and blood.

In this study, we used citosol to sacrifice the mice. However, a previous study found citosol injection negatively affects

cardiovascular function [54]. In this study, we injected citosol in both OVA and PBS-challenged mice, and found that the levels of inflammatory indicators and cardiovascular injury were significantly differed in both groups. Moreover, citosol injection was performed on the left side of the mice, and the biomarkers of adipose tissue were analyzed on the right side of the mice to refrain prevent other interference. Up to now, no study has demonstrated citosol injection can affect the characteristics of adipose tissues. Therefore, this study was able to exclude the effects of citosol injection on adipose tissues. Additionally, the levels of

inflammatory markers in adipose tissues were associated with the levels of inflammatory markers in serum. The level of biomarkers with cardiovascular injury was also associated with the levels of inflammation and adipokines in serum. It means that the changes of levels of inflammatory mediators were affected by adipose tissues, and further involve the pathogenesis of cardiovascular injury. The results showed that allergen exposure is an important risk factor for cardiovascular injury, and citosol injection had lesser impact in this study.

5. Conclusions

This is the first study to demonstrate a correlation between allergen-induced adipose tissue inflammation, inflammation of the circulatory system and cardiovascular injury, leading to our conclusion that allergen exposure might be one risk factor for cardiovascular injury and inflammation of the circulatory system. Based on this study, the same analysis on human subjects would be highly desirable. Moreover, there needs to be an increase in knowledge about the biological mechanisms associated with allergen exposure, adipose tissue inflammation, and cardiovascular injury in order to develop therapeutic approaches.

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References

- [1] V.L. Roger, A.S. Go, D.M. Lloyd-Jones, E.J. Benjamin, J.D. Berry, W.B. Borden, D. M. Bravata, S. Dai, E.S. Ford, C.S. Fox, H.J. Fullerton, C. Gillespie, S.M. Hailpern, J. A. Heit, V.J. Howard, B.M. Kissela, S.J. Kittner, D.T. Lackland, J.H. Lichtman, L.D. Lisabeth, D.M. Makuc, G.M. Marcus, A. Marelli, D.B. Matchar, C.S. Moy, D. Mozaffarian, M.E. Mussolino, G. Nichol, N.P. Paynter, E.Z. Soliman, P.D. Sorlie, N. Sotoodehnia, T.N. Turan, S.S. Virani, N.D. Wong, D. Woo, M.B. Turner, A.H.A. S. Comm, S.S. Subcomm, Heart disease and stroke statistics-2012 update a report from the American heart association, *Circulation* 125 (2012) E2–E220.
- [2] Minister of Health and Welfare, Leading Causes of Death in Taiwan, Minister of Health and Welfare, Taipei, 2013.
- [3] M.L. Bell, K. Ebisu, R.D. Peng, J.M. Samet, F. Dominici, Hospital admissions and chemical composition of fine particle air pollution, *Am. J. Resp. Crit. Care* 179 (2009) 1115–1120.
- [4] Y.F. Bi, T.G. Wang, M. Xu, Y. Xu, M.A. Li, J.L. Lu, X.L. Zhu, G. Ning, Advanced research on risk factors of type 2 diabetes, *Diabetes-Metab. Res.* 28 (2012) 32–39.
- [5] T.L.M. Hectors, C. Vanparys, K. van der Ven, G.A. Martens, P.G. Jorens, L.F. Van Gaal, A. Covaci, W. De Coen, R. Blust, Environmental pollutants and type 2 diabetes: a review of mechanisms that can disrupt beta cell function, *Diabetologia* 54 (2011) 1273–1290.
- [6] F.B. Hu, Globalization of diabetes the role of diet, lifestyle, and genes, *Diabetes Care* 34 (2011) 1249–1257.
- [7] A. Navas-Acien, E.K. Silbergeld, R.A. Streeter, J.M. Clark, T.A. Burke, E. Guallar, Arsenic exposure and type 2 diabetes: a systematic review of the experimental and epidemiologic evidence, *Environ. Health Perspect.* 114 (2006) 641–648.
- [8] J.F. Thayer, S.S. Yamamoto, J.F. Brosschot, The relationship of autonomic imbalance, heart rate variability and cardiovascular disease risk factors, *Int. J. Cardiol.* 141 (2010) 122–131.
- [9] A. Zanobetti, M. Franklin, P. Koutrakis, J. Schwartz, Fine particulate air pollution and its components in association with cause-specific emergency admissions, *Environ. Health-Global* 8 (2009).
- [10] P. Libby, Inflammation in atherosclerosis, *Nature* 420 (2002) 868–874.
- [11] P. Libby, Inflammation and cardiovascular disease mechanisms, *Am. J. Clin. Nutr.* 83 (2006) 456s–460s.
- [12] T.B. Harris, L. Ferrucci, R.P. Tracy, M.C. Corti, S. Wacholder, W.H. Ettinger, H. Heimovitz, H.J. Cohen, R. Wallace, Associations of elevated interleukin-6 and C-reactive protein levels with mortality in the elderly, *Am. J. Med.* 106 (1999) 506–512.
- [13] P.M. Ridker, C.H. Hennekens, J.E. Buring, N. Rifai, C-reactive protein and other markers of inflammation in the prediction of cardiovascular disease in women, *N. Engl. J. Med.* 342 (2000) 836–843.
- [14] R.B.S. Harris, T.D. Mitchell, E.W. Kelso, W.P. Flatt, Changes in environmental temperature influence leptin responsiveness in low- and high-fat-fed mice, *Am. J. Physiol.-Regul. I* (293) (2007) R106–R115.
- [15] D. Ikeoka, J.K. Mader, T.R. Pieber, Adipose tissue, inflammation and cardiovascular disease, *Rev. Assoc. Med. Bras.* 56 (2010) 116–121.
- [16] M.E.F. Vazquez-Vela, N. Torres, A.R. Tovar, White adipose tissue as endocrine organ and its role in obesity, *Arch. Med. Res.* 39 (2008) 715–728.
- [17] E. Lubos, C.M. Messow, R. Schnabel, H.J. Rupperecht, C. Espinola-Klein, C. Bickel, D. Peetz, F. Post, K.J. Lackner, L. Tired, T. Munzel, S. Blankenberg, Resistin, acute coronary syndrome and prognosis results from the AtheroGene study, *Atherosclerosis* 193 (2007) 121–128.
- [18] C. Menzaghi, S. Bacci, L. Salvemini, C. Mendonca, G. Palladino, A. Fontana, C. De Bonis, A. Marucci, E. Goheen, S. Prudente, E. Morini, S. Rizza, A. Kanagaki, G. Fini, D. Mangiacotti, M. Federici, S. De Cosmo, F. Pellegrini, A. Doria, V. Trischitta, Serum resistin, cardiovascular disease and all-cause mortality in patients with type 2 diabetes, *PLoS One* 8 (2013).
- [19] H. Osawa, Y. Doi, H. Makino, T. Ninomiya, K. Yonemoto, R. Kawamura, J. Hata, Y. Tanizaki, M. Iida, Y. Kiyohara, Diabetes and hypertension markedly increased the risk of ischemic stroke associated with high serum resistin concentration in a general Japanese population: the Hisayama Study, *Cardiovasc. Diabetol.* 8 (2009).
- [20] S. Pilz, G. Weihrauch, U. Seelhorst, B. Wellnitz, B.R. Winkelmann, B.O. Boehm, W. Marz, Implications of resistin plasma levels in subjects undergoing coronary angiography – the Ludwigshafen Risk and Cardiovascular Health (LURIC) study, *Clin. Endocrinol.* 66 (2007) 380–386.
- [21] C. Weikert, S. Westphal, K. Berger, J. Dierkes, M. Mohlig, J. Spranger, E.B. Rimm, S.N. Willich, H. Boeing, T. Pischon, Plasma resistin levels and risk of myocardial infarction and ischemic stroke, *J. Clin. Endocrinol. Metab.* 93 (2008) 2647–2653.
- [22] M.H. Zhang, B. Na, N. Schiller, M.A. Whooley, Resistin, exercise capacity, and inducible ischemia in patients with stable coronary heart disease: data from the heart and soul study, *Atherosclerosis* 213 (2010) 604–610.
- [23] W. Zhu, K.K.Y. Cheng, P.M. Vanhoutte, K.S.L. Lam, A. Xu, Vascular effects of adiponectin: molecular mechanisms and potential therapeutic intervention, *Clin. Sci.* 114 (2008) 361–374.
- [24] S.S. Huang, P.H. Huang, Y.H. Chen, K.H. Chiang, J.W. Chen, S.J. Lin, Association of adiponectin with future cardiovascular events in patients after acute myocardial infarction, *J. Atheroscler. Thromb.* 17 (2010) 295–303.
- [25] Y. Nakamura, K. Shimada, D. Fukuda, Y. Shimada, S. Ehara, M. Hirose, T. Kataoka, K. Kamimori, S. Shimodozono, Y. Kobayashi, M. Yoshiyama, K. Takeuchi, J. Yoshikawa, Implications of plasma concentrations of adiponectin in patients with coronary artery disease, *Heart* 90 (2004) 528–533.
- [26] T. Pischon, C.J. Girman, G.S. Hotamisliligil, N. Rifai, F.B. Hu, E.B. Rimm, Plasma adiponectin levels and risk of myocardial infarction in men, *JAMA* 291 (2004) 1730–1737.
- [27] F.Y.L. Li, K.K.Y. Cheng, K.S.L. Lam, P.M. Vanhoutte, A. Xu, Cross-talk between adipose tissue and vasculature: role of adiponectin, *Acta Physiol.* 203 (2011) 167–180.
- [28] Y. Okamoto, S. Kihara, N. Ouchi, M. Nishida, Y. Arita, M. Kumada, K. Ohashi, N. Sakai, I. Shimomura, H. Kobayashi, N. Terasaka, T. Inaba, T. Funahashi, Y. Matsuzawa, Adiponectin reduces atherosclerosis in apolipoprotein E-deficient mice, *Circulation* 106 (2002) 2767–2770.
- [29] C.C. Jung, C.C. Chang, Y.S. Tsai, H.J. Su, Allergen exposure induces inflammation and affects adiponectin levels in adipose tissue, *Toxicol. Lett.* (2013).
- [30] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(–Delta Delta C) method, *Methods* 25 (2001) 402–408.
- [31] M. Gueders, G. Paulissen, C. Crahay, F. Quesada-Calvo, J. Hacha, C. Van Hove, K. Tournoy, R. Louis, J.-M. Foidart, A. Noël, D. Cataldo, Mouse models of asthma: a comparison between C57BL/6 and BALB/c strains regarding bronchial responsiveness, inflammation, and cytokine production, *Inflamm. Res.* 58 (2009) 845–854.
- [32] B.D. Medoff, Y. Okamoto, P. Leyton, M. Weng, B.P. Sandall, M.J. Rahe, S. Kihara, K.D. Bloch, P. Libby, A.D. Luster, Adiponectin deficiency increases allergic airway inflammation and pulmonary vascular remodeling, *Am. J. Respir. Cell Mol. Biol.* 41 (2009) 397–406.
- [33] S.A. Shore, I.N. Schwartzman, M.S. Mellema, L. Flynt, A. Imrich, R.A. Johnston, Effect of leptin on allergic airway responses in mice, *J. Allergy Clin. Immunol.* 115 (2005) 103–109.
- [34] S.A. Shore, R.D. Terry, L. Flynt, A. Xu, C. Hug, Adiponectin attenuates allergen-induced airway inflammation and hyperresponsiveness in mice, *J. Allergy Clin. Immunol.* 118 (2006) 389–395.
- [35] K.J. Baines, J.L. Simpson, N.A. Bowden, R.J. Scott, P.G. Gibson, Differential gene expression and cytokine production from neutrophils in asthma phenotypes, *Eur. Respir. J.* 35 (2010) 522–531.
- [36] R. Djukanovic, M. Uddin, G. Nong, J. Ward, G. Seumois, L.R. Prince, S.J. Wilson, V. Cornelius, G. Dent, Prosurvival activity for airway neutrophils in severe asthma, *Thorax* 65 (2010) 684–689.
- [37] R.B. Clampitt, R.J. Hart, The tissue activities of some diagnostic enzymes in ten mammalian species, *J. Comp. Pathol.* 88 (1978) 607–621.
- [38] B. Jw, The mechanisms relating to increases in plasma enzymes and isoenzymes in diseases of animals, *Vet. Clin. Pathol.* 12 (1983) 9–24.
- [39] P.J. O'Brien, Cardiac troponin is the most effective translational safety biomarker for myocardial injury in cardiotoxicity, *Toxicology* 245 (2008) 206–218.
- [40] D.B. Walker, Serum chemical biomarkers of cardiac injury for nonclinical safety testing, *Toxicol. Pathol.* 34 (2006) 94–104.
- [41] A.H. Berg, P.E. Scherer, Adipose tissue, inflammation, and cardiovascular disease, *Circ. Res.* 96 (2005) 939–949.
- [42] G.K. Hansson, Mechanisms of disease – inflammation, atherosclerosis, and coronary artery disease, *N. Engl. J. Med.* 352 (2005) 1685–1695.

- [43] D. Kawanami, K. Maemura, N. Takeda, T. Harada, T. Nojiri, Y. Imai, I. Manabe, K. Utsunomiya, R. Nagai, Direct reciprocal effects of resistin and adiponectin on vascular endothelial cells: a new insight into adipocytokine-endothelial cell interactions, *Biochem. Biophys. Res. Commun.* 314 (2004) 415–419.
- [44] D.D. McManus, A. Lyass, E. Ingelsson, J.M. Massaro, J.B. Meigs, J. Aragam, E.J. Benjamin, R.S. Vasan, Relations of circulating resistin and adiponectin and cardiac structure and function: the Framingham offspring study, *Obesity* 20 (2012) 1882–1886.
- [45] N. Ouchi, J.L. Parker, J.J. Lugus, K. Walsh, Adipokines in inflammation and metabolic disease, *Nat. Rev. Immunol.* 11 (2011) 85–97.
- [46] D. Rothenbacher, H. Brenner, W. Marz, W. Koenig, Adiponectin, risk of coronary heart disease and correlations with cardiovascular risk markers, *Eur. Heart J.* 26 (2005) 1640–1646.
- [47] Y.J. Wang, W.B. Lau, E.H. Gao, L. Tao, Y.X. Yuan, R. Li, X.L. Wang, W.J. Koch, X.L. Ma, Cardiomyocyte-derived adiponectin is biologically active in protecting against myocardial ischemia-reperfusion injury, *Am. J. Physiol. Endocrinol. Metab.* 298 (2010) E663–E670.
- [48] E.J.B. de Chantemele, J.D. Mintz, W.E. Rainey, D.W. Stepp, Impact of leptin-mediated sympatho-activation on cardiovascular function in obese mice, *Hypertension* 58 (2011) 271–U285.
- [49] P. Palatini, L. Thijs, J.A. Staessen, R.H. Fagard, C.J. Bulpitt, D.L. Clement, P.W. de Leeuw, M. Jaaskivi, G. Leonetti, C. Nachev, E.T. O'Brien, G. Parati, J.L. Rodicio, E. Roman, C. Sarti, J. Tuomilehto, S.H. Europe, Predictive value of clinic and ambulatory heart rate for mortality in elderly subjects with systolic hypertension, *Arch. Intern. Med.* 162 (2002) 2313–2321.
- [50] R. Kolloch, U.F. Legler, A. Champion, R.M. Cooper-DeHoff, E. Handberg, Q. Zhou, C.J. Pepine, Impact of resting heart rate on outcomes in hypertensive patients with coronary artery disease: findings from the International VErapamil-SR/trandolapril Study (INVEST), *Eur. Heart J.* 29 (2008) 1327–1334.
- [51] C.N. Lumeng, J.L. Bodzin, A.R. Saltiel, Obesity induces a phenotypic switch in adipose tissue macrophage polarization, *J. Clin. Invest.* 117 (2007) 175–184.
- [52] K. Rabe, M. Lehrke, K.G. Parhofer, U.C. Broedl, Adipokines and insulin resistance, *Mol. Med.* 14 (2008) 741–751.
- [53] L.P.A. Dourado, M.D.M. Noviello, D.M. Alvarenga, Z. Menezes, D.A. Perez, N.V. Batista, G.B. Menezes, A.V.M. Ferreira, D.D. de Souza, D.C. Cara, Experimental food allergy leads to adipose tissue inflammation, systemic metabolic alterations and weight loss in mice, *Cell. Immunol.* 270 (2011) 198–206.
- [54] D. Turner, J. Ilkiw, Cardiovascular and respiratory effects of three rapidly acting barbiturates in dogs, *Am. J. Vet. Res.* 51 (1990) 598–604.