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NLRP3 inflammasome mediate palmitate-induced endothelial dysfunction

Jun-hui Xing[a,**,1], Rui Li[b,1], Yue-qiao Gao[c], Meng-yu Wang[a], Yu-zhou Liu[a], Jin Hong[a], Jian-zeng Dong[a], He-ping Gu[a], Ling Li[a,*]

[a] Department of Cardiology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan, China
[b] Department of Health Care, China-Japan Friendship Hospital, Ministry of Health, Beijing, China
[c] Department of Endocrinology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan, China

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ABSTRACT

Aims: Free fatty acids (FFA) is a key contributor to insulin resistance and endothelial dysfunction. However, the precise mechanism underlying the role of FFA remains elusive. This study aimed to investigate the role of NLRP3 (NOD-like receptor pyrin domain containing-3) inflammasome in FFA induced endothelial dysfunction.

Main methods: HUVECs were transfected with NLRP3 siRNA and then stimulated with LPS and palmitate. C57 BL/6 J mice transfected with NLRP3 Lenti-Virus were fed with a high-fat diet (HFD). The levels of NLRP3 inflammasome, AMPKα (AMP-activated protein kinase), endothelial nitric oxide synthase (eNOS) and the activity of the insulin signal pathway, in endothelial cells were determined via Western blotting. Endothelial function was determined by measuring the level of endothelium-dependent vasodilatation.

Key findings: FFA could activate NLRP3 inflammasome and induce IL-1β release both *in vitro*. and *in vivo*. Using siRNA and Lenti-Virus to inhibit NLRP3 abolished palmitate-induced IL-1β release and restored impaired phosphorylation of IRS-1 (Tyr), Akt (Ser473) and eNOS (Ser1177) and ACh-mediated endothelium-dependent vasorelaxation induced by palmitate. AMPKα activator AICAR(5-aminoimidazole-4-carbox-amide-1-β-d-ribofuranoside) inhibited NLRP3 inflammasome activation and decreased IL-1β release and restored impaired insulin signal pathway induced by palmitate.

Significance: NLRP3 inflammasome activation via AMPKα inactivation mediated palmitate-induced endothelial dysfunction through involves IL-1β-induced insulin signal pathway.

1. Introduction

Endothelial dysfunction characterized by a reduction of endothelium-dependent vasodilation is associated with a number of disorders, such as obesity, diabetes, atherosclerosis and hypertension [1,2]. Usually, endothelial dysfunction and insulin resistance are present simultaneously and can affect each other. Several pathogenic factors including hyperglycemia, hyperlipemia, inflammation factors and oxidative stress have been identified as risk factors of endothelial dysfunction [3,4]. Among them, Increased plasma FFA concentrations cause endothelial dysfunction and insulin resistance. FFA-induced endothelial dysfunction is mediated by endoplasmic reticulum (ER) stress, reactive oxygen species (ROS) production, activated oxidative stress, apoptosis, insulin resistance and Inflammatory pathways. FFA has been proposed to promote inflammatory responses by directly engaging toll-like receptors (TLRs) and inducing NF-κB–dependent production of inflammatory cytokines such as tumor necrosis factor (TNF) and IL-6. Elevated plasma FFA concentration has been proven to damage the insulin signaling pathways (IRS-PI3K-Akt) and thus result in the reduction of endothelial nitric oxide synthase (eNOS) activity and nitric oxide (NO) production in endothelial cells [5–7] by inducing proinflammatory signaling pathways and oxidative stress. However, the role of inflammatory mediators in FFA-induced endothelial dysfunction are not completely understood.

The NLRP3 inflammasome, which is the best characterized and most extensively studied inflammasome, contains NLRP3, the apoptosis associated speck like protein (ASC) and the serine protease caspase-1 [8–10]. It could be activated by several pathogenic factors such as hyperglycemia, hyperlipemia, inflammation factors and oxidative stress. Activated NLRP3 oligomerises with the ASC is responsible for the activation of caspase-1 and for the processing of pro-IL-1β into its mature active form and its subsequent release [9–12], thus induce the

* Corresponding author. Department of Cardiology, The First Affiliated Hospital of Zhengzhou University, No.1 Jianshe East Road, Zhengzhou, 450052, China.
** Corresponding author. Department of Cardiology, The First Affiliated Hospital of Zhengzhou University, No.1 Jianshe East Road, Zhengzhou, 450052, China.
E-mail addresses: xjunhui404@163.com (J.-h. Xing), liling63035@sina.com (L. Li).
[1] Junhui-Xing and Rui Li contributed equally to this work.

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inflammation reaction. IL-1β is a potent proinflammatory cytokine that is related to insulin resistance and has a key proatherogenic effect in vascular diseases [13,14]. It had been reported that NLRP3 inflammasome plays an important role in insulin resistance, obesity, diabetes [15], metabolic diseases [16] and atherosclerosis [17,18].

Recently, palmitate, the most abundant saturated FFA, has been implicated as a secondary signal in the activation of the NLRP3 inflammasome in the obesity model [19–21]. However, the relationship between NLRP3 inflammasome and palmitate-induced endothelial dysfunction has yet to be explored. In this study, the potent role of NLRP3 inflammasome in palmitate-induced endothelial dysfunction and its possible mechanism were investigated.

2. Materials and methods

2.1. Materials

Antibodies specific to NLRP3, ASC, cleaved-caspase-1 (10KD), t-IRS-1, p-IRS-1 (Tyr), t-Akt, p-Akt (Ser473), t-eNOS, p-eNOS (Ser1177), AMPKa, p-AMPKa (Thr172) and β-actin were obtained from Cell Signaling Technology (CST) (Danvers, MA); Anti-Caspase-1 (45KD) and anti-IL-1β (17KD) were bought from Santa Cruz Biotechnology (Santa Cruz, CA); Protease inhibitor cocktail was from Sigma (St Louis, MO); Lipopolysaccharides (LPS), Palmitate (PA), fatty acid-free BSA and AICAR were obtained from Sigma (St. Louis, MO); SiRNA against human NLRP3 and a control siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); Others will be described below.

2.2. Preparation of PA-BSA complex

Palmitate was added to the culture medium as PA-BSA complex. Briefly, sodium palmitate was dissolved in NaOH solution (0.1 M) at 70 °C to yield a stock concentration of 100 mM and kept at −20 °C. Palmitate was then conjugated with 10% fatty acid–free BSA at a 1:1 M ratio to make the PA-BSA complex (50 mM). The PA solution was filter-sterilized and stored at .20 °C. In this study, PA was added to the culture medium at final concentrations of 50 μM, 100 μM, 200 μM.

2.3. Cell culture and treatment

HUVECs were purchased from the American Type Cell Collection (ATCC, Manassas, VA). As previously reported [22], They were cultured in endothelial cell medium (ECM; Sciencell, USA) containing 10% fetal bovine serum (FBS) and penicillin (100u/ml)/streptomycin (100μg/ml) in a humidified atmosphere of 5% CO_2 and 95% O_2 at 37 °C. 80% confluence of HUVECs (3–5 passages) were used for the experiments. Cells were treated with the following reagents: LPS (100 ng/ml) was pretreated for 4 h before palmitate (50 μM, 100 μM, 200 μM) treatment for another 24 h, then Insulin (100 nM) was stimulated for another 10 min. The Cells were collected for other experiments.

2.4. Animals

The animal protocols were reviewed and approved by the Animal Care and Use Committee of Zhengzhou University, in accordance with AAALAC guidelines. The regular and high-fat diet (HFD) (60% fat calories) were purchased from TestDiet. Male C57BL/6 J mice(8 weeks of age, 20–30 g) were used for the experiments. All Mice were housed in a constant temperature (22 °C) with a 12/12 h light/dark cycles and had free access to water.

The mice were divided into 4 groups (n = 10): Vehicle group (Lenti-GFP with regular diet), HFD group (Lenti-GFP with HFD), Lenti-NLRP3 group (Lenti-NLRP3 with regular diet) and Lenti-NLRP3 + HFD group (Lenti-NLRP3 with HFD). We use Lenti-NLRP3 (NLRP3 Lenti-Virus) to knockdown NLRP3. After 10 weeks of diet, the mice received a jugular-vein injection of a total Lenti-Virus(GFP and NLRP3) dose of 1×10^7 PFU/mouse for one time. Sequences for mice NLRP3 Lenti-virus were: sense, 5′- GGTGAAATGTACTTAAATC-3'; antisense, 5′-GATTTAAGTACATTTCACC-3'. The lenti-virus were obtained from GenePharma (Shanghai, China). After another 2 weeks of transfection, all mice were sacrificed.

2.5. Small Interfering RNA (siRNA)

HUVECs were transfected with the NLRP3 siRNA or Control siRNA duplexes purchased from Santa Cruz Biotechnology (Santa Cruz, CA) using the transfection reagent and medium (Santa Cruz, CA) according to the manufacturer's instructions as described previously [23]. The expression of target proteins were examined by Western Blot analysis.

2.6. Western blot

Western blotting and band densitometry were performed as previously reported [22,23]. Tissues and cultured cells were homogenized on ice in 1 × lysis buffer. The protein expression was assayed with the same quantity of protein samples using the BCA method. Proteins were separated by 10% or 15% SDS-PAGE gels and transferred to nitrocellulose membranes. After blocking for 2 h in 5% non-fat milk the membranes were incubated with primary antibodies overnight at 4 °C. On the second day, the membranes were incubated with rabbit anti-goat (1:5000) or goat anti-rabbit IgG (1:5000) for 2 h at room temperature. The bands were identified by a standard enhanced chemiluminescence method.

2.7. Enzyme-linked immunosorbent assay (ELISA)

Cell culture supernatants and tissues lysates were assayed for human and mouse IL-1β according to manufacturer's instructions (Jiancheng, Nanjing, China).

2.8. Assessment of endothelial function

The isolated thoracic aortas were placed in Krebs-Henseleit bicarbonate buffer (K–H solution) to measure endothelial function. The aortas were cut into 3-mm ring segments and placed in an organ bath chamber containing 10 ml K–H solution that was bubbled with 5% CO2/95% O2 gas mixture and maintained at 37 °C. The aortic rings were stretched to 1.0 g tension and allowed to equilibrate for 60 min. Then the rings were precontracted with 1 μmol/L norepinephrine (NE). Endothelium-dependent vessel relaxation was determined as the response to acetylcholine (ACh, 10^{-9} to 10^{-4} mol/L). Endothelium-independent vessel relaxation was measured as the response to sodium nitroprusside (SNP, 10^{-9} to 10^{-4} mol/L). NE, Ach and SNP were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.9. Statistical analysis

All Values were presented as mean ± SEM, and all the data were analysed using SPSS 19.0 software. Data were analysed by one-way ANOVA followed by Tukey's post hoc test. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Palmitate activated NLRP3 inflammasome in HUVECs

It was reported that palmitate could be the second signal for the activation of NLRP3 inflammasome in the bone marrow-derived macrophages (BMMs) [20]. To confirm whether palmitate could activate NLRP3 inflammasome in endothelial cells, we pretreated HUVECs with LPS for 4 h followed by different concentrations of PA treatment for another 24 h. As expected, LPS or palmitate did not affect NLRP3, ASC

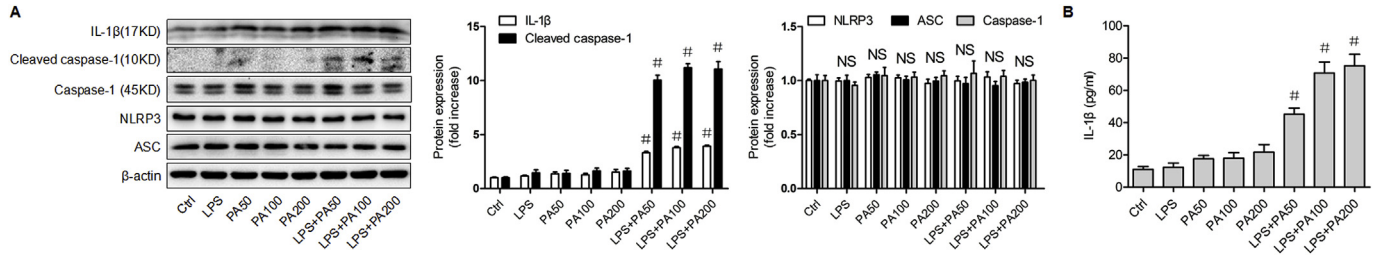

Fig. 1. Palmitate activate NLRP3 inflammasome in HUVECs. HUVECs were pretreated with LPS (100 ng/ml) for 4 h followed by PA (50 μM, 100 μM, 200 μM) treatment for another 24 h as indicated; (A) Representative blots and relative expression of IL-1β, Cleaved caspase-1, NLRP3, ASC and caspase-1 protein levels; (B) ELISA for IL-1βlevels in supernatants. The shown Western blots were representative of at least 3 independent experiments with similar results. * represent $p < 0.05$ vs control ($n = 3$), # represent $p < 0.05$ vs PA ($n = 3$), otherwise, not significant (NS). Ctrl, control.

or caspase-1 protein expression. Palmitate treatment alone did not increase cleaved caspase-1 the mature form of caspase-1 or IL-1β protein expression in HUVECs, but the two combined did. With LPS, palmitate could incease cleaved caspase-1 expression and thus induce IL-1β secretion in HUVECs in concentration-dependent fashion, supporting the two-signal hypothesis of inflammasome activation (Fig. 1).

3.2. Palmitate inhibited insulin mediated IRS-1, Akt and eNOS phosphorylation in HUVECs

In order to determine the role of palmitate in impaired insulin signal transduction in vascular endothelium, we evaluated the phosphorylation of IRS-1 (Tyr), Akt (Ser473) and eNOS (Ser1177). As shown in Fig. 2A, palmitate alone inhibited phosphorylation of IRS-1 (Tyr), Akt (Ser473) and eNOS (Ser1177) in HUVECs, which was consistent with previous studies [7]. With LPS, palmitate inhibit phosphorylation of IRS-1 (Tyr), Akt (Ser473) and eNOS (Ser1177) in concentration-dependent manner in HUVECs compared with the palmitate treatment group. While protein expressions of IRS-1, Akt and eNOS were not changed in either LPS or palmitate group.

As an important inflammatory cytokine, IL-1β was reported to induce insulin resistance in obesity. We also detected the effects of IL-1β on the insulin signal pathway. Surprisingly, IL-1β did inhibit phosphorylation of IRS-1 (Tyr), Akt (Ser473) and eNOS (Ser1177) in concentration-dependent manner in HUVECs which was consistent with the palmitate experiment (Fig. 2B).

Since IL-1β was released by NLRP3 inflammasome activation which was induced by palmitate, these results indicated that NLRP3 inflammasome activation could inhibit IRS-1, Akt and eNOS phosphorylation in HUVECs.

3.3. NLRP3 protein inhibition restored palmitate induced depression of IRS-1, Akt and eNOS phosphorylation in HUVECs

To confirm the role of NLRP3 inflammasome in endothelial dysfunction, we performed NLRP3 siRNA knockdown experiments in HUVEC. Indeed, inhibition of NLRP3 decreased cleaved caspase-1 protein expression and also abolished palmitate-induced IL-1β release in HUVECs (Fig. 3). Also, we found that inhibition of NLRP3 protein expression restored the insulin mediated IRS-1, Akt and eNOS phosphorylation depression in palmitate plus LPS treated HUVECs, without changing IRS-1, Akt and eNOS protein expressions (Fig. 4).

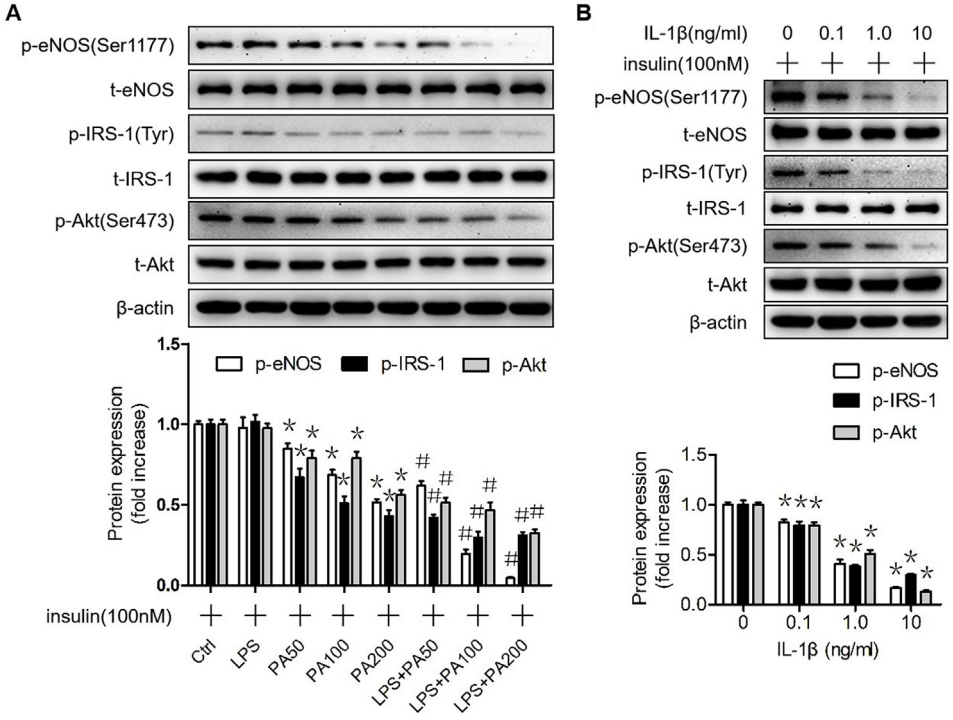

Fig. 2. Palmitate inhibit insulin mediated IRS-1, Akt and eNOS phosphorylation in HUVECs. (A) HUVECs were pretreated with LPS (100 ng/ml) for 4 h, and then exposed with PA (50 μM, 100 μM, 200 μM) for 24 h, followed by insulin (100 nM) stimulation for another 10 min. Representative blots and relative expression of p-IRS-1(Thr), p-Akt (Ser473) and p-eNOS (Ser1177) protein levels; (B) HUVECs were treated with IL-1β (0.1, 1, 10 ng/ml) for 24 h followed by insulin (100 nM) stimulation for 10 min, Representative blots and relative expression of p-IRS-1(Thr), p-Akt (Ser473) and p-eNOS (Ser1177) protein levels. The shown Western blots were representative of at least 3 independent experiments with similar results. * represent $p < 0.05$ vs control ($n = 3$), # represent $p < 0.05$ vs PA ($n = 3$), otherwise, not significant. Ctrl, control.

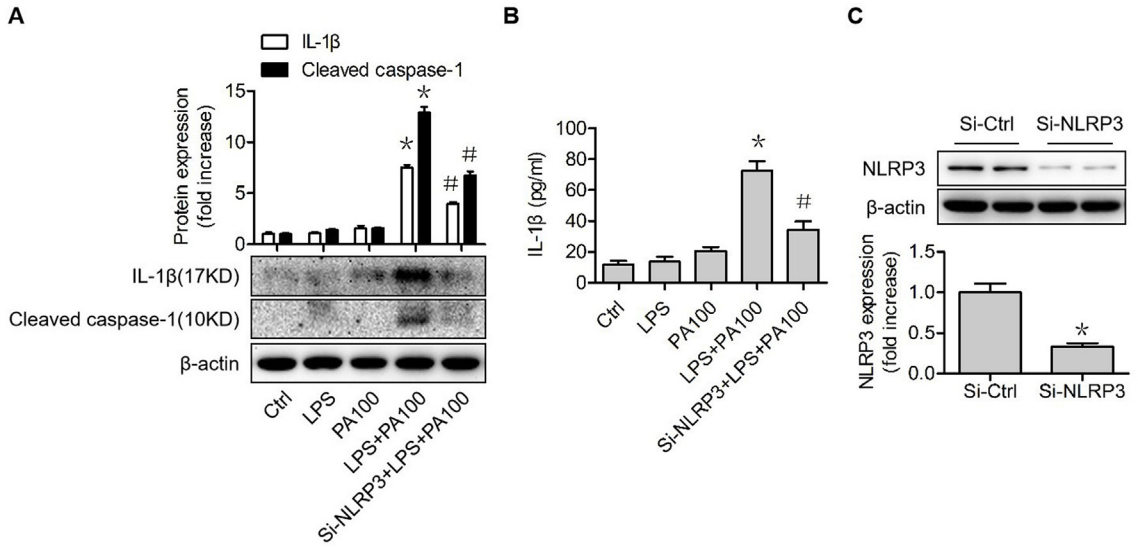

Fig. 3. NLRP3 inflammasome inhibition downregulate IL-1β expression induced by Palmitate in HUVECs. HUVECs were transfected with Control or NLRP3 SiRNA for 24 h, and then pretreated with LPS (100 ng/ml) for 4 h followed by PA (100 μM) treatment for another 24 h. (A) Representative blots and relative expression of IL-1β, Cleaved caspase-1 protein levels; (B) ELISA for IL-1β levels in supernatants; (C) Representative blots and relative expression of NLRP3 protein levels; .The shown Western blots were representative of at least 3 independent experiments with similar results. * represent $p < 0.05$ vs PA100 (n = 3), # represent $p < 0.05$ vs LPS + PA 100 (n = 3), otherwise, not significant. Ctrl, control; Si-Ctrl, Control SiRNA; Si-NLRP3, NLRP3 SiRNA.

3.4. AMPKα was required for palmitate-induced NLRP3 inflammasome activation in HUVECs

The mechanisms of palmitate-induced activation of NLRP3 inflammasome were further explored in this study. Palmitate alone decreased AMPKα (Thr172) phosphorylation in HUVECs which was consistent with previous studies. Palmitate plus LPS treatment could inhibit phosphorylation of AMPKα (Thr172) in concentration-dependent manner in HUVECs compared with the palmitate treatment group. While protein expression of AMPKα was not changed in either LPS or palmitate group (Fig. 5A). To determine whether AMPKα could regulate the activation of NLRP3 inflammasome, we performed experiments with AMPK agonist AICAR. Notably, AICAR, which could enhance the phosphorylation of AMPKα, abolished cleaved caspase-1 and IL-1β expression and restored IRS-1 (Tyr), Akt (Ser473) and eNOS (Ser1177) phosphorylation induced by palmitate plus LPS (Fig. 5B).

3.5. HFD activated NLRP3 inflammasome in mouse aorta

To further confirm the role of NLRP3 inflammasome in endothelial dysfunction, mice were infected with the NLRP3 Lenti-Virus. It was found that HFD increased cleaved caspase-1 and IL-1β protein expression without changing the protein levels of NLRP3, ASC and caspase-1 in mouse aorta. NLRP3 Lenti-Virus abolished HFD-induced cleaved caspase-1 and IL-1β release which was consistent with the cell experiments (Fig. 6).

3.6. NLRP3 inhibition restored the decreased insulin mediated IRS-1, Akt and eNOS phosphorylation in mouse aorta

In addition, we assayed the effects of NLRP3 on the insulin signal pathway in mouse aorta. Similar to the effects of palmitate on HUVECs, HFD inhibit phosphorylation of IRS-1 (Tyr), Akt (Ser473) and eNOS (Ser1177). NLRP3 Lenti-Virus restored the phosphorylation of IRS-1, Akt and eNOS decreased by HFD (Fig. 7).

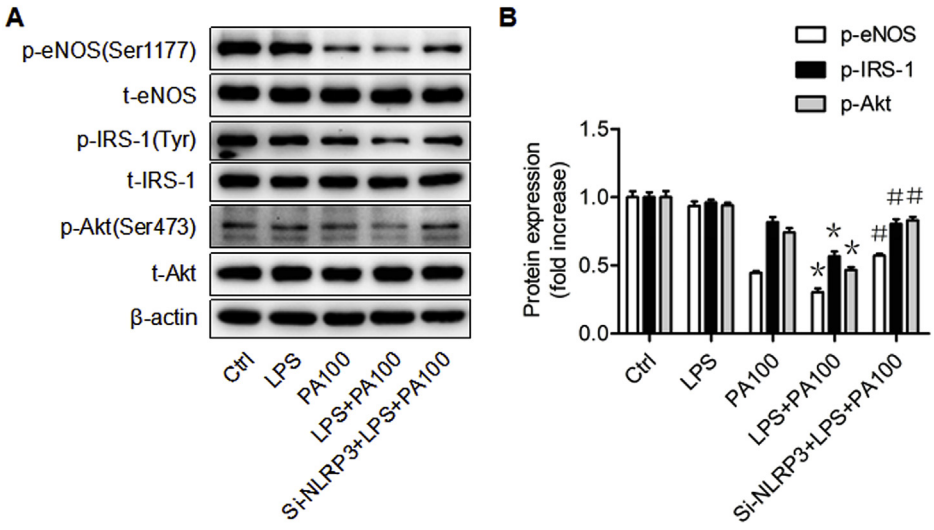

Fig. 4. NLRP3 protein inhibition restored insulin mediated IRS-1, Akt and eNOS phosphorylation depression in palmitate treated HUVECs. HUVECs were transfected with Control or NLRP3 SiRNA for 24 h, and then pretreated with LPS (100 ng/ml) for 4 h followed by PA (100 μM) treatment for 24 h, then insulin (100 nM) was stimulated for another 10 min. (A) Western blots for p-IRS-1(Thr), p-Akt (Ser473) and p-eNOS (Ser1177) protein levels; (B) Relative expression of p-IRS-1(Thr), p-Akt (Ser473) and p-eNOS (Ser1177) protein levels. The shown Western blots were representative of at least 3 independent experiments with similar results. * represent $p < 0.05$ vs PA100 (n = 3), # represent $p < 0.05$ vs LPS + PA 100 (n = 3), otherwise, not significant. Ctrl, control; Si-NLRP3, NLRP3 SiRNA.

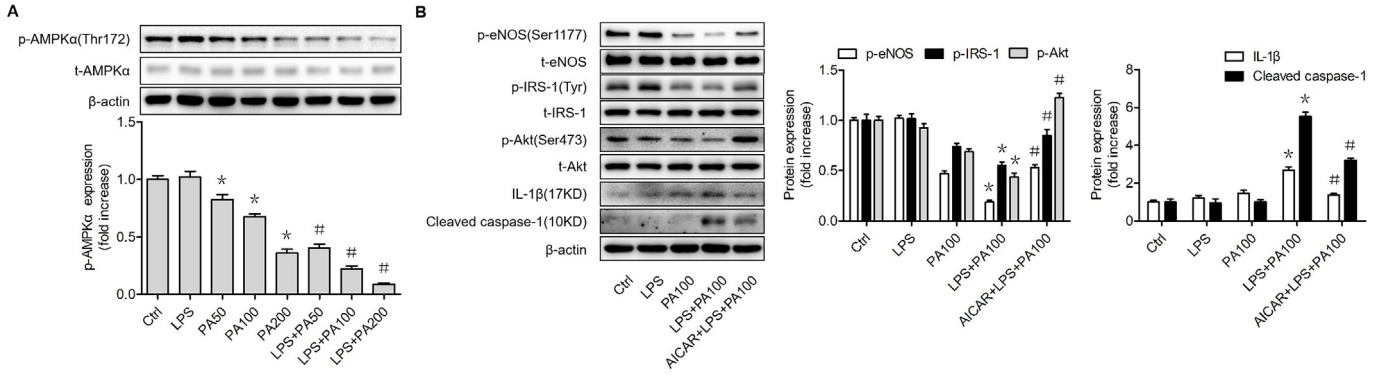

Fig. 5. AMPKα is required for palmitate-induced NLRP3 inflammasome activation in HUVECs. (A) HUVECs were pretreated with LPS (100 ng/ml) for 4 h, followed by PA (50 μM, 100 μM, 200 μM) treatment for another 24 h. Representative blots and relative expression of p-AMPKα (Thr172) protein levels. * represent $p < 0.05$ vs control (n = 3), # represent $p < 0.05$ vs PA (n = 3), otherwise, not significant; (B) HUVECs were pretreated with LPS (100 ng/ml) for 4 h, followed by PA (100 μM) treatment for 24 h in the absence or presence of AICAR. Representative blots and relative expression of p-IRS-1(Thr), p-Akt (Ser473) and p-eNOS (Ser1177), IL-1β and Cleaved caspase-1 protein levels.* represent $p < 0.05$ vs PA100 (n = 3), #represent $p < 0.05$ vs LPS + PA 100 (n = 3), otherwise, not significant. The presented blots were representative of at least 3 independent experiments with similar results. Ctrl, control.

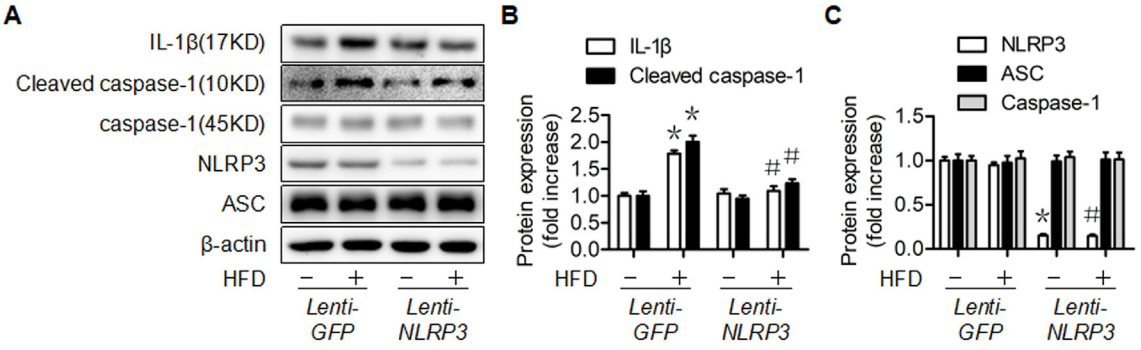

Fig. 6. HFD activate NLRP3 inflammasome in mouse aorta. Male C57BL/6 J mice were fed with regular and high-fat diet (HFD) (60% fat calories) for 10 weeks, and then transfected with GFP and NLRP3 Lenti-Virus for another 2 weeks. (A) Western Blots for IL-1β, Cleaved caspase-1, NLRP3, ASC and caspase-1 protein levels; (B) Relative expression of IL-1βand Cleaved caspase-1 protein levels; (C) Relative expression of NLRP3, ASC and caspase-1 protein levels. * represent $p < 0.05$ vs Vehicle (n = 5), # represent $p < 0.05$ vs HFD (n = 5), otherwise, not significant. HFD, High-fat diet; Lenti-GFP, GFP Lenti-Virus; Lenti-NLRP3, NLRP3 Lenti-Virus.

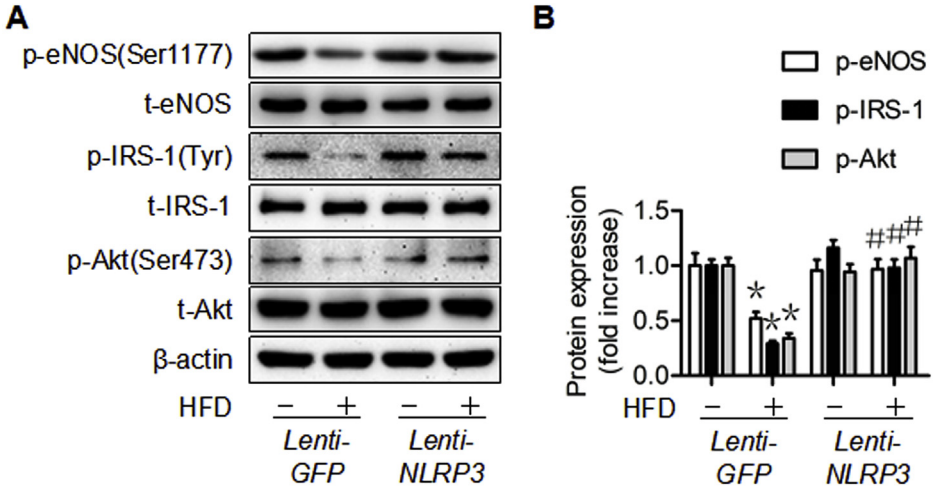

Fig. 7. NLRP3 protein inhibition restored decreased insulin mediated Akt and eNOS phosphorylation in mouse aorta. Male C57BL/6 J mice were fed with regular and high-fat diet (HFD) (60% fat calories) for 10 weeks, and then transfected with GFP and NLRP3 Lenti-Virus for another 2 weeks, followed by insulin (100 nM) stimulation for 10 min. (A) Western blots for p-IRS-1(Thr), p-Akt (Ser473) and p-eNOS (Ser1177) protein levels; (B) Relative expression of p-IRS-1(Thr), p-Akt (Ser473) and p-eNOS (Ser1177) protein levels. * represent $p < 0.05$ vs Vehicle (n = 5), # represent $p < 0.05$ vs HFD (n = 5), otherwise, not significant. HFD, High-fat diet; Lenti-GFP, GFP Lenti-Virus; Lenti-NLRP3, NLRP3 Lenti-Virus.

3.7. NLRP3 inhibition restored impaired endothelium-dependent vasorelaxation induced by HFD in mouse aorta

Next, the role of NLRP3 in HFD-induced impairment of endothelial functions was investigated in mouse aorta. As shown in Fig. 8A, ACh (10^{-9}–10^{-4} M) induced vasodilation in a concentration-dependent manner in preconstricted aortic rings. HFD decreased ACh-mediated vasodilation. NLRP3 protein inhibition reversed ACh-mediated vasodilation impaired by HFD. We also studied sodium nitroprusside (SNP, 10^{-9}–10^{-4} M) induced endothelium-independent relaxation and showed that there was no statistically significant difference (Fig. 8B).

4. Discussion

The present study provided new evidence to show the role of NLRP3 inflammasome in FFA induced endothelial dysfunction. It was found

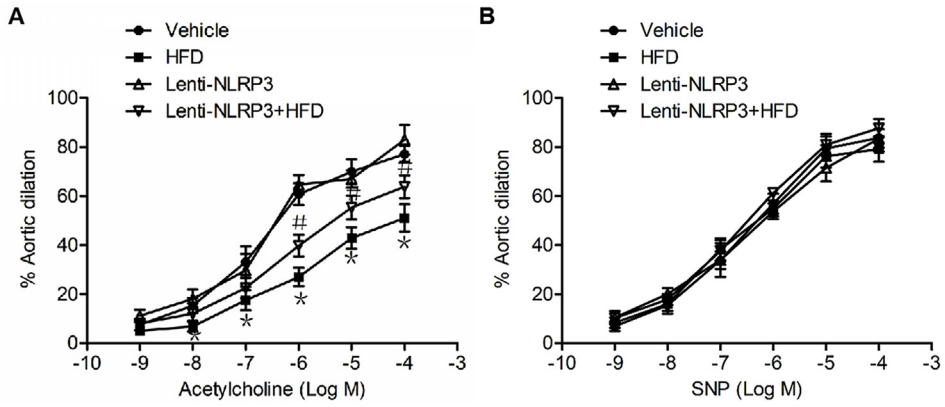

Fig. 8. NLRP3 inhibition restored impaired endothelium-dependent vasorelaxation induced by HFD in mouse aorta. Vasodilator responses of isolated aortic rings to (A) Ach in an endothelium-dependent manner and to (B) SNP in an endothelium independent manner in the studied groups. HFD, High-fat diet; Lenti-NLRP3, NLRP3 Lenti-Virus; Ach, acetylcholine; SNP, sodium nitroprusside. * represent $p < 0.05$ vs Vehicle ($n = 5$), $^{\#}$represent $p < 0.05$ vs HFD ($n = 5$), otherwise, not significant. HFD, High-fat diet; Lenti-GFP, GFP Lenti-Virus; Lenti-NLRP3, NLRP3 Lenti-Virus.

that palmitate could activate NLRP3 inflammasome and induce IL-1β release through the inhibition of AMPKα. In contrast, NLRP3 inhibition restored palmitate-induced impaired of insulin signal pathway and endothelium-dependent vasorelaxation, which was associated with IL-1β-related insulin resistance. Thus, It was demonstrated that NLRP3 inflammasome mediated palmitate induced endothelial dysfunction.

It is well known that elevated FFA in obesity could cause insulin resistance not only in liver and skeletal muscle, but also in endothelial cells [24]. FFA is associated with insulin resistance and endothelial dysfunction. The underlying mechanism is vascular inflammation which is a key point linking cardiovascular disease to obesity and other metabolic disorders. IL-1β is an important proinflammatory cytokine that links insulin resistance and endothelial dysfunction. It had been demonstrated that IL-1β cytokine is processed by caspase-1 which can be activated by NLRP3 inflammasome. NLRP3 inflammasome activation requires two steps. The priming step could be provided by pattern recognition or cytokine receptors which induce expression and synthesis of pro-IL-1β. In the second step, a range of pathogenic factors, named second signals, such as diverse substances of microbial, environmental, or endogenous origin, triggers NLRP3 inflammasome activation [25,26].

Palmitate is one of the most abundant saturated fatty acids in plasma and is substantially elevated following an HFD. It had been know that palmitate could induce endothelial dysfunction and polyunsaturated fatty acids could improve endothelial function. Recently, the saturated fatty acid palmitate, but not unsaturated fatty acid, has been implicated to activate NLRP3 inflammasome in bone marrow-derived macrophages (BMMs) in the obesity model [19–21]. Thus, we did not detect the effects of polyunsaturated fatty acids on NLRP3 inflammasome in this study. To test whether palmitate could activate NLRP3 inflammasome in endothelial cells, LPS-primed HUVECs were incubated with palmitate to induce a robust increase of cleaved caspase-1 and release of IL-1β in a concentration-dependent manner without changing NLRP3, ASC or caspase-1 protein expression. Of note, without LPS, palmitate alone did not increase the levels of cleaved caspase-1 or IL-1β release. Furthermore, HFD could increase circulating FFA levels, which cause inflammation and lipotoxicity [27–29]. The HFD-induced proinflammatory responses, including production of cytokines, are implicated as a mechanism for insulin resistance and endothelial dysfunction [30–33]. Also, we performed mice with HFD, and found that HFD increased cleaved caspase-1 expression and induced IL-1β release without changing NLRP3, ASC or caspase-1 protein expression in mouse aorta. These indicate that HFD could activate NLRP3 inflammasome. These indicated that palmitate acted as a secondary signal in the activation of NLRP3 inflammasome in endothelial cells.

Insulin resistance is characterized by endothelial dysfunction, and endothelial dysfunction is a hallmark of insulin resistance [34,35]. FFA has been proven to damage the insulin signaling pathways (IRS-PI3K-Akt) to induce insulin resistance and thus reduce eNOS phosphorylation in endothelial cells which in turn induce endothelial dysfunction by inducing the expressions of proinflammatory cytokines [5–7]. In the present study, It was found that both palmitate and IL-1β inhibited the IRS-1-Akt-eNOS signaling pathway, which is consistent with the previous studies. NLRP3 inhibition also suppressed the release of IL-1β and alleviated palmitate-induced impairment of insulin signaling in endothelial cells. Our study further exhibited that ACh-mediated vasodilation in mouse aortic rings was reduced by HFD, while the inhibition of NLRP3 could alleviate HFD-induced impairment of vasodilation. These results demonstrated that NLRP3 inflammasome mediate palmitate induced endothelial dysfunction through the regulation of proinflammatory response and insulin signaling pathway.

It was reported that AMPKα, which is another kinase upstream of phosphorylated Akt and eNOS [36], negatively regulates FFA induced reactive oxygen species (ROS) generation, which results in an increased level of β-oxidation of FFAs in mitochondria [37]. FFA could also decrease AMPKα activity which leads to mitochondrial ROS accumulation. Previous studies [38,39] suggested that NLRP3 inflammasome activation could be positively regulated by ROS. This study confirmed the hypothesis that palmitate stimulation may activate NLRP3 inflammasome through AMPKα inhibition. It was also found that palmitate decreased AMPKα phosphorylation, while the AMPKα agonist AICAR inhibited NLRP3 inflammasome activation and alleviated palmitate-induced impairment of insulin signal pathway.

5. Conclusion

In summary, this study demonstrated that NLRP3 inflammasome activation via AMPKα inactivation mediated palmitate-induced endothelial dysfunction, the mechanism involves IL-1β release induced insulin signal pathway. These findings may provide a new target for the treatment of palmitate induced endothelial dysfunction.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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References

[1] E. Cersosimo, R. DeFronzo, Insulin resistance and endothelial dysfunction: the road map to cardiovascular diseases, Diabetes Metab. Res. Rev. 22 (2006) 423–436.
[2] D.H. Endemann, E.L. Schiffrin, Nitric oxide, oxidative excess, and vascular complications of diabetes mellitus, Curr. Hypertens. Rep. 6 (2004) 85–89.
[3] M. Hamaty, M. Lamberti, J.R. Sowers, Diabetic vascular disease and hypertension, Curr. Opin. Cardiol. 13 (1998) 298–303.
[4] M. Shimabukuro, N. Higa, T. Asahi, Y. Oshiro, N. Takasu, T. Tagawa, et al.,

Hypoadiponectinemia is closely linked to endothelial dysfunction in man, J. Clin. Endocrinol. Metab. 88 (2003) 3236–3240.
[5] A. Aljada, P. Dandona, Effect of insulin on human aortic endothelial nitric oxide synthase, Metabolism 49 (2000) 147–150.
[6] G. Zeng, F. Nystrom, L. Ravichandran, et al., Roles for insulin receptor, PI3-kinase, and Akt in insulin-signaling pathways related to production of nitric oxide in human vascular endothelial cells, Circulation 101 (2000) 1539–1545.
[7] H. Li, H.L. Li, Y.G. Bao, X.X. Zhang, Y.R. Yu, Free fatty acids induce endothelial dysfunction and activate protein kinase C and nuclear factor-κB pathway in rat aorta, Int. J. Cardiol. 152 (2011) 218–224.
[8] M. Lamkanfi, T.D. Kanneganti, Nlrp3: an immune sensor of cellular stress and infection, Int. J. Biochem. Cell Biol. 42 (2010) 792–795.
[9] K. Schroder, R. Zhou, J. Tschopp, The NLRP3 inflammasome: a sensor for metabolic danger? Science 327 (2010) 296–300.
[10] I.N. Mohamed, S.S. Hafez, A. Fairaq, A. Ergul, J.D. Imig, A.B. El-Remessy, Thioredoxin-interacting protein is required for endothelial NLRP3 inflammasome activation and cell death in a rat model of high-fat diet, Diabetologia 57 (2014) 413–423.
[11] F. Martinon, A. Mayor, J. Tschopp, The inflammasomes: guardians of the body, Annu. Rev. Immunol. 27 (2009) 229–265.
[12] O. Gross, C.J. Thomas, G. Guarda, J. Tschopp, The inflammasome: an integrated view, Immunol. Rev. 243 (2011) 136–151.
[13] X. Wang, G.Z. Feuerstein, J.L. Gu, P.G. Lysko, T.L. Yue, Interleukin-1 beta induces expression of adhesion molecules in human vascular smooth muscle cells and enhances adhesion of leukocytes to smooth muscle cells, Atherosclerosis 115 (1) (1995) 89–98.
[14] K. Kanno, Y. Hirata, T. Imai, F. Marumo, Induction of nitric oxide synthase gene by interleukin in vascular smooth muscle cells, Hypertension 22 (1) (1993) 34–39.
[15] S. Legrand-Poels, N. Esser, L. L'homme, Andre Scheen, N. Paquot, J. Piette, Free fatty acids as modulators of the NLRP3 inflammasome in obesity/type 2 diabetes, Biochem. Pharmacol. 92 (2014) 131–141.
[16] E.L. Goldberg, V.D. Dixit, Drivers of age-related inflammation and strategies for healthspan extension, Immunol. Rev. 265 (1) (2015) 63–74.
[17] P. Duewell, H. Kono, K.J. Rayner, C.M. Sirois, G. Vladimer, F.G. Bauernfeind, et al., NLRP3 inflammasomes are required for atherogenesis and activated by cholesterol crystals, Nature 464 (2010) 1357–1361.
[18] W. Liu, Y. Yin, Z. Zhou, M. He, Y. Dai, OxLDL-induced IL-1 beta secretion promoting foam cells formation was mainly via CD36 mediated ROS production leading to NLRP3 inflammasome activation, Inflamm. Res. 63 (1) (2014) 33–43.
[19] B. Vandanmagsar, Y.H. Youm, A. Ravussin, J.E. Galgani, K. Stadler, R.L. Mynatt, et al., The NLRP3 inflammasome instigates obesity-induced inflammation and insulin resistance, Nat. Med. 17 (2011) 179–188.
[20] H. Wen, D. Gris, Y. Lei, S. Jha, L. Zhang, M.T. Huang, et al., Fatty acid-induced NLRP3-ASC inflammasome activation interferes with insulin signaling, Nat. Immunol. 12 (2011) 408–415.
[21] N.N. Pejnovic, J.M. Pantic, I.P. Jovanovic, et al., Galectin-3 deficiency accelerates high-fat diet-induced obesity and amplifies inflammation in adipose tissue and pancreatic islets, Diabetes 62 (2013) 1932–1944.
[22] S.J. Wei, J.H. Xing, B.L. Wang, Li Xue, J.L. Wang, R. Li, et al., Poly(ADP-ribose) polymerase inhibition prevents reactive oxygen species induced inhibition of aldehyde dehydrogenase2 activity, Biochim. Biophys. Acta 1833 (3) (2013) 479–486.
[23] J.H. Xing, H.T. Liu, H.B. Yang, R. Chen, Y.G. Chen, J. Xu, Upregulation of Unc-51-like kinase 1 by nitric oxide stabilizes SIRT1, independent of autophagy, PLoS One 9 (12) (2014) e116165.
[24] G. Boden, Fatty acid-induced inflammation and insulin resistance in skeletal muscle and liver, Curr. Diabetes Rep. 6 (3) (2006) 177–181.
[25] F. Bauernfeind, A. Ablasser, E. Bartok, S. Kim, J. Schmid-Burgk, T. Cavlar, V. Hornung, Inflammasomes: current understanding and open questions, Cell. Mol. Life Sci. 68 (2011) 765–783.
[26] V.A. Rathinam, S.K. Vanaja, K.A. Fitzgerald, Regulation of inflammasome signaling, Nat. Immunol. 13 (2012) 333–342.
[27] H.S. Kim, M.S. Han, K.W. Chung, S. Kim, E. Kim, M.J. Kim, et al., Toll-like receptor 2 senses beta-cell death and contributes to the initiation of autoimmune diabetes, Immunity 27 (2007) 321–333.
[28] J.A. Kim, K.K. Koh, M.J. Quon, The union of vascular and metabolic actions of insulin in sickness and in health, Arterioscler. Thromb. Vasc. Biol. 25 (2005) 889–891.
[29] J.A. Kim, M. Montagnani, S. Chandrasekran, M.J. Quon, Role of lipotoxicity in endothelial dysfunction, Heart Fail. Clin. 8 (2012) 589–607.
[30] G.S. Hotamisligil, Role of endoplasmic reticulum stress and c-Jun NH2-terminal kinase pathways in inflammation and origin of obesity and diabetes, Diabetes 54 (Suppl 2) (2005) S73–S78.
[31] H.J. Jang, H.S. Kim, D.H. Hwang, M.J. Quon, J.A. Kim, Toll-like receptor 2 mediates high-fat diet-induced impairment of vasodilator actions of insulin, Am. J. Physiol. Endocrinol. Metab. 304 (2013) E1077–E1088.
[32] F. Kim, K.A. Tysseling, J. Rice, M. Pham, L. Haji, B.M. Gallis, et al., Free fatty acid impairment of nitric oxide production in endothelial cells is mediated by IKKbeta, Arterioscler. Thromb. Vasc. Biol. 25 (2005) 989–994.
[33] M. Yuan, N. Konstantopoulos, J. Lee, L. Hansen, Z.W. Li, M. Karin, S.E. Shoelson, Reversal of obesity- and diet-induced insulin resistance with salicylates or targeted disruption of Ikkbeta, Science 293 (2001) 1673–1677.
[34] A.D. Baron, Insulin resistance and vascular function, J. Diabet. Complicat. 16 (2002) 92–102.
[35] J.A. Kim, M. Montagnani, K.K. Koh, M.J. Quon, Reciprocal relationships between insulin resistance and endothelial dysfunction: molecular and pathophysiological mechanisms, Circulation 113 (2006) 1888–1904.
[36] H. Chen, M. Montagnani, T. Funahashi, I. Shimomura, M.J. Quon, Adiponectin stimulates production of nitric oxide in vascular endothelial cells, J. Biol. Chem. 278 (2003) 45021–45026.
[37] S.X. Wang, M. Zhang, B. Liang, J. Xu, Z.L. Xie, C. Liu, et al., AMPKα2 deletion causes aberrant expression and activation of NAD(P)H oxidase and consequent endothelial dysfunction *in vivo*: role of 26S proteasomes, Circ. Res. 106 (2010) 1117–1128.
[38] K. Schroder, J. Tschopp, The inflammasomes, Cell 140 (2010) 821–832.
[39] R. Zhou, A.S. Yazdi, P. Menu, J. Tschopp, A role for mitochondria in NLRP3 inflammasome activation, Nature 469 (7329) (2011) 221–225.