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Improvement of hyperglycemia in a murine model of insulin resistance and high glucose- and inflammasome-mediated IL-1β expressions in macrophages by silymarin

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ABSTRACT

Macrophages and inflammasome pathway are involved in high-glucose toxicity and development of insulin resistance. Silymarin (SMR) was known to modulate glucose homeostasis and reduce inflammation. However, it is still unknown whether SMR possess anti-hyperglycemic effects in diabetic-like knockout mice (*Hnf-1α*$^{kin/-}$/Ins.cre mice) with insulin resistance and also unclear how SMR regulates LPS induced stress markers and pro-inflammatory cytokines under stresses of high glucose (HG) or NLRP3 inflammasome activation. Current results show that oral administration of SMR (100 mg/kg) reduced hyperglycemia in the mouse model of maturity-onset diabetes of the young type 3-like mice. In cultured macrophages, SMR (5–20 μg/ml) reduces high glucose (HG)-enhanced expressions of inducible nitric oxide synthase, nitric oxide generation stimulated by LPS; however, no effects on COX-2 expressions. The enhanced interleukin-1β (IL-1β) secretions in the presence of HG or palmitate were also significantly down regulated by SMR in dose-dependent manner in LPS-treated macrophages. Such observations may result from the decreased extracellular signal-regulated kinase 1/2 phosphorylation, while without affecting protein kinase C-α phosphorylation and nuclear factor-κB activation. These findings together show that SMR acts as a protector against HG-related stresses not only by lowering hyperglycemia but also suppressing HG- and inflammasome-mediated IL-1β expressions to improve insulin resistance.

1. Introduction

Hyperglycemia is the most prominent sign that characterizes diabetes. Under hyperglycemic states, inflammatory response mediates glucose toxicity and insulin resistance. Livers with inflamed hepatocytes become less sensitive to insulin and then showed insulin resistance since liver is one of the primary metabolic tissues that are normally sensitive to insulin [1]. A role for hepatocyte nuclear factor (HNF)-1 alpha (HNF-1α) in controlling development and metabolism has been suggested by analysis of HNF-1α-null mice [2]. A novel maturity-onset diabetes mouse model of the young 3-like (MODY3-like) with pancreas-reactivation, *Hnf-1α*$^{kin/-}$/Ins.cre mice (PR *Hnf-1α*-knockout mice), generated by disruption of liver glucose metabolism contributes to the MODY disease [3], has been developed then to hyperglycemia with normal insulin levels and can be used as animal model of insulin resistance.

Both macrophages and inflammasome pathway are involved in development of insulin resistance. Interleukin (IL)-1beta toxicity to islet beta cells [4]. High glucose (HG) diets not only increase interleukin-1β (IL-1β) expression [5] but also augment tumor necrosis factor-α (TNF-

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α) and nitric oxide (NO) production in lipopolysaccharide (LPS)-activated monocytes and macrophages [6–8]. In addition to HG stress, a diet in high fat and systemic prolonged inflammation are considered to increase the risk of insulin resistance and type 2 diabetes [9]. Palmitate, a saturated fatty acid, can induce caspase-1, IL-1β and IL-18 production via activating the NLR family, pyrin domain containing 3 (NLRP3) inflammasome [10] and promotes insulin resistance [10–12].

Silymarin (SMR), a hepatoprotective agent, extracted from seeds and fruit of milk thistle (*Silybum marianum*) and is traditionally used in herbal medicines. Early studies have suggested that the use of SMR is beneficial for diabetic patients with cirrhosis or hepatic dysfunction, particularly those who were considered insulin resistance [13]. Silybin, the major active component of SMR, exhibits antioxidant activities [14], anti-inflammatory and anti-fibrogenic effects on human hepatic stellate cells [15]. More recent *in vivo* studies have indicated that SMR at doses of 20–100 mg/kg show anti-inflammatory in animal models of diabetes [16,17]. SMR also exerts anti-inflammatory effects in various chemically-induced inflammation models. SMR's mechanisms of inhibitions on the cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS)/NO pathways in LPS-induced inflammation are through the nuclear factor-κB (NF-κB) pathways [18–20], which is responsible for the T-cell-produced transcriptional activation of pro-inflammatory cytokines such as TNF-α and IL-1β [21–23]. Inhibition of SMR on acute inflammations is also through PKC in LPS-activated macrophages [24].

Accumulating evidence [25] supports a role for SMR to modulate glucose homeostasis, including *in vivo* by preventing pancreas damage in rat models [26] and *in vitro* by reducing IL-1β-induced NO production as well as pancreatic β-cell damages in human islets [27]; however, it is still indefinite whether SMR possess anti-hyperglycemic effects in MODY3-like mice with insulin resistance. Our previous study has demonstrated that HG enhanced LPS-induced oxidative stress and inflammatory responses by increasing expression of iNOS/NO and cytokine secretions such as IL-1β [28] in macrophages. However, it is still unknown the effects of SMR on HG-mediated responses of and cytokines. Moreover, IL-1β secretion was regulated through either the NF-κB or NLRP3 inflammasome pathways [29] and was also considered to play an important role in diabetic cardiomyopathy [30].

Here, we aimed to determine effects of SMR in PR *Hnf-1α*-knockout mice and also employed this HG-mediated stress assay system to determine how iNOS/NO stress markers, pro-inflammatory cytokines respond to SMR in macrophages under HG stress. Insulin resistance modulation by SMR was approached by determining IL-1β secretion in palmitate activated NLRP3 inflammasome.

2. Materials and methods

2.1. Materials

Chemicals including SMR (S-0292), LPS (from *Escherichia coli* 0111:B4), palmitate, antibodies against phospho-extracellular signal-regulated kinase 1/2 and actin were purchased from Sigma (St. Louis, MO, USA). Antibodies against iNOS, COX-2, phospho-PKC-α and HRP-second antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All ELISA kits were purchased from eBioscience (San Diego, CA, USA). ATP was purchased from InvivoGen (San Diego, CA, USA).

2.2. Maturity-onset diabetes of the young 3 (MODY3, *Hnf-1α*$^{kin/-}$/*Ins.cre*)-like animal model of diabetes mellitus

Mice were generated by disruption of liver glucose metabolism contributes to the MODY disease [3] and housed under standard conditions. All experiments were performed in accordance with the Institute for Laboratory Animal Research Guide for the Care and Use of Laboratory Animals and protocol was fully approved by the Institutional Animal Research Review Committee of the Taipei Medical University (IACUC registration number COA-051 and approval number LAC-2016-0224). 100 mg/kg SMR was chosen to test oral effects of anti-hyperglycemia according to previous studies [16,17]. Mice were 18 h fasted before experimentations. The ACCU-CHEK Advantage Meter (Roche, Basel, Switzerland) was used, and measurements were recorded at fasting (as baseline) as well as the 2-h and 4-h postprandial states.

Table 1
Percentages of glycemic change from baselines with or without silymarin treatments in PR Hnf-1α-knockout mice mice.

Treatments in types of mice	percentages of glycemic change (%)	
	2 h-post dose	4 h-post dose
Silymarin in MODY3/PR (n = 4)	−42.5 ± 22.9*	−17.5 ± 19.3
vehicle in MODY3/PR (n = 3)	−21.0 ± 8.7	−3.0 ± 6.5
Silymarin in WT (n = 5)	5.0 ± 19.6	−8.9 ± 19.7
vehicle in WT (n = 5)	−4.8 ± 15.6	−9.3 ± 11.7

Data were expressed as the means ± SD; dose of silymarin: 100 mg/kg (water as the vehicle).
*Indicate significant differences at the $p < 0.05$ while compared to the other groups within the same observation time.
Abbreviations; MODY3/PR: maturity-onset diabetes of the young 3-like with pancreas-reactivation; WT: wide-type.

2.3. Cell culture

The RAW 264.7 macrophages (ATCC account number: TIB-71) and the human THP-1 monocytes (ATCC account number: TIB-202) were purchased from the American Type Culture Collection (Rockville, MD, USA). NF-κB reporter cells (RAW-Blue™ cells) were purchased from InvivoGen (San Diego, CA, USA). All the cells were cultured in glucose-free RPMI-1640 medium purchased from Life Technologies Corporation (Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal calf serum purchased from Biological Industries (Kibbutz Beit Haemek, Israel). In the normal and high glucose condition (NG and HG), cells were incubated with the culture medium supplemented with 5.5 mM and 25 mM D-(+)-glucose (Sigma-Aldrich, St. Louis, MO, USA), respectively. To induce monocyte-to- macrophage differentiation, THP-1 macrophages were differentiated from THP-1 monocytes by cultured in the culture medium supplemented with 50 nM phorbol 12-myristate 13-acetate (Sigma-Aldrich, St. Louis, MO, USA) for 48 h.

2.4. Enzyme-linked immunosorbent assay (ELISA)

RAW 264.7 cells (2×10^5 in 0.5 ml NG or HG medium) were cultured in 24-well plate overnight. The cells were incubated for 30 min with or without SMR and then for 48 h with or without LPS (1 μg/ml). For the NLRP3 inflammasome assay, the THP-1 macrophages (2×10^5 in 0.5 ml NG medium) were incubated for 5.5 h with or without LPS, then for 30 min with or without SMR, and then with or without 500 μM palmitate for 24 h. The expression levels of the cytokines in the condition medium were measured via ELISA according to the manufacturer's protocol that published in our previous report [28].

2.5. NO production assay

The assay precudure was followed our previous study [28]. RAW 264.7 cells were seeded in 24-well plates at a density of 2×10^5 cells in 0.5 ml NG or HG medium and then incubated with or without LPS (1 μg/ml) in the absence or presence of SMR for 24 h. The expression level of NO in the condition medium was measured using the Griess reaction.