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•Research Articles•

Mangiferin ameliorates insulin resistance by inhibiting inflammation and regulatiing adipokine expression in adipocytes under hypoxic condition

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[ABSTRACT] Adipose tissue hypoxia has been recognized as the initiation of insulin resistance syndromes. The aim of the present study was to investigate the effects of mangiferin on the insulin signaling pathway and explore whether mangiferin could ameliorate insulin resistance caused by hypoxia in adipose tissue. Differentiated 3T3-L1 adipocytes were incubated under normal and hypoxic conditions, respectively. Protein expressions were analyzed by Western blotting. Inflammatory cytokines and HIF-1-dependent genes were tested by ELISA and q-PCR, respectively. The glucose uptake was detected by fluorescence microscopy. HIF-1α was abundantly expressed during 8 h of hypoxic incubation. Inflammatory reaction was activated by up-regulated NF-κB phosphorylation and released cytokines like IL-6 and TNF-α. Glucose uptake was inhibited and insulin signaling pathway was damaged as well. Mangiferin substantially inhibited the expression of HIF-1α. Lactate acid and lipolysis, products released by glycometabolism and lipolysis, were also inhibited. The expression of inflammatory cytokines was significantly reduced and the damaged insulin signaling pathway was restored to proper functional level. The glucose uptake of hypoxic adipocytes was promoted and the dysfunction of adipocytes was relieved. These results showed that mangiferin could not only improve the damaged insulin signaling pathway in hypoxic adipocytes, but also ameliorate inflammatory reaction and insulin resistance caused by hypoxia.

[KEY WORDS] Mangiferin; Hypoxia; Inflammation; Insulin resistance; Insulin signaling pathway
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Introduction

Hypoxia is regarded as the initiation of inflammation and insulin resistance syndromes [1-2]. Inflammation significantly contributes to the development of insulin resistance. Adipose tissue produces hundreds of hormones known as adipokines that act in paracrine and endocrine fashion to regulate the body's homeostasis. Obesity is characterized by the hypertrophic adipocytes whose diameters are 150−200 μm [3], longer than the diffusion distance of oxygen 100−200 μm [4]. In addition, obesity may cause vascularization of the adipose tissue. Both hypertrophic adipocytes and vascularization of the adipose tissue lead to hypoxia, in which hypoxia-inducible factor 1α (HIF-1α) is abundantly expressed. HIF-1α regulates fibronectin and collagens [5], together with other extracellular matrix proteins during normal transcriptional activity, but excessive expression of HIF-1α will lead to adipose tissue fibrosis. Then the adipocytes may suffer from apoptosis and necrosis [6], a degenerative condition of tumor progression, resulting in dysfunctional adipose tissue.

Insulin promotes glucose uptake in muscle and adipose tissues by facilitating glucose transporter 4 (GLUT4) translocation, which plays an important role in the body glucose homeostasis. Insulin-signaling pathway leading to GLUT4 translocation is mediated through the activation of phosphatidylinositol 3-kinase (PI3K), which subsequently triggers the activation of Akt. However, insulin-mediated GLUT4 translocation together with glucose uptake will be inhibited when insulin resistance occurs. It is already established that inflammation is closely associated with insulin resistance [7], and therefore, inflammation inhibition may contribute to ameliorating insulin resistance.

Mangiferin, a natural polyphenol extracted from *rhizome*

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anemarrhenae, plays an important role in anti-diabetes, anti-inflammation, cardiovascular protection, and antioxidant treatment [8]. Mangiferin increases glucose utilization and 2-DG uptake of 3T3-L1 cells in a dose-dependent manner [9] and improves glucose metabolism by activation of AMP-activated protein kinase [10]. However, the protective role of mangiferin on hypoxia-induced alternations of glucose homeostasis in adipocytes remains an unexplored area of research. In the present study, we analyzed the inflammatory changes and the glucose uptake of 3T3-L1 cells under hypoxic condition. And the protective role of mangiferin was evaluated.

Materials and Methods

Materials

Mangiferin (purity, ≥ 98%) and Compound-C were purchased from Sigma (St. Louis, MO, USA). 5-Aminoimidazole-4-carboxamidel-*β*-D-ribofuranoside (AICAR) was purchased from Beytime Institute of Biotechnology (Shanghai, China). These agents were dissolved in DMSO (final concentration was < 0.1% *V/V*). Other reagents used in the present study were obtained as follows: insulin, Wanbang Biochemical Pharmaceutical Company (Xuzhou, Jiangsu, China); 3-isobutyl-1-methylxanthine (IBMX) and dexamethasone, Sigma; anti-HIF-1*α* antibody (PA1-16601), anti-Phospho-NF-*κ*B (p-p65) (#3033), anti-NF-*κ*B (p65) (#4764); anti-p-AMPK*α* (#2535), anti-AMPK (#2532), GAPDH (MB001), antiphospho-IRS-1 (Ser307) (BS4104), anti-IRS-1 (R301) (BS1408), and PY99 (sc-7020), Santa Cruz Biotechonlogy (CA, USA); anti-phospho-Akt (Thr308) (#9275), anti-Akt (A444) (BS1810), anti-GLUT4 (R271) polyclonal (BS3680), anti-Na^+/K^+-ATPase *α*1 (G19) (BS3002), goat anti-rabbit IgG (H + L) HRP (BS13278), and goat anti-mouse IgG (H + L) HRP (BS12478), Bioworld Technology (St. Paul, MN, USA); Western blotting detection reagent, Beyotime Institute of Biotechnology; and 2-[*N*-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl) amino]-2-deoxy-D-glucose (2-NBDG) (N13195), Invitrogen (Eugene, OR, USA).

Cell culture and differentiation

3T3-L1 preadipocytes, obtained from the cell bank of Chinese Academy of Sciences (Shanghai, China) were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, MA, USA) supplemented with 10% (*V/V*) fetal bovine serum (FBS) and antibiotics (100 U penicillin/mL, and 100 U streptomycin/mL) at 37 ºC in 5% CO_2 atmosphere. To induce differentiation, the cells were incubated in DMEM (10% FBS) containing 0.5 mmol·L^{-1} of IBMX, 1 μmol·L^{-1} of dexamethasone (Dex), and 10 μg·mL^{-1} of insulin for 48 h, and then in DMEM (10% FBS) containing 10 μg·mL^{-1} of insulin alone for 48 h. The differentiated cells were maintained in DMEM (10% FBS) for 7 days.

Hypoxia induction and treatment

The differentiated cells were incubated in a hypoxic chamber on the 8th day (HERAcell150i, Thermo scientific, China) at an atmosphere of 1% O_2, 94% N_2, and 5% CO_2, at 37 ºC for 8 h. The control cells were incubated in an atmosphere of 21% O_2, and 5% CO_2 at 37 ºC. The cells were treated with mangiferin (0.1, 1, and 10 μmol·L^{-1}), mangiferin plus Compound-C (25 μmol·L^{-1}) or AICAR (500 μmol·L^{-1}) during the hypoxia period.

Lactate and glycerol release

The concentrations of lactate and glycerol in conditioned medium were determined using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), according to the manufacturer's instructions.

Estimation of inflammatory cytokines

Inflammatory cytokines IL-6 and TNF-*α* were estimated in conditioned medium using enzyme-linked immune-sorbent assay (ELISA) kits (Nanjing Jiancheng Bioengineering Institute).

Western blot analysis

The differentiated 3T3-L1 cells were seeded onto 6-well plates and deprived of serum for 4 h in DMEM. The cells were pretreated for 30 min with mangiferin (0.1, 1, and 10 μmol·L^{-1}) and mangiferin plus Compound-C (25 μmol·L^{-1}) or AICAR (500 μmol·L^{-1}), respectively, before being incubated in the hypoxic chamber for 8 h, followed by the insulin stimulation for another 0.5 h. The cells were washed with ice-cold PBS and lysed in ice-cold Radio-Immunoprecipitation Assay (RIPA) buffer. The lysates were centrifuged at 12 000 *g* for 15 min at 4 ºC and the supernatants were collected. For the assay of membrane GLUT4, membrane protein was prepared with plasma membrane using cytoplasmic protein extraction kit (Sangon Biotech Co., Ltd., Shanghai, China). The protein was separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (0.45-μm, Millipore Co., Ltd., USA) by semidry electrophoretic transfer. The PVDF membranes were blocked with 6% nonfat milk in Tris-buffered saline containing Tween-20 (TBST) (20 mmol·L^{-1} of Tris-HCl, pH 7.6, 150 mmol·L^{-1} of NaCl, 0.1% Tween-20) for 2.5 h at room temperature, and then incubated with primary antibody (1 : 800 dilution in TBST) at 4 ºC overnight. After 2-h incubated with HRP-conjugated secondary antibody at room temperature, the signals were detected by ECL (Beyotime Institute of Biotechnology) and quantified by densitometry with IPP 6.0 software (Media cybernetics, MD, USA).

Real time quantitative PCR analysis

The differentiated 3T3-L1 cells were pretreated for 30 min with mangiferin (0.1, 1, and 10 μmol·L^{-1}) and mangiferin plus Compound-C (25 μmol·L^{-1}) or AICAR (500 μmol·L^{-1}), respectively, and washed thrice with cold PBS after being incubated in the hypoxic chamber for 8 h. The total RNA was extracted by TRIzol (Sunshine Biotechnology Nanjing, Co., Ltd, China), according to the manufacturer's instructions. The extracted RNA was dissolved in diethyl pyrocarbonate (DEPC) treated water and the concentrations were determined by optical density measurement at 260 nm on a spectropho-