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Butein induction of HO-1 by p38 MAPK/Nrf2 pathway in adipocytes attenuates high-fat diet induced adipose hypertrophy in mice

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ABSTRACT

Adipose tissue inflammation and oxidative stress are key components in the development of obesity and insulin resistance. Heme oxygenase (HO)−1 in adipocytes protects against obesity and adipose dysfunction. In this study, we report the identification of butein, a flavonoid chalcone, as a novel inducer of HO-1 expression in adipocytes *in vitro* and *in vivo*. Butein upregulated HO-1 mRNA and protein expression in 3T3-L1 adipocytes, accompanied by Kelch-Like ECH-Associated Protein (Keap) 1 degradation and increase in the nuclear level of nuclear factor erythroid 2-related factor 2 (Nrf2). Butein modulation of Keap1 and Nrf2 as well as HO-1 upregulation was reversed by pretreatment with p38 MAPK inhibitor SB203580, indicating the involvement of p38 MAPK in butein activation of Nrf2 in adipocytes. In addition, HO-1 activation by butein led to the inhibitions of reactive oxygen species and adipocyte differentiation, as evidenced by the fact that butein repression of reactive oxygen species and adipogenesis was reversed by pretreatment with HO-1 inhibitor SnPP. Induction of HO-1 expression by butein was also demonstrated in the adipose tissue of C57BL/6 mice fed a high-fat diet administered along with butein for three weeks, and correlated with the inhibitions of adiposity and adipose tissue inflammation, which were reversed by co-administration of SnPP. Altogether, our results demonstrate that butein activates the p38 MAPK/Nrf2/HO-1 pathway to act as a potent inhibitor of adipose hypertrophy and inflammation in a diet-induced obesity model and thus has potential for suppressing obesity-linked metabolic syndrome.

1. Introduction

Oxidative stress in adipose tissue has been implicated in the pathogenesis of obesity and type 2 diabetes (T2D). The importance of reactive oxygen species production in adipocytes and associated insulin resistance is highlighted by the fact that reactive oxygen species increases in adipose tissue induce an inflammatory response in adipocytes (Lin et al., 2005; Wellen and Hotamisligil, 2005).

Adipose tissue expansion in obesity leads to increased secretion of inflammatory cytokines as well as chemokines, which trigger increased infiltration and accumulation of immune cells in adipose tissue. Adipose-infiltrated immune cells such as macrophages, basophils and regulatory T cells, in collaboration with adipocytes, mediate adipose tissue inflammation by secretion of pro-inflammatory cytokines and free fatty acids by which obesity mediates systemic insulin resistance (Nishimura et al., 2009). Indeed, anti-inflammatory agents that mitigate adipose tissue inflammation lead to therapeutic effects in a variety of models of insulin resistance (Luck et al., 2015; Tobar et al., 2011; Ying et al., 2014).

Heme oxygenase (HO)-1, along with other phase II antioxidant enzymes, serves as a defense system against oxidative stress. HO-1 converts heme to biliverdin, carbon monoxide, and ferrous ions but exerts pleiotropic functions far beyond heme metabolism. HO-1 protects the cardiovascular system and acts an anti-inflammatory, immunomodulatory, and proangiogenic protein, also regulating the cell cycle (Deshane et al., 2007; Kim et al., 2011; Peyton et al., 2002; Wu et al., 2008). In addition, a body of evidence has emerged indicating a role for HO-1 in metabolic homeostasis. HO-1 is highly expressed in the white adipose tissue of genetic or high-fat diet (HFD)-induced obese mice (Huang et al., 2012; Soukas et al., 2000). Although there is controversy, many previous studies have shown that HO-1 plays a protective role against adipose tissue dysfunction that is linked with obesity and insulin resistance. For example, systemic induction of HO-1 by treatment with HO-1 inducers hemin or cobalt protoporphyrin reduces adiposity and improves insulin sensitivity in *ob/ob* mice or Zucker diabetic rats (Li et al., 2008; Ndisang et al., 2009; Nicolai et al., 2009).

Butein is a flavonoid chalcone derived from *Toxicodendron vernicifluum* and has various antioxidant, anti-inflammatory, anti-adipogenic,

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anti-cancer, and anti-fibrogenic activities (Song et al., 2013). Butein has also been used to induce HO-1 expression in various cells to play anti-oxidative roles (Cho et al., 2011; Lee et al., 2013). Recently, we reported that butein has anti-inflammatory functions in adipocytes (Wang et al., 2014). Based on our previous findings and in view of the important role of adipose HO-1 in the development of obesity and insulin resistance, we explored whether butein induces HO-1 in adipocytes and investigated the underlying mechanisms. We also determined the *in vivo* pharmacological function of butein after 3-week administration to mice fed a HFD.

2. Materials and methods

2.1. Materials

Butein and TNFα were purchased from Sigma-Aldrich (St. Louis, MO). Primary antibodies used were: HO-1 (sc-136961), NQO-1 (sc-32793), Nrf2 (sc-722), PCNA (sc-7909) and Lamin B (sc-6216) from Santa Cruz Biotechnology (Santa Cruz, CA), Keap1 (ab66620) from Abcam (Cambridge, MA), β-tubulin (PA1-16947) from Thermo scientific (Waltham, MA), actin (A5441) from Sigma-Aldrich (St. Louis, MO), p-JNK (#9251), p-p38 MAPK (#4631), p-ERK (#9101), ERK (#9102), and p-PKCδ (#9374) from Cell Signaling Technology (Beverly, MA).

2.2. 3T3-L1 Preadipocytes culture, differentiation and oil red o (ORO) staining

Murine 3T3-L1 cells were cultured in the growth medium (GM) containing Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum (FBS), 50 U/ml penicillin and 50 μg/ml streptomycin and 2 mM glutamine at 37 °C in a humidified atmosphere with 10% CO_2, and induced to differentiate as previously described (Wang et al., 2014). Briefly, 2 day-post-confluent preadipocytes (day 0) were cultured in the GM supplemented with 0.5 μM 3-isobutyl-1-1-methyl-xanthine, 1 μM dexamethasone and 1 μg/ml insulin for 3 days. The cells were further incubated in the growth medium supplemented with 1 μg/ml insulin for additional 3 days, and thereafter medium was replaced with fresh GM every other day. Mature adipocytes at day 8 or more were used in the experiments. For induction of inflammatory genes in adipocytes, mature adipocytes were treated with TNFα (10 ng/ml), LPS (10 ng/ml), IFNγ (500 U/ml) (TLI) for 6 h in the presence or absence of butein (30 μM) or SnPP (20 μM).

For the measurement of the effect of HO-1 inhibitor SnPP (tin protoporphyrin IX) on the butein inhibition of adipogenesis, 3T3-L1 cells were differentiated with or without 30 μM butein and 20 μM SnPP treatment for 7 days. Differentiated cells were stained with ORO, and lipid accumulation was quantified. ORO stock solution (0.35% in isopropanol) was prepared by stirring-solubilization overnight at room temperature and subsequent filtration through 0.2 μm pore filter paper. Cells in the culture plates were fixed with 10% formalin for 30 min and wells were washed with 60% isopropanol. After air dry, wells were stained with 60% water diluted ORO stock solution at room temperature for 30 min. Then cells were washed with water several times and air dried. Stained cells were examined under bright field microscope and the pictures were taken at 60X magnification. For quantification, ORO in stained cells in culture plates was eluted in 1 ml of 100% isopropanol for 10 min and colorimetric intensity was measured by spectrometry at 510 nm.

2.3. In vitro *macrophage migration assay*

For migration assays, RAW264.7 ($5{\times}10^5$) cells were seeded in the 24-well upper chamber of a cell culture insert with 8-μm pore membrane (BD Life Sciences, Franklin Lakes, NJ, USA) in FBS-free media. Adipocyte CM was added to the lower chamber, and cells were incubated for 3 h. Macrophages found on the bottom of filter were counted as cells that had performed chemotaxis. Cells were quantified from 5 fields/condition; each condition was performed in triplicate.

2.4. Isolation of primary macrophages

Peritoneal macrophages were obtained according to the methods reported previously (Kim et al., 2015). Briefly, peritoneal macrophages were obtained from C57BL/6 mice by peritoneal lavage 3 days after injection of 3 ml of 3% thioglycolate (Difco, Sparks, MD, USA). Then mice were killed and 7 ml PBS was injected into the abdominal and the abdomen was massaged gently for 3 min. Harvest the PBS with cells. After centrifugation at 1000*g* for 5 min, cells were plated in 12-well plates at $2{\times}10^5$ cells/well.

2.5. Cell lysis, nuclear fractionation and western blot analysis

Whole cell or tissue lysates were prepared and western blot analysis was performed as described previously (Wang et al., 2014). Briefly, the cells or the aliquots of tissues were lysed in the buffer containing10 mM Tris–HCl (pH 7.1), 100 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% Triton X-100, 0.5% Nonidet P-40, 1 mM dithiothreitol and 0.5 mM phenylmethylsulfonyl fluoride, supplemented with inhibitors of proteinase and phosphatase. For isolation of nuclear fraction, we used NE-PER Nuclear and Cytoplasmic Extraction kits obtained from Thermo scientific. Protein concentrations in cell or tissue lysates were determined by a Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). The aliquots of lysates were separated by 6–10% sodium dodecyl sulfate-polyacrylamide gels electrophoresis (20 μg of protein/lane). The separated proteins were transferred to nitrocellulose membranes (GE Healthcare). The membranes were blocked for 30 min at room temperature in Tris-buffered saline containing 1% Tween 20 and 4% skim milk and incubated with the primary antibodies against interest proteins, followed by incubation with secondary antibodies. Immunoreactive protein was visualized by ECL chemiluminescence detection kit (Amersham Biosciences, Buckinghamshire, UK). Image was obtained using ChemiDoc™ XRS+ System (Bio-rad, Hercules, CA).

2.6. RNA isolation, real time quantitative (qPCR) and reverse transcription (RT)-PCR

Total RNA was extracted from cells or adipose tissues with TRIzol reagent (Invitrogen). The total RNA (2 μg) was reverse-transcribed using random primer (Promega, Madison, WI). PCR amplification conditions were optimized for each pair of primer. Specific primers were designed using qPrimerDepot (http://mouseprimerdepot.nci.nih.gov, Table 1). RT-PCR products were separated on an agarose gel. qPCR was performed as previously described using an ABI7000 and Stratagene3000 MXP PCR cycler with the SybrGreen detection system 6. The mRNA expression of all genes tested is normalized to the *Rps3* gene expression.

2.7. Analysis of reactive oxygen species

For the measurement of intracellular level of reactive oxygen species, the cells were pretreated with or without SnPP at 20 μM for 1 h then treated with butein at 30 μM for 10, 30 min, 3, 10 h and washed with PBS for three times, then incubated with 10 μM cell-permeable fluorogenic probe 2', 7'-dichlorodihydrofluorescin diacetate (DCFH-DA) (Invitrogen D-399) for 1 h at 37 °C in the dark. Reactive oxygen species were analyzed by Spectrophotometer at 502/523 nm.

2.8. Animals and drug treatment

Six week-old male C57BL/6 mice were purchased from SamtakoBio (Osan, Korea) and housed under pathogen-free conditions and fed *ad*

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