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Bardoxolone methyl prevents fat deposition and inflammation in the visceral fat of mice fed a high-fat diet

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ABSTRACT

Key features of diet-induced obesity are visceral fat deposition, macrophage infiltration and inflammation that can lead to metabolic disorders. This study examined the effects of bardoxolone methyl (BARD) in preventing obesity and inflammation in the visceral fat of mice fed high-fat diet. Male C57BL/6J mice were fed a high-fat diet (HFD), a low-fat diet (LFD, i.e., lab chow diet) or a high-fat diet supplemented with BARD (HFD/BARD) for 21 weeks. BARD at a dosage of 10 mg/kg body weight was administered orally in drinking water. Histology, immunohistochemistry and Western blot were used for the analysis of epididymal adipose tissue. Morphological results demonstrated that HFD fed mice treated with BARD had smaller adipocytes and fewer macrophages present in epididymal adipose tissue than the HFD group. Furthermore, BARD administration reduced the inflammatory profile in this tissue by increasing the expression of nuclear factor of kappa-light-polypeptide gene enhancer in B-cells inhibitor, alpha (IκB-α) protein and decreasing the protein expression of tumour necrosis factor alpha (TNF-α). BARD also prevented oxidative stress reflected by a reduction in stress activated proteins, including signal transducer and activator of transcription 3 (STAT3), protein kinase B (Akt), extracellular-signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK). BARD administration activated the sympathetic nervous system in epididymal adipose tissue assessed by the increased synthesis of tyrosine hydroxylase (TH) and uncoupling protein 2 (UCP2). The expression of inflammatory and sympathetic nervous system proteins in BARD mice fed a HFD was equivalent to that of the LFD control mice, indicating the anti-inflammatory and anti-obesity properties of this drug. In conclusion, the oral administration of BARD in HFD mice prevented fat deposition, inflammation and oxidative stress, and improved sympathetic activity in visceral fat. This study suggests a potential therapeutic role of BARD in preventing the development of obesity.

1. Introduction

The incidence of obesity has reached epidemic proportions. This disease is associated with serious diseases such as type 2 diabetes, cardiovascular disease and cancer. A characteristic of overweight or obese people is excessive visceral fat deposition. High energy intake, particularly the consumption of a high-fat diet, promotes the development of obesity in humans and rodents [1–6]. The chronic consumption of food high in fat produces a state of positive energy balance where the energy consumed from food (energy intake) is higher than the energy used for work and rest (energy expenditure), leading to excess body fat, weight gain and obesity. An excess of visceral fat is correlated to metabolic dysfunction including insulin resistance, diabetes, and inflammatory and obesity related disorders [7–10].

Obesity results in numerous changes in visceral adipose tissue including the deposition of fat, enlargement of adipocytes and inflammation. In obese humans and mice, white adipose tissue is characterised by the infiltration of inflammatory macrophages [11–13]. Adipose tissue inflammation during obesity is also associated with an increased production of pro-inflammatory cytokines, such as tumour necrosis factor alpha (TNF-α), and activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) inflammatory signalling pathway [14–16]. Chronic inflammation in this tissue leads to the development of insulin resistance and obesity related metabolic syndromes [14,17–19].

Oxidative stress resulting from the increased production of oxidant species or reactive oxygen species causes tissue injury and pathological conditions such as inflammation, and liver and vascu-

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lar disease [20,21]. Increased oxidative stress has been found in the white adipose tissue of obese mice, which is associated with increased pro-inflammatory cytokines, reduced adipocytokines, hyperlipidaemia and diabetes [20]. A cell culture study using 3T3-L1 cells revealed that oxidative stress may produce these impairments through increased expression of protein kinases including protein kinase B (Akt), extracellular-signal-regulated kinase (ERK), signal transducer and activator of transcription (STAT), c-Jun N-terminal kinase (JNK) [22]. Further, Akt, STAT3, ERK and JNK have been identified as stress activated proteins and shown to promote pathology including insulin resistance, inflammatory disease in the prostate, and β-cell dysfunction [23–25]. The expression of ERK and JNK mRNA, phosphorylated protein and total protein levels are significantly higher in the visceral fat of obese compared to non-obese women [26]. In addition, mice in which JNK has been knocked out ($JNK^{-/-}$) are resistant to the development of obesity [27].

Human studies have shown that obesity results in dysfunction of the sympathetic nervous system (SNS) [28,29]. In obese humans and animals there is reduced adipose lipolysis and sympathetically mediated energy expenditure, as well as a decrease in the β_3 adrenergic receptor, and other catecholamine related mediators, including the rate limiting enzyme tyrosine hydroxylase (TH) and uncoupling protein 2 (UCP2) [29–34].

Bardoxolone methyl (BARD), also known as RTA 402 or CDDO-methyl ester, is a synthetic triterpenoid compound derived from oleanolic acid. BARD has been shown to induce weight loss, suppress inflammation and oxidative stress, and have potential for treating chronic diseases in humans [35–37]. For example, clinical trials have been used to investigate BARD function in chronic kidney disease and type 2 diabetes mellitus. BARD has been orally administered to type 2 diabetic (db/db) mice resulting in reduced body fat mass and liver injury, and an improvement of glucose homeostasis and insulin sensitivity [35]. Although BARD has been studied for the treatment of a number of chronic diseases, the preventive effect of BARD in adipose tissue during HFD-induced obesity has not been investigated.

In this study we examined the anti-obesity effects of BARD in the visceral fat tissue of mice fed a HFD (an established model of diet-induced obesity) [36–38]. We found that oral BARD prevents fat deposition, inflammation and oxidative stress signalling, and improves the sympathetic innervation of visceral fat tissue. The study suggested the therapeutic potential of BARD in preventing HFD-induced obesity.

2. Materials and methods

2.1. Animals

A total of 21 male C57BL/6J mice were purchased from Animal Resource Centre (Perth, Australia). They were acclimatised in the institutional animal facility under controlled conditions (temperature 22 °C, 12 h light/dark cycle) for one week before experimentation. All procedures were approved by the Animal Ethics Committee, University of Wollongong, NSW, Australia, and complied with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Mice were divided into three groups ($n = 7$), and fed a high-fat diet (HFD), a high-fat diet supplemented with bardoxolone methyl (HFD/BARD), and a low-fat diet (LFD, i.e., lab chow). The animals were fed either a HFD (HFD group and HFD/BARD group) containing 40% of calories from fat (SF11-095; Speciality Feeds, WA), or a LFD (LFD group) (AIN93M, Speciality Feeds, WA) for the duration of the experiment. The BARD dosage (10 mg/kg body weight) was based upon previous studies [35,39], and either BARD or saline vehicle was administered in drinking water for 21 weeks. Body weight of the animals was measured before (LFD:HFD:HFD/BARD = 22.1 ± 0.3 g: 21.2 ± 0.5 g: 22.3 ± 0.5 g, $p = 0.23$) and after (LFD:HFD:HFD/BARD = 27.2 ± 0.3 g: 40.8 ± 1.4 g: 28.1 ± 0.6 g, $p < 0.01$) the experiment. At 21 weeks of treatment the mice were asphyxiated in chambers prefilled with CO_2. A portion of the epididymal fat mass was fixed in 4% paraformaldehyde for histology and immunohistochemistry, while another portion was collected, snap-frozen in liquid nitrogen and stored at −80 °C for Western blotting.

2.2. Histology and immunohistochemistry

Paraformaldehyde fixed visceral fat pads were dehydrated, embedded in paraffin and 5 μm tissue sections cut on a microtome. The tissue sections were stained with haematoxylin and eosin and photographed at 20× magnification. The adipocytes were measured quantitatively using the software ImageJ 1.46r (http://imagej.nih.gov/ij/download.html) as per our previous study [40]. Three fields per section and three sections per fat mass were analysed to quantify the area and the number of adipocytes. Immunohistochemistry was used to quantify the number of F4/80 positive crown like structures and macrophages. Tissue sections (5 μm) were deparaffinised and rehydrated in graduated alcohol to ultrapure distilled water. Antigens were retrieved by microwaving in sodium citrate buffer (10 mM, pH 6.0) for 5 min. The sections were then washed with 3% H_2O_2 in methanol, blocked with 5% normal rabbit serum and incubated overnight at 4 °C with anti-F4/80 primary antibody (ab6640, Abcam Inc., Cambridge, MA). Samples were incubated consecutively for 30 min at room temperature with rabbit anti-rat IgG biotinylated secondary antibody (ab6733, Abcam Inc., Cambridge, MA) diluted at 1:50, and then streptavidin-HRP polymer conjugate (2438, Sigma–Aldrich Pty Ltd, Sydney, Australia) diluted at 1:1000. ImmPACT DAB peroxidase substrate kit (4100, Vector laboratories Inc., Burlingame, CA) was used to develop the stained sections before counterstaining with Harris' haematoxylin (POCD Scientific, Artarmon, Australia). Microphotographs were taken as described for H&E stained sections, and ImageJ 1.46r (National Institutes of Health, USA) used to quantify positive F4/80 stained cells and crown like structures.

2.3. Western blot analysis

Western blot was used to detect protein expression, as described previously [41]. Briefly, tissue was homogenised in lysis buffer containing NP-40, protease inhibitor cocktail, β-glycerol phosphate and phenylmethanesulfonyl fluoride. Protein lysates were then centrifuged for 10 min at 4 °C. The concentration of extracted protein was determined using the bicinchoninic acid Protein Assay Kit (Pierce Chemical Co., Rockford, IL). Samples were diluted in laemmli buffer containing 5% 2-mercaptoethanol and heated at 95 °C for 5 min. Protein samples (25 μg per sample) were resolved using 4–12% Bis Tris–HCl gels (Bio-Rad, Gladesville, Australia) before being transferred to Immun-BlotTM PVDF membranes (Bio-Rad). The antibodies used to detect specific proteins are described below. Horseradish peroxidase conjugated secondary antibodies and enhanced chemiluminescence reagent (GE Healthcare, Piscataway, NJ, USA) were used to detect proteins of interest. Quantity One software (Bio-Rad) was used to quantify the Western blot bands. Detected proteins were normalised to β-actin as described previously [41].

2.3.1. Antibodies

The primary antibodies used for Western blot were: TNF-α (sc-8301), UCP2 (sc-6525), JNK (sc-571), β_3-AR (sc-1473), Akt1/2/3 (sc-8312) from Santa Cruz Biotechnology (Dallas, TX); hydroxylase