Journal of Steroid Biochemistry & Molecular Biology xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry & Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Des-acyl ghrelin inhibits the capacity of macrophages to stimulate the expression of aromatase in breast adipose stromal cells

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ARTICLE INFO

Article history:
Received 12 May 2016
Received in revised form 11 July 2016
Accepted 12 July 2016
Available online xxx

Keywords:
Des-acyl ghrelin
Aromatase
Adipose tissue macrophages
Breast cancer
Tumor necrosis factor
Obesity

ABSTRACT

Des-acyl ghrelin is the unacylated form of the well-characterized appetite-stimulating hormone ghrelin. It affects a number of physiological processes, including increasing adipose lipid accumulation and inhibiting adipose tissue inflammation. Breast adipose tissue inflammation in obesity is associated with an increase in the expression of the estrogen biosynthetic enzyme, aromatase, and is hypothesized to create a hormonal milieu conducive to tumor growth. We previously reported that des-acyl ghrelin inhibits the expression and activity of aromatase in isolated human adipose stromal cells (ASCs), the main site of aromatase expression in the adipose tissue. The current study aimed to examine the effect of des-acyl ghrelin on the capacity of mouse macrophages (RAW264.7 cells) and human adipose tissue macrophages (ATMs) to stimulate aromatase expression in primary human breast ASCs. RAW264.7 cells were treated with 0, 10 and 100pM des-acyl ghrelin following activation with phorbol 12-myristate 13-acetate, and cells and conditioned media were collected after 6 and 24 h. The effect of des-acyl ghrelin on macrophage polarization was examined by assessing mRNA expression of pro-inflammatory M1-specific marker *Cd11c* and anti-inflammatory M2-specific marker *Cd206*, as well as expression of *Tnf* and *Ptgs2*, known mediators of the macrophage-dependent stimulation of aromatase. TNF protein in conditioned media was assessed by ELISA. The effect of RAW264.7 and ATM-conditioned media on aromatase expression in ASCs was assessed after 6 h. Results demonstrate des-acyl ghrelin significantly increases the expression of *Cd206* and suppresses the expression of *Cd11c*, *Tnf* and *Ptgs2* in activated RAW264.7 cells. Treatment of RAW264.7 and ATMs with des-acyl ghrelin also significantly reduces the capacity of these cells to stimulate aromatase transcript expression in human breast ASCs. Overall, these findings suggest that in addition to direct effects on aromatase in ASCs, des-acyl ghrelin also has the capacity to inhibit the macrophage-dependent induction of aromatase, and provides a novel mechanism for potential effects of des-acyl ghrelin to break the linkage between obesity and breast cancer.

Abbreviations: ASCs, adipose stromal cells; ATMs, adipose tissue macrophages; CD, cluster of differentiation; Ptgs2, Prostaglandin-Endoperoxide Synthase 2; DAG, des-acyl ghrelin; FCS, fetal calf serum; GHSR1a, growth hormone secretagogue receptor; IL, interleukin; MCP-1, monocyte chemoattractant protein-1; PGE_2, prostaglandin E_2; PMA, Phorbol 12-myristate 12 acetate; qRT-PCR, quantitative real-time polymerase chain reaction; TNF, tumor necrosis factor.

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http://dx.doi.org/10.1016/j.jsbmb.2016.07.005
0960-0760/

1. Introduction

Aromatase is the rate-limiting enzyme in estrogen biosynthesis, responsible for catalyzing the conversion of androgens into estrogens. Prior to menopause, estrogens are mainly produced by the ovaries and fluctuate with every menstrual cycle [1]. They affect reproductive function, mammary gland development, as well as the normal functioning of a number of tissues and organ systems, including maintaining bone and joint health, cognitive function and cardio-metabolic health [1]. After menopause, the ovaries no longer produce measurable levels of estrogens and normal functioning of these tissues is ensured via the local expression of aromatase, with estrogens acting locally in an autocrine and paracrine manner [1]. Aromatase expression at extra-gonadal sites is responsive to a number of stimuli. Of all sites to produce estrogens after menopause, the adipose tissue is by far the most important in terms of tissue volume. In the adipose tissue, aromatase is elevated with obesity, and this is thought to occur, at least in part, due to chronic low grade inflammation that is associated with obesity [2]. Inflammatory mediators, including prostaglandin E_2 (PGE_2) and tumor necrosis factor-α (TNF) stimulate aromatase expression in adipose stromal cells (ASCs), the main cell type responsible for aromatase expression in the adipose tissue [3,4].

Obesity is also associated with inflammation in breast white adipose tissue and an increase in breast aromatase expression [5]. The local production of estrogens in the breast fat is believed lead to a hormonal milieu conducive to cancer development and growth [6]. In obesity, white adipose tissue inflammation occurs as a consequence of the recruitment of immune cells, e.g. macrophages, to dead or dying adipocytes. Macrophages can be classified into two major populations: the classically activated M1 macrophage, characterized by CD11c surface expression and responsible for the production of pro-inflammatory factors including IL-6, TNFα, and IL-1β, and the alternatively activated M2 macrophage, that expresses CD206 and CD301 and secretes anti-inflammatory cytokines [7]. In obesity, the number of M1 macrophages increases in the adipose tissue and correlates with the degree of tissue inflammation [7].

Ghrelin is a 28-amino acid peptide hormone, predominantly produced from the stomach, which has well-characterized roles in the stimulation of appetite and growth hormone release [8]. It is known to act via the growth hormone secretagogue receptor (GHSR1a) to mediate effects in the hypothalamus [8]. Recently, ghrelin has been shown to play a crucial role in glucose and energy homeostasis and bone metabolism, and to inhibit the production of pro-inflammatory cytokines IL-1β and TNFα in lipopolysaccharide-stimulated RAW264.7 macrophages, effects that were antagonized in the presence of a GHSR1a-specific antagonist [9]. Conversely, signaling involving GHSR1a has been shown to stimulate M1 macrophage polarization and adipose tissue inflammation during aging [10]. The physiological levels of ghrelin and its unacylated form, des-acyl ghrelin, are in the range 0.1–0.5 nM [11]. Des-acyl ghrelin circulates more abundantly than ghrelin and does not bind to GHSR1a at physiological concentrations. Despite this, it has been shown to exert a number of biological effects, including regulating adipocyte differentiation and function, reducing adipose tissue inflammation, inhibiting skeletal muscle atrophy, promoting glucose uptake, and stimulating osteoblast proliferation [11]. Effects of des-acyl ghrelin to prevent the dysregulation of glucose metabolism in a diet-induced mouse model of obesity are also associated with a switch in macrophage polarization in epididymal white adipose tissue [12].

Recently, we have demonstrated that des-acyl ghrelin inhibits aromatase expression in isolated ASCs [13]. In the current study, we aimed to determine whether des-acyl ghrelin also had direct effects on macrophage polarization and impacted their capacity to stimulate aromatase expression in isolated ASCs.

2. Material and methods

2.1. Isolation of ASCs, ATMs and tissue culture

Primary human breast adipose stromal cells (ASCs) were isolated from breast reduction surgery and cultured as previously described [14]. Immune cells, including adipose tissue macrophages (ATMs), were isolated after ASC preparation was allowed to adhere for 1 h and characterized based on CD14 and CD16 expression (data not shown). ATMs were plated at a density of 100,000 cells/mL, 10 mL per dish, and allowed to adhere overnight. Mouse macrophages RAW264.7 cells were purchased from American Type Culture Collection (ATCC; USA). RAW264.7 cells and ATMs were grown as an adherent monolayer in DMEM (Sigma), supplemented with 10% fetal calf serum (FCS) (Life Technologies) and 5% penicillin/streptomycin and 1% sodium pyruvate. Tissue culture flasks and dishes were incubated at 37 °C in 5% CO_2 in a humidifying incubator.

2.2. Conditioned media and RNA from RAW264.7 and ATMs

Prior to treatment, RAW264.7 cells or ATMs were serum-starved for 24 h. RAW264.7 cells were pre-treated with 10pM and 100pM des acyl ghrelin (DAG; NeoMPS) for one hour and then activated using 20 nM phorbol 12-myristate 13-acetate (PMA; Sigma). Activated RAW264.7 and human ATMs were treated with 10pM or 100pM des-acyl ghrelin for 24 h. Conditioned media negative controls included incubation of treatment-containing media for 24 h in the absence of cells to account for direct effects of

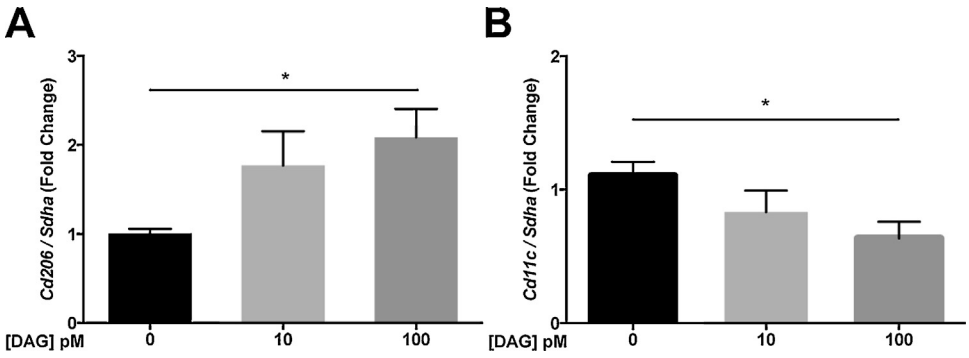

Fig. 1. Des-acyl ghrelin treatment causes a shift in macrophage polarization from M1 to M2.
Mouse macrophages (RAW264.7) were treated with 20 nM phorbol 12-myristate 13-acetate (PMA) and 10pM or 100pM des-acyl ghrelin (DAG) for 6 h. Cells were collected for qRT-PCR. Results demonstrate that DAG (A) inhibits *Cd11c* and (B) stimulates *Cd206* mRNA expression. Data are mean ± SEM; n = 3, experiment repeated twice. * $P < 0.05$.

Please cite this article in press as: C.M.C. Au, et al., Des-acyl ghrelin inhibits the capacity of macrophages to stimulate the expression of aromatase in breast adipose stromal cells, J. Steroid Biochem. Mol. Biol. (2016), http://dx.doi.org/10.1016/j.jsbmb.2016.07.005