Biochemical and Biophysical Research Communications 477 (2016) 678–684

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Adiponectin corrects premature cellular senescence and normalizes antimicrobial peptide levels in senescent keratinocytes

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ARTICLE INFO

Article history:
Received 22 June 2016
Accepted 24 June 2016
Available online 25 June 2016

Keywords:
Adiponectin
Keratinocyte
Senescence
Antimicrobial peptide
SIRT1

ABSTRACT

Stress-induced premature senescence or aging causes dysfunction in the human somatic system. Adiponectin (Acrp30) plays a role in functional recovery, especially with adenosine 3′,5′-monophosphate (AMP)-activated protein kinase (AMPK) and silent mating type information regulation 2 homolog 1 (SIRT1). Acrp30 stimulation reduced the premature senescence positive ratio induced by hydrogen peroxide (H_2O_2) and restituted human β-defensin 2 (hBD-2) levels in senescent keratinocytes. Acrp30 recovered AMPK activity in senescent keratinocytes and increased SIRT1 deacetylation activity. As a result, FoxO1 and FoxO3 transcription activity was recovered. Additionally, Acrp30 stimulation suppresses NFκB p65, which induces abnormal expression of hBD-2 induced by H_2O_2. In the present study, we have shown that Acrp30 reduces premature senescence and recovers cellular function in keratinocytes. These results suggest a role for Acrp30 as an anti-aging agent to improve impaired skin immune barriers.

1. Introduction

White adipose tissue is a structural component of multiple organs in the human body. It is a multifunctional tissue that participates in energy storage and hormonal cytokine production. Adipocytes function as an endocrine organ to control triglyceride storage and energy release.

Adipokines, or cytokines released by adipocytes, have recently been studied due to their unique physiologic properties [1–4]. Adiponectin (Acrp30) is an adipokine that is secreted from adipocytes and sebocytes at a concentration of 5–10 μg/ml in the plasma. Recent studies have shown a negative correlation between Acrp30 levels with body weight, fat mass and insulin sensitivity [2,3]. Acrp30 regulates insulin sensitivity, which improves glucose uptake by cells and regulates energy expenditure more efficiently. Additionally, it stimulates adenosine 3′,5′-monophosphate (AMP)-activated protein kinase (AMPK) activity, which plays a key role in energy homeostasis regulation and metabolic stress [5]. Through AMPK stimulation, Acrp30 suppresses essential enzymes involved in adenosine triphosphate (ATP)-consuming anabolic pathways, such as nicotinamide adenine dinucleotide phosphate (NAD^+/NADPH). This results in an increase in cellular ATP supplies, and enhancement of silent mating type information regulation 2 homolog 1 (SIRT1) deacetylation activity [6].

SIRT1 is a class III histone deacetylase (HDAC3), which is involved with many cellular process regulations such as apoptosis, differentiation, cellular senescence, endocrine signaling, glucose homeostasis, aging and longevity [7]. SIRT1 has various targets including acetylated p53, p300, forkhead box O (FoxO), peroxisome proliferator-activated receptor (PPARα), PPARγ and PPARγ coactivator-1α (PGC-1α) [6]. SIRT1 promotes keratinocyte differentiation through transcription factors PPARα, PPARγ and CCAAT/enhancer-binding protein alpha (CEBPα) [8].

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http://dx.doi.org/10.1016/j.bbrc.2016.06.119
0006-291X/

Additionally, antimicrobial peptides play an integral part in the innate immunity of the skin. β-defensins, a family of mammalian antimicrobial peptides, have antimicrobial activity against bacterial, fungal and viral pathogens in the skin [9]. The antimicrobial peptides attack invading pathogens by breaking down their cell walls [10]. They are produced by keratinocytes in the skin and increase in concentration with keratinocyte differentiation or as a defense mechanism of the innate immune system. However, there can be abnormal increases in antimicrobial peptides levels with ultraviolet B (UVB) or reactive oxygen species (ROS) stress. For example, recent studies in HaCaT cells, or immortal keratinocytes, treated with UVB have shown abnormally high levels of antimicrobial peptides expression [11–13]. There are also clinical reports indicating abnormally high levels of antimicrobial peptides in aged skin, which were predicted to be expose by cumulative UVB and ROS stress. Abnormal levels of AMPs lead to innate immune related skin diseases such as psoriasis or immune skin diseases [13,14].

Acrp30 also has multiple beneficial anti-apoptotic, anti-inflammatory and anti-oxidative effects [14]. However, there are few reports on the effects of Acrp30 on keratinocytes except with cellular proliferation and wound healing. It has been extrapolated that Acrp30 may have anti-aging benefits such as cellular damage repair, enhancement of keratinocyte differentiation and moisturization, and improvement of antimicrobial barriers. Therefore, we have developed an *in vitro* premature senescence model using hydrogen peroxide (H_2O_2) treatment to study the effects of Acrp30 in restituting the antimicrobial barrier.

2. Material and methods

2.1. Chemicals and reagents

Full-length recombinant human Acrp30 was obtained from Biobud (Seongnam, South Korea). H_2O_2 was purchased from Junsei chemicals (Tokyo, Japan). H_2O_2 was diluted with deuterium-depleted water (DDW) to a designated concentration and filtrated using a Millex GV 0.22 μm pore disk filter unit, which was obtained from Millipore (Carrigtwohill, Ireland). SIRT1, FoxO3, phosphor FoxO3 (S253), FoxO1A, phosphor FoxO1A (S256), AMPKα, phosphor AMPKα (T172), p38, phosphor p38, c-Jun N-terminal protein kinase (JNK), phosphor JNK, extracellular signal-regulated kinase (ERK), phosphor ERK, nuclear factor kappa light chain enhancer of activated B cells (p65-NFκB), phosphor p65-NFκB (S536), phosphor p65-NFκB (S468), Rel B, histone H2A.X and phosphor histone H2A.X (S139) antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA); human β-defensin 2 (hBD-2) antibody was purchased from abCam (Cambridge, UK); phosphor FoxO1A (T24), Cu/Zn superoxide dismutase (SOD1), lamin B and β-actin antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA); $p16^{INK4A}$ antibody was obtained from R&D Systems (Minneapolis, MN, USA); and magnesium superoxide dismutase (SOD2) antibody was obtained from ABfrontier (Seoul, South Korea).

2.2. Cell culture

Human keratinocytes were obtained from Life Technologies (Carlsbad, CA, USA). Keratinocytes were maintained under Epi-life growth medium (Life Technologies) at 37 °C in a humidified atmosphere containing 5% CO_2-95% air. Epi-life growth medium was refreshed every two days until a sub-confluent or designated cell population was achieved. All experiments were performed using cells on the third or fourth passage.

2.3. Senescence associated β-galactosidase assay

Senescent status was verified by an *in situ* senescence-associated β-galactosidase (SA-β gal) immunostaining assay. Keratinocytes were grown on 12-well or 6-well cell culture cluster plates. Keratinocytes were treated with designated concentrations of H_2O_2 for 2 h. After exposure to H_2O_2, each group was treated with Acrp30. SA-β gal staining was performed after each treatment, as described previously [15]. Cells were observed and photographed by an Olympus CKX41 microscope (Olympus, Japan) for senescent ratio analysis of 5 regions per treatment group. Stained and unstained cells were counted and the senescent ratio was compared with the cellular viability ratio of each treatment group.

2.4. Immunoblotting analysis

The regulation of AMP expression levels by Acrp30 was measured by immunoblotting analysis. Keratinocytes were treated with candidate compounds and washed twice in ice cold phosphate buffered saline (PBS). The samples were lysed in lysis buffer containing 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 150 mM sodium chloride (NaCl), 1 mM sodium orthovanadate (Na_3O_4V), 1% Triton X-100 and DDW. One tablet of complete protease inhibitor cocktail (Roche Applied Science, Penzberg, Germany) was added before cell lysis. Protein concentrations were determined using a bicinchoninic acid solution kit (Sigma-Aldrich, St. Louis, MO, USA). Proteins were separated by an 8%, 12.5% or 15% SDS-polyacrylamide/bis-acrylamide gel and transferred onto a poly vinylidene difluoride (PVDF) membrane (Millipore Co, Bedford, MA, USA). The membrane was blocked for 1 h by 5% or 10% non-fat dry milk (BD Biosciences, San Jose, CA, USA). After blocking the membrane, it was rinsed with tris buffered saline-tween 20 (TBS-T, 0.1% tween 20) and incubated with primary antibodies for 1 h at room temperature or overnight at 4 °C. The membrane was washed five times with TBS-T for 7 min, and then incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibodies. After extensive washing, the bands were detected by Enhanced Chemi-Luminescence (ECL) reagent (ATTO Corporation, Tokyo, Japan). All immunoblotting data were quantified with β-actin or lamin B.

2.5. SIRT1 direct activity assay

SIRT1 direct activity levels were detected using a deacetylase Fluor-metric activity assay kit (Enzo Life Sciences, Farmingdale, NY, USA). The fluorescence intensity was read at Ex: 360 nm and Em: 460 nm with a Victor 3 multi-counting reader (Perkin-Elmer, San Jose, CA, USA).

2.6. Reverse transcriptase polymerase chain reaction (RT-PCR) analysis

RT-PCR was used to measure the expression levels of the AMPs. Keratinocytes were seeded on 60 mm diameter cell culture dishes and treated with Acrp30 or H_2O_2. Cells were harvested after treatment termination and total ribonucleic acid (RNA) was isolated using TRIzol reagent with chloroform/isopropyl alcohol capture (Geneall Inc., Seoul, Korea). RNA concentrations were estimated based on absorbance at 260 nm using the Nanodrop ND 1000 spectrophotometer (Thermo Fisher Scientific, Carlsbad, CA, USA). 1 μg of total RNA was reverse transcribed with 200 units of Moloney Murine Leukemia (M-MLV) reverse transcriptase (Fermentas, Carlsbad, CA, USA) for 60 min using oligo(dt)12–18 in a 20 μl reaction. The first-strand cDNA (2 μl) was amplified by PCR master mix (Fermentas, Carlsbad, CA, USA) with 10 pmol of primer