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Ubiquitin specific protease 2 acts as a key modulator for the regulation of cell cycle by adiponectin and leptin in cancer cells

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ABSTRACT

Adiponectin and leptin, both produced from adipose tissue, cause cell cycle arrest and progression, respectively in cancer cells. Ubiquitin specific protease-2 (USP-2), a deubiquitinating enzyme, is known to impair proteasome-induced degradation of cyclin D1, a critical cell cycle regulator. Herein, we investigated the effects of these adipokines on USP-2 expression and its potential role in the modulation of cell cycle. Treatment with globular adiponectin (gAcrp) decreased, whereas leptin increased USP-2 expression both in human hepatoma and breast cancer cells. In addition, overexpression or gene silencing of USP-2 affected cyclin D1 expression and cell cycle progression/arrest by adipokines. Adiponectin and leptin also modulated *in vitro* proteasomal activity, which was partially dependent on USP-2 expression. Taken together, our results reveal that modulation of USP-2 expression plays a crucial role in cell cycle regulation by adipokines. Thus, USP-2 would be a promising therapeutic target for the modulation of cancer cell growth by adipokines.

1. Introduction

Adipose tissue acts as a dynamic endocrine organ via secretion of a wide range of hormone-like substances, which are collectively called adipokines (Havel, 2002). Among the various adipokines, adiponectin and leptin are known to generate many biological actions potently. Adiponectin has been shown to play diverse protective roles ranging from control of glucose and lipid metabolism to suppression of inflammation, steatohepatitis, insulin resistance, cardiovascular disease, and a wide array of cancers (Perrier and Jarde, 2012; Lim et al., 2014). Once secreted from adipose tissue, adiponectin circulates in blood stream and produces its responses by binding to adipoR1, adipoR2 or T-cadherin localized in target tissues throughout the body (Lee et al., 2008). Recently, the role of adiponectin in the modulation of cell death or survival, particularly in cancer cells, has been extensively studied. Although adiponectin has been shown to cause cell proliferation (Ogunwobi and Beales, 2006), many other recent studies support the notion that adiponectin possesses anti-proliferative properties (Man et al., 2010; Nepal and Park, 2014). For the suppression of cancer cell proliferation, adiponectin induces programmed cell death (apoptosis) (Saxena et al., 2010) and/or cell cycle arrest (Wang et al., 2006). Leptin also harbors metabolic functions, including regulation of appetite and control of body fat mass (Kelesidis et al., 2010). In addition, it is well established that leptin stimulates cell proliferation in many cancer cells (Garofalo and Surmacz, 2006). It is well established that adiponectin and leptin have opposing roles in the modulation of cancer cell death or survival. However, detailed mechanisms underlying are still largely unknown.

The ubiquitin–proteasome system (UPS) is one of the major protein degradation pathways required for the maintenance of intracellular protein homeostasis. UPS is a process wherein one or more ubiquitin moieties are attached to the substrate that undergoes proteasomal degradation in 26S proteasome in an ATP-dependent manner (Ding et al., 2014). For the degradation of intracellular proteins through UPS, a number of enzymes such as ubiquitin activating enzyme (E1), ubiquitin conjugating enzymes (E2), and an ubiquitin protein ligase (E3) are involved (Johnson, 2015). In addition, the overall proteasomal activity depends on the activity of deubiquitinases, as well as the proteasome (Ding et al., 2014). Increasing evidence suggests that UPS plays a cardinal role in many pathological conditions, such as cancer, neurodegeneration, and metabolic disorders (Schmidt and Finley, 2014). The relationship between UPS and cancer has hit fever pitch in recent years. In particular, it has been reported that ubiquitination is involved in the control of cell death, survival and/or proliferation through modulation of apoptosis, autophagy and cell cycle progression (Ding et al., 2014; Teixeira and Reed, 2013). Also, many tumor suppressors and oncogenes interact with enzymes involved in UPS and ubiquitin-mediated signaling pathways are therefore found to be altered in the tumor tissue (Ding et al., 2014).

Deubiquitinating enzymes (deubiquitinases or DUBs), a group of enzymes removing ubiquitin from proteins and other molecules,

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are classified into two classes, including cysteine proteases and metalloproteases (Reyes-Turcu et al., 2009). Recent reports suggest the involvement of DUBs in cancer biology, making it a potential therapeutic target for cancer therapeutics (Hussain et al., 2009). Approximately 100 DUBs have been characterized and found to be involved in the control of various biochemical pathways. Among them, ubiquitin specific protease-2 (USP-2) is a cysteine protease which removes ubiquitin from substrates and therefore could spare substrates from proteasomal degradation. USP-2 is specifically implicated in TNF-α induced cytoprotection in hepatocytes (Haimerl et al., 2009; Metzig et al., 2011), circadian regulation (Molusky et al., 2012), and myoblast differentiation (Park et al., 2002). A growing body of evidence additionally suggests that USP-2 plays a role in the control of oncogenic behavior in cancers via controlling degradation of various proteins. For example, USP-2 interacts and stabilizes fatty acid synthase which is overexpressed in prostate cancer cells (Graner et al., 2004) or causes murine double minute 2 (MDM2) accumulation and enhance MDM2-mediated p53 degradation, wherein p53 affects expression of several genes involved in apoptosis in cancer cells (Stevenson et al., 2007). Importantly, USP-2 has been also shown to impair ubiquitination of the proteins involved in cell cycle, including cyclin A1 and cyclin D1 and thereby, stabilize these cell cycle regulators (Kim et al., 2012; Shan et al., 2009). In particular, USP2 acts as a specific deubiquitinase for cyclin D1. USP2 promotes stabilization of cyclinD1 via direct interaction with cyclin D1 and antagonizes ubiquitin-dependent degradation (Shan et al., 2009). This abnormal overexpression of cyclin D1 is closely related with development of tumorigenesis in many cancer types (Lee et al., 2010).

Cyclin D1 is known as a crucial regulator of cell cycle progression. It is well known that overexpression (or activation) of cyclin D1 is closely involved in tumorigenesis. During cell proliferation, cyclin D1 together with cyclin dependent kinase 4 and 6 form complexes, which stimulate cell cycle progression by phosphorylating retinoblastoma protein (Alao, 2007). It is being increasingly understood that cyclin D1 undergoes ubiquitination-dependent proteasomal degradation, which could be controlled by E3 ligase or USP-2 (Shan et al., 2009). In particular, it has been recently shown that USP-2 deubiquitinates cyclin D1, prevents degradation, accumulates cyclin D1 and finally leads to cell cycle progression from G1 to S phase (Lee et al., 2010).

Based on previous reports, it is well known that adiponectin and leptin differentially modulate cyclin D1 expression and cell cycle in various different types of cancer cells (Chen et al., 2007; Saxena et al., 2010), and USP-2 plays an important role in the modulation of cyclin D1 expression (Shan et al., 2009). However, the involvement of USP-2 in the modulation of cell cycle by these adipokines has not been explored. Thus, to better understand mechanisms underlying cyclin D1 expression and cell cycle modulation by adiponectin and leptin, we examined the effect of these adipokines on USP-2 expression and its potential role in cell cycle regulation in cancer cells. Herein, we demonstrate for the first time that adiponectin decreases and leptin increases USP-2 expression. Moreover, we also show that alterations in USP-2 expression by adipokines play a crucial role in the regulation of cell cycle progression, at least in part, via changes in cyclin D1 expression.

2. Materials and methods

2.1. Materials

All the cell culture reagents were obtained from Hyclone Laboratories (South Logan, Utah, USA). Recombinant human globular adiponectin (gAcrp) was obtained from Peprotech Inc. (Rocky Hill, NJ, USA) and recombinant mouse leptin was purchased from Sigma-Aldrich (St. Louis, MO, USA). Cell proliferation assay kit (MTS) and Proteasome-Glo Cell based assay kit were purchased from Promega Corporation (Madison, WI, USA). Antibodies against USP-2 and β-actin were obtained from Cell Signaling Technology Inc (Beverly, MA, USA). CyclinD1 polyclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horse radish peroxidase (HRP) conjugated goat anti-rabbit IgG was obtained from Pierce Biotechnology (Rockford, IL, USA). USP-2 plasmid was a gift from Cheryl Arrowsmith (Addgene plasmid #36894). MG132, a proteasome inhibitor, and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless mentioned elsewhere.

2.2. HepG2 and MCF-7 cell culture

HepG2 and MCF-7 cell lines were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA) and routinely cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS and 1% penicillin–streptomycin in the presence or absence of 0.1% amphotericin, respectively.

2.3. MTS assay

For the determination of cell number, cells were seeded in 96 well plates at the density of 4×10^4 cells per well essentially as described previously (Nepal and Park, 2014). After incubation with MTS (3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) for 2 h at 37 °C, the resultant cell viability/number was monitored via a Versamax microplate reader (Sunnyvale, CA, USA) by measuring absorbance at 490 nm.

2.4. Proteasomal activity assay

Cells were seeded at the density of 2×10^4 cells per well in white-walled 96 well plates. After overnight incubation, cells were either treated with gAcrp, leptin, betulinic acid (BA) or MG-132 as indicated in figure legends. Finally, 50 μl of Proteasomal–Glo solution (Promega), which measures the enzymatic activity in isolated proteasome using a luminogenic substrate, was added and cells were incubated for 1 h. Proteasomal activity was finally assessed by measurement of luminescence from luminogenic proteasome substrate, Suc-LLVY-Glo, with a micro-plate reader (Fluostar Optima, BMG Labtech, Ortenberg, Germany).

2.5. RNA isolation, reverse transcription (RT) and quantitative PCR (qPCR)

To measure the mRNA levels of genes of interest, total RNAs were isolated using Qiagen lysis solution (Qiagen, MD, USA) according to the manufacturer's instructions and reverse transcribed for the synthesis of cDNA. Real time-PCR amplification was then carried out with a Roche LightCycler 2.0 (Mannheim, Germany) using the absolute QPCR SYBR green capillary mix AB gene system (Thermoscientific, UK) at 95 °C for 15 s, 56 °C for 30 s and 72 °C for 45 s. The primer sequences used for amplification of target genes are listed in Table 1. Comparative threshold (C_t) method was used for the determination of target mRNA after normalizing target mRNA C_t values to those for glyceraldehyde-3- phosphate dehydrogenase GAPDH (ΔCt).

Table 1
Sequences of human primers used in quantitative RT-PCR.

Target gene	Primer	Nucleotide sequence
GAPDH	F	5′-ACCACAGTCCATGCCATCAC-3′
	R	5′-TCCACCACCCTGTTGCTGTA-3′
USP-2	F	5′-CTTCTGGGACCTCTCACTGC-3′
	R	5′-TCTTTGGGAACCTCTGGATG-3′