Original article

MOLECULAR METABOLISM

Adiporedoxin, an upstream regulator of ER oxidative folding and protein secretion in adipocytes

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ABSTRACT

Objective: Adipocytes are robust protein secretors, most notably of adipokines, hormone-like polypeptides, which act in an endocrine and paracrine fashion to affect numerous physiological processes such as energy balance and insulin sensitivity. To understand how such proteins are assembled for secretion we describe the function of a novel endoplasmic reticulum oxidoreductase, adiporedoxin (Adrx).

Methods: Adrx knockdown and overexpressing 3T3-L1 murine adipocyte cell lines and a knockout mouse model were used to assess the influence of Adrx on secreted proteins as well as the redox state of ER resident chaperones. The metabolic phenotypes of *Adrx* null mice were characterized and compared to WT mice. The correlation of Adrx levels BMI, adiponectin levels, and other inflammatory markers from adipose tissue of human subjects was also studied.

Results: Adiporedoxin functions *via* a CXXC active site, and is upstream of protein disulfide isomerase whose direct function is disulfide bond formation, and ultimately protein secretion. Over and under expression of Adrx *in vitro* enhances and reduces, respectively, the secretion of the disulfide-bonded proteins including adiponectin and collagen isoforms. On a chow diet, *Adrx* null mice have normal body weights, and glucose tolerance, are moderately hyperinsulinemic, have reduced levels of circulating adiponectin and are virtually free of adipocyte fibrosis resulting in a complex phenotype tending towards insulin resistance. Adrx protein levels in human adipose tissue correlate positively with adiponectin levels and negatively with the inflammatory marker phospho-Jun kinase.

Conclusion: These data support the notion that Adrx plays a critical role in adipocyte biology and in the regulation of mouse and human metabolism *via* its modulation of adipocyte protein secretion.

Keywords Adipocyte; Adipokine; Protein secretion; Endoplasmic reticulum; Oxidoreductase; Disulfide bond formation

1. INTRODUCTION

In addition to fat storage and release, white adipose tissue (WAT) makes and secretes adipokines, hormone-like polypeptides, which act in an endocrine and paracrine fashion to affect numerous physiological processes such as energy balance and insulin sensitivity [1,2]. In addition, fat cells in adipose tissue are maintained as an organ by extracellular matrix proteins including multiple collagen isoforms whose cellular origin is not completely clear [3]. The initial folding and assembly of all secreted and integral membrane proteins, takes place in the endoplasmic reticulum (ER) and is mediated by the actions of numerous enzymes and chaperones [4–6]. About 1/3 of all cellular proteins pass through the ER and the vast majority of these have inter- and/or intra-molecular disulfide bonds, the formation of which also takes place in the ER mediated by oxidoreductases of the thioredoxin superfamily [6–8]. Improper disulfide bond formation leads to protein misfolding and, potentially, ER stress, which can have deleterious consequences for cells, for example, apoptosis in the endocrine pancreas due to insulin misfolding [9,10]. Therefore, while normal ER function is essential in all cells, it is especially important in secretory cells such as adipocytes and pancreatic beta cells.

Adiponectin assembly and disulfide bond formation impose a particular burden on the adipocyte ER because it circulates at high concentrations, ca. 30 nM, yet is turned over quite rapidly in blood [11]. Adiponectin has a complicated tertiary structure with post-translational modifications that include the ER-dependent formation of intermolecular disulfide bonds that are required for oligomerization into its most physiologically relevant form, an octadecamer (the high molecular weight or HMW form) [12–14], as well as hydroxylation, glycosylation and proline isomerization all of which occur in its collagenous domain [15–17]. Adiponectin must therefore be properly and efficiently assembled and secreted at high rates, and malfunctions of this process will lead to ER stress in the adipocyte and subsequent

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perturbation of fat cell and organismal metabolism such as insulin resistance and type 2 diabetes [18]. The assembly and secretion of collagens from adipocytes (and other cells) also requires disulfide bonds [17], and excess collagen deposition leads to a fibrotic pathology in adipose tissue [19].

What little is known about disulfide bond formation in the adipocyte ER involves adiponectin assembly, which requires the action of the enzyme endoplasmic reticulum oxidoreductase 1α (ERo1α), a primary electron acceptor that uses molecular oxygen and a flavin nucleotide to initiate oxidative protein folding [20]. ERo1α does not interact directly with substrates such as adipokines to mediate S—S bond formation, rather it oxidizes members of the protein disulfide isomerase (PDI) family, which interact with substrates and mediate correct disulfide bond formation and protein folding [20—22]. ERo1α has also been shown to interact with endoplasmic reticulum protein, ERp44, to mediate what has been called dynamic ER retention of substrates [23]. The combined actions of ERo1α and ERp44 have been shown to play a role in adiponectin assembly and secretion [24,25]. A third PDI relative implicated in adiponectin multimerization is the disulfide bond A oxidoreductase-like protein (DsbA-L) [26]. Unlike ERo1α and ERp44, it lacks both active site cysteines required for redox function and a classical ER localization signal, but it may instead have a chaperone function in protein folding. None of the above proteins has a notable tissue-specific distribution although the expression of DsbA-L is somewhat higher in adipose than in other tissues examined, and it is induced upon differentiation of 3T3-L1 cells [26].

We performed a proteomic analysis of GLUT4 storage vesicles (GSVs) that were selectively immunoadsorbed with anti-GLUT4-coupled beads following depletion of GLUT4-precursor compartments [27]. Amongst the proteins we discovered in the GSV-precursor membranes were several potential ER residents including one that was known at the time only as a possible secreted protein (c10orf58) whose mRNA was expressed in human testes [28]. Its sequence revealed a CXXC motif, which is the active site sequence of an oxidoreductase [6,21]. We determined by qPCR that its mRNA was expressed in mouse testes, but we also saw that mRNA expression in brown and white fat was an order of magnitude higher than in any other mouse tissue except testes (data not shown), which we confirm here for protein expression (Figure 1). Consequently, we are calling this molecule adiporedoxin (Adrx), and we show here that it plays an important role in the assembly and secretion of disulfide-bond containing proteins, in particular, adiponectin in murine and human cells and adiponectin and collagens in murine cells. Its absence in mice leads to a complex and mixed phenotype of lower adipokine secretion and lower adipocyte collagen deposition along with adipocyte ER stress and hyperinsulinemia.

2. MATERIALS AND METHODS

2.1. Animals

Primary adipocytes from male Sprague—Dawley rats (175—200 g, Harlan Laboratories, Indianapolis, IN) were isolated and fractionated as described [27]. The same fractionation protocol was performed for 3T3-L1 cultured murine adipocytes. Age matched, 8 week old C7BL/6 male and female mice were purchased from Jackson Laboratory (Farmington, CT), maintained at 22 °C with a light—dark cycle (light from 0700 to 1900h), and allowed free access to food and water. At 10 weeks of age, mice were body weight matched into groups fed either HF (45% of calories, mainly as lard) or LF (15%) diets, then sacrificed as described [29]. All females were euthanized at the pro-estrus phase. For Adrx tissue distribution, mice were perfused with PBS, tissues were carefully dissected with particular attention to removing any fat, immediately mechanically homogenized in a tissue grinder with RIPA buffer containing a protease inhibitor cocktail and subjected to a 1000 × g spin to remove debris. The supernatant was collected for SDS-PAGE and Western blot analysis. For fat tissue, the infranatant below the fat cake was collected for analysis.

The *Adrx* knockout mice were generated by microinjecting targeted ES cells (KOMP, derived from C57BL/6N, VG15730, clone 15730A-H5) into C57BL/6N blastocysts, and these gave rise to male chimeras with significant ES cell contribution (as determined by coat color). By mating with C57BL/6N females and genotyping the offspring by PCR analysis, germ line transmission was confirmed. Male and female heterozygous F1 animals were interbred to obtain *Adrx* knockout (*Adrx* KO) animals. Male animals only were analyzed at >8 weeks of age. The animals were maintained in a pathogen-free animal facility at 22 °C under a 12-h light/12-h dark cycle with access to a standard rodent chow. Except when specifically noted, all mice used for *in vivo* or *in vitro* studies were fasted for 4—6 h starting from early morning prior to use. For tissue harvesting, mice were sacrificed under CO_2 anesthesia, and tissues were rapidly taken and immediately frozen in liquid nitrogen and stored at −80 °C until biochemical analysis. Tissue and cell secreted collagen content was measured using Sirius Red Total Collagen Detection Kit (Chondrex, Redmond, WA, #9062). Mouse serum total and HMW adiponectins were measured by using ELISA kit from ALPCO (Salem, NH, #47-ADPMS-E01).

2.2. Primary human adipose cells

Human adipose stromal vascular cells (SVC) were isolated from adipose tissue by collagenase digestion, grown and differentiated as previously described [30]. Adipocytes (day 10 post differentiation) were transfected with control or a combination of two Adrx-directed siRNA (SI04213587 and SI04959066, Qiagen, Hilden, Germany) using Lipofectamine and PLUS reagents (Life Technologies, Carlsbad, CA).

2.3. Cell culture

Murine 3T3-L1 cells were cultured, differentiated, and maintained as described previously [27]. Human embryonic kidney (HEK)-293T cells were cultured in DMEM with 10% FBS. For Adrx overexpression, confluent 3T3-L1 fibroblasts were infected with a pBABE retrovirus construct driving Adrx cDNA using the following oligo primers: sense sequence containing BamH1 restriction site, 5′-ttttggatccatgtcttttctccaggac-3′ and an antisense sequence containing an EcoR1 restriction site, 5′ttttgaattctcagcttctcccggacgctggagt-3′, then differentiated for subsequent analysis as fat cells. Site directed mutagenesis of Adrx was performed using a Quickchange Mutagenesis Kit (Agilent, Santa Clara, CA) following the manufacturer's instructions.

For Adrx silencing, mouse lentiviral vectors driving several shRNA sequences were purchased from Open Biosystems (Huntsville, AL) and transfected into HEK-293T cell with trans-IT 293 (Mirus, Madison, WI) according to the manufacturer's instructions. Supernatant containing retrovirus was harvested 48 h later and used to infect confluent 3T3-L1 fibroblasts prior to their differentiation. Four pLKO.1 c10orf58 mouse lentiviral vectors were tested. All sequences were effective with target # 3 showing the best knockdown efficiency (>90%), and it was used for all of the adiporedoxin studies.

2.4. QPCR

Total RNA was isolated, quantified and reverse-transcribed using a commercial cDNA synthesis kit. Quantitative PCR (qPCR) was performed with commercially available TaqMan probes (Thermo Fisher

