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Cell death–inducing DFF45-like effector C is reduced by caloric restriction and regulates adipocyte lipid metabolism

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Abstract

Members of the cell death–inducing DFF45-like effector (CIDE) gene family have been shown to regulate lipid metabolism. In this article, we report that the third member of the human CIDE family, CIDEC, is down-regulated in response to a reduced caloric intake. The down-regulation was demonstrated by microarray and real-time polymerase chain reaction analysis of subcutaneous adipose tissue in 2 independent studies on obese patients undergoing treatment with a very low calorie diet. By analysis of CIDEC expression in 65 human tissues, we conclude that human CIDEC is predominantly expressed in subcutaneous adipocytes. Together, these observations led us to investigate the effect of decreased CIDEC expression in cultured 3T3-L1 adipocytes. Small interfering RNA–mediated knockdown of CIDEC resulted in an increased basal release of nonesterified fatty acids, decreased responsiveness to adrenergic stimulation of lipolysis, and increased oxidation of endogenous fatty acids. Thus, we suggest that CIDEC is a regulator of adipocyte lipid metabolism and may be important for the adipocyte to adapt to changes in energy availability.

1. Introduction

A core function of adipose tissue is to store and release energy in the form of lipids, and it responds promptly to the nutritional status of the body. Impairment in this function seems to be partly responsible for the development of obesity and obesity-related metabolic disturbances, such as insulin resistance and type 2 diabetes mellitus [1,2].

Recent reports indicate that the cell death–inducing DFF45-like effector (CIDE) gene family is involved in the regulation of lipid metabolism. The CIDE gene family consists of 3 members: CIDEA, CIDEB, and CIDEC (also referred to as *CIDE-3*). CIDEA knockdown increases the lipolytic capacity of mouse and human adipocytes, and CIDEA-deficient mice are lean and resistant to diet-induced obesity [3,4]. We have shown that levels of human CIDEA in subcutaneous adipose tissue are regulated by energy intake and associated with basal metabolic rate, body fat content, and serum insulin levels [5]. CIDEB-deficient mice are also less susceptible to diet-induced obesity and display a significant decrease in liver triglyceride content and secretion [6]. The mouse homologue of the CIDEC gene (fat-specific protein 27) is localized to intracellular lipid droplets and was recently shown to affect lipolysis [7]. However, no report on the function or expression of human CIDEC in relation to metabolism has been published.

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This study aimed to investigate the regulation of CIDEC during caloric restriction, its tissue distribution, and its role in adipocyte lipid metabolism.

2. Material and methods

All study subjects received written and oral information before giving written informed consent. These studies were approved by ethical review boards at Göteborg University and the University of Cape Town.

2.1. Subjects and samples

To determine the tissue distribution of CIDEC expression, microarray expression profiles of 65 human tissues were acquired from the GEO database (data set GSE3526). Each tissue in this data set is represented by Human Genome U133 plus 2.0 DNA microarray (Affymetrix, Santa Clara, CA) expression profiles from 3 to 9 different individuals.

For real-time polymerase chain reaction (rtPCR) analysis of CIDEC tissue distribution, RNA from adipose tissue and isolated adipocytes from 3 healthy women (body mass index [BMI] 23.0-27.6 kg/m^2) was used together with the Human Total RNA Master Panel II (Clontech, Mountain View, CA).

For the microarray study on adipose tissue depots, deep subcutaneous, superficial subcutaneous, and omental adipose tissue biopsies were obtained from 6 women with an average BMI of 27.4 ± 3.5 kg/m^2.

In the very low calorie diet (VLCD; 450 kcal/d) microarray study, 6 women and 18 men (starting BMI of 37.6 ± 4.9 kg/m^2; age, 25-61 years) were given a VLCD for 16 weeks. Subcutaneous adipose tissue biopsies were obtained at the start of VLCD treatment (week 0), twice during the VLCD phase (weeks 8 and 16), and 2 weeks after normal food was reintroduced (week 18). On average, patients lost 27.7 kg during the diet [5,8].

In the VLCD rtPCR study, 8 men and 20 women (starting BMI of 36.3 ± 3.7 kg/m^2; age, 18-59 years) were given a VLCD for 12 weeks. Subcutaneous adipose tissue biopsies were obtained at the start of VLCD treatment (week 0) and 3 times during the VLCD phase (weeks 2, 6, and 12). On average, patients lost 19.6 kg during the diet.

2.2. RNA preparation

Adipose tissue biopsies were stored at −80°C until analysis. Adipocytes were isolated as previously described [9]. Total RNA from human tissues was prepared with the RNeasy Lipid Tissue kit, and RNA from 3T3-L1 cells was prepared with the RNeasy kit (both from Qiagen, Germantown, MD).

2.3. Real-time PCR

Human low-density lipoprotein receptor–related protein 10 was used as reference to normalize the expression levels between adipose tissue samples, and peptidyl-prolyl isomerase A was used as reference in the tissue distribution panel. These genes have consistently shown small interindividual and intertissue variation, respectively [10].

Assay-On-Demands were used for detection of mouse CIDEC, low-density lipoprotein receptor–related protein 10, and peptidyl-prolyl isomerase A. Human CIDEC was analyzed with an Assay-on Demand targeted to exon 3 (detects full-length isoform) and an Assay-by-Design detecting the full-length and alternatively spliced α-isoform (primer sequences GGCTGCCTGCCTGTGA and GGGACTTCATGGCGTATTCCA, probe sequence ACTGCGTTGGACCTCT). Assays and reagents for rtPCR analysis were from Applied Biosystems (Foster City, CA) and were used according to the manufacturer's protocol. All standards and samples were analyzed in triplicates.

2.4. Microarray

Preparation of complementary RNA and hybridization to microarrays were performed as previously described [9,11,12]. The RNA from the VLCD study was analyzed with the Human Genome U133A DNA microarray, and the RNA from the adipose tissue depot was analyzed with the Human Genome U133 plus 2.0 DNA microarray (both from Affymetrix).

2.5. Cell culture and CIDEC gene silencing

Cell culture reagents were from PAA Laboratories (Pasching, Austria), Novonordisk (Bagsvaerd, Denmark), and Sigma-Aldrich (St Louis, MO). The 3T3-L1 cells were cultured in Dulbecco modified Eagle medium (high glucose) supplemented with sodium pyruvate, penicillin, streptomycin, nonessential amino acids, L-glutamine, and 10% fetal calf serum. Differentiation was started 2 days postconfluence by addition of 500 μmol/L 3-isobutyl-1-methylxanthine, 250 nmol/L dexamethasone, and 860 nmol/L insulin to cell culture medium. After 4 days, 3-isobutyl-1-methylxanthine and dexamethasone were excluded; and cells were cultured for 2 more days in 860 nmol/L insulin. Finally, cells were cultured in original culture medium for 4 more days.

Differentiated 3T3-L1 cells were electroporated with stealth small interfering RNA (siRNA) (Invitrogen, Carlsbad, CA) using the Amaxa Nucleofector (Amaxa, Cologne, Germany) according to the instructions of the manufacturer. Experiments were performed 24 hours after electroporation. All siRNA experiments were performed twice, with similar results. In each experiment, 2 separate anti-CIDEC siRNA oligos were used to control for nonspecific siRNA effects.

2.6. Analysis of lipolysis and fatty acid oxidation

Cells were kept for 3 hours in Dulbecco modified Eagle medium without phenol red ± 10 μmol/L isoproterenol. After incubation, medium was collected and analyzed with a nonesterified fatty acid (NEFA)–C kit (Wako Chemicals, Richmond, VA). To determine oxidation of fatty acids, cells were labeled with 3H-palmitic acid (4 hours; GE Healthcare, Uppsala, Sweden) and chased for 4 hours. Pulse and chase

media were analyzed for bovine serum albumin–nonprecipitable radioactivity (in 3.5% perchloric acid, 2% fatty acid–free bovine serum albumin, 3 precipitations). Total protein was analyzed with the bicinchoninic acid kit (Pierce, Rockford, IL). Total protein concentrations in all samples for measurement of lipolysis and fatty acid oxidation, respectively, were measured in the same BCA assay, with a mean intraassay coefficient of variation of 3.4%.

2.7. Statistics

Skewed variables were log-transformed before statistical analyses. Differences between pairs of observations within person were analyzed with paired *t* tests. Means from unpaired data were compared with 2-sample *t* tests. Testing for correlation between peroxisome proliferator–activated receptor (PPAR) γ messenger RNA (mRNA) or BMI and CIDEC mRNA levels with multiple time points was initially performed with univariate standard linear regression. We then used the method of generalized estimating equations to obtain standard errors of parameter estimates that correctly adjusted for the dependence of multiple observations within each subject [13]. Significance of the correlation was then determined from Wald tests using the correct standard errors [14].

3. Results

3.1. Human CIDEC expression is decreased in response to caloric restriction

Members of the CIDE family have previously been reported to be important in lipid metabolism. Thus, we decided to analyze the expression of human CIDEC in microarray data from subcutaneous adipose tissue from obese subjects undergoing a VLCD for 16 weeks.

After the first 8 weeks of VLCD treatment, the average reduction of CIDEC expression was 25% ($P = .0001$). After 16 weeks of VLCD treatment, the average CIDEC reduction was 10% ($P = .028$). At week 18, 2 weeks after reintroduction of normal food, CIDEC levels no longer showed a significant difference from baseline ($P = .22$).

To verify the decrease in CIDEC expression in response to caloric restriction, we analyzed CIDEC expression with rtPCR in subcutaneous adipose tissue biopsies from 28 obese patients undergoing a 12-week VLCD treatment. After 2 weeks of VLCD treatment, CIDEC levels were significantly reduced (Fig. 1A). Between weeks 2 and 12, the CIDEC expression was not altered significantly. In contrast, average body weight was continuously reduced throughout the study (Fig. 1B).

The CIDEC expression did not correlate significantly to BMI during any of the VLCD treatments ($P = .097$ for microarray study and 0.196 for the rtPCR study).

The transcription factor PPARγ has been suggested to regulate CIDEC expression [15,16]. We therefore used data from the VLCD microarray study to test the association

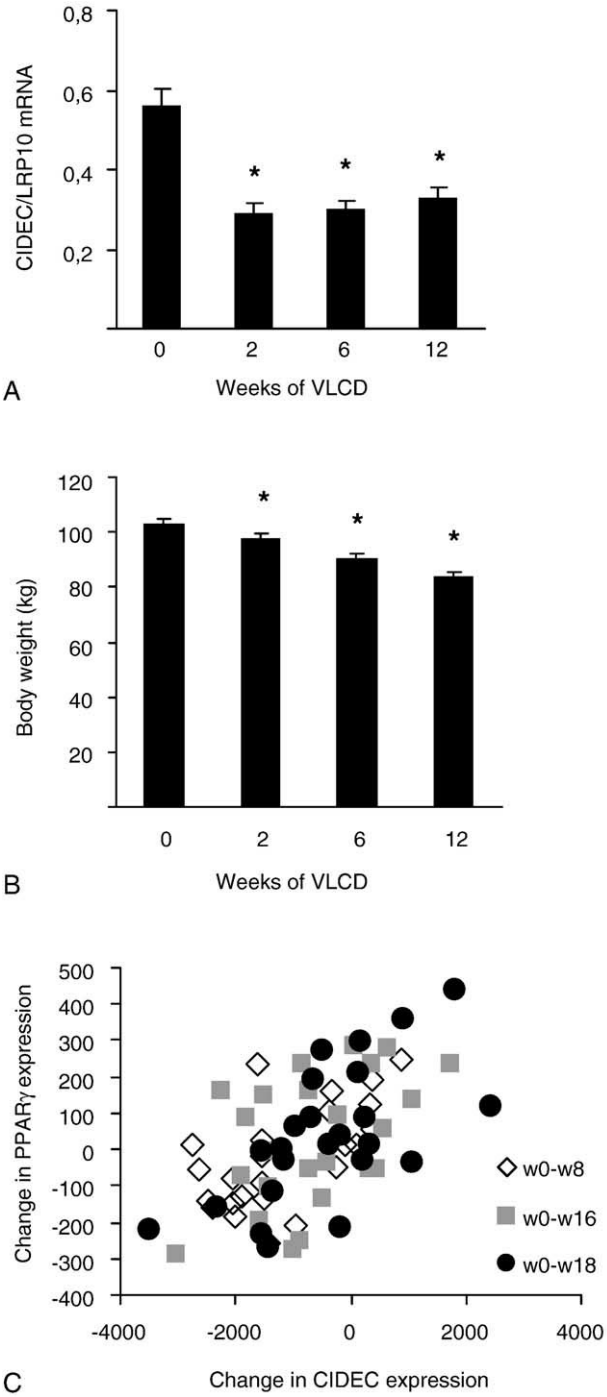


Fig. 1. CIDEC expression in subcutaneous adipose tissue during VLCD. A, Real-time PCR analysis of CIDEC expression in subcutaneous adipose tissue during a 12-week VLCD. Mean value ± SEM. *$P \leq .0001$ (paired *t* tests vs week 0). No significant differences between weeks 2, 6, and 12 (paired *t* tests). B, Body weight during a 12-week VLCD. Mean value ± SEM. *$P \leq .0001$ (paired *t* tests vs previous time point). C, Correlation between changes in PPARγ and CIDEC mRNA levels during the microarray VLCD study (week [w] 0-w8 and w0-w16) and 2 weeks after reintroduction of normal food (w0-w18). Differences are shown for each subject. $P < .0001$ for correlation (Wald test).

between PPARγ and CIDEC expression levels. Indeed, PPARγ mRNA levels positively correlated to CIDEC mRNA levels during the VLCD treatment and after the reintroduction of food, both when absolute signal values (not shown) and when Δ values (change from week 0) for each gene were analyzed (Fig. 1C, $P < .0001$ in both). The microarray analysis did not distinguish between PPARγ 1 and 2.

3.2. Human CIDEC is predominantly expressed in adipocytes

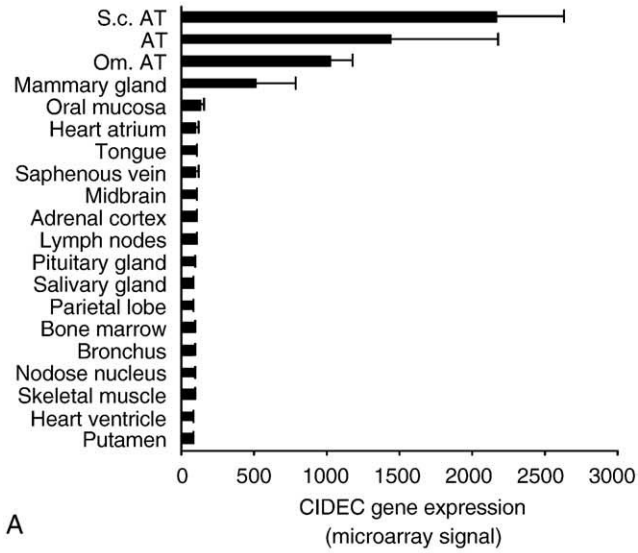
We postulated that genes that are predominantly expressed in adipocytes will be important for adipocyte function and have previously developed a microarray-based strategy to identify such genes [17,18]. In this article, we used the GEO database GSE3526 data set, which includes microarray gene expression data from 65 human tissues, to find genes with an adipose-restricted expression pattern. The highest CIDEC expression was found in subcutaneous adipose tissue, followed by adipose tissue of unspecified origin and omental adipose tissue (Fig. 2A). An intermediate CIDEC expression level was observed in mammary gland, which is a fat-rich tissue. All other tissues displayed very low expression of CIDEC.

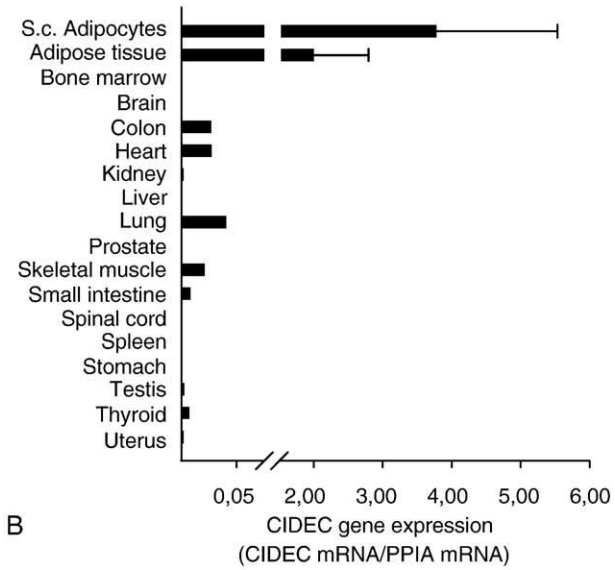
The adipose-restricted expression pattern of CIDEC was verified in an rtPCR analysis of isolated adipocytes, adipose tissue, and a tissue panel consisting of 17 human tissues (Fig 2B). The highest expression level was observed in isolated adipocytes, followed by adipose tissue. All other tissues displayed levels that were less than 2% of the adipocyte signal.

The difference in CIDEC expression between subcutaneous and omental fat tissue was verified in a microarray adipose depot study. The CIDEC expression in both deep and superficial subcutaneous adipose tissues was significantly higher compared with that in omental adipose tissue (Fig. 2C).

3.3. Analysis of the alternative CIDECα isoform

Liang et al [19] reported an alternative splicing of the human CIDEC mRNA, resulting in the CIDECα isoform. To investigate the contribution of each isoform, we used 2 different assays in our rtPCR studies: one that detected both isoforms (Fig. 2B) and one that detected the full-length form only (data not shown). However, the results from the 2





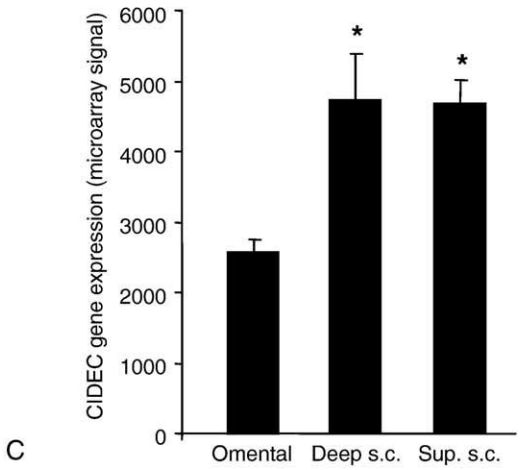


Fig. 2. Tissue distribution of human CIDEC gene expression. A, The DNA microarray analysis of human CIDEC expression in 65 tissues. Mean expression values from the 20 tissues with highest CIDEC expression are presented. B, Real-time PCR analysis of CIDEC expression in human isolated adipocytes and subcutaneous adipose tissue and the Human Total RNA Master Panel II. C, The DNA microarray analysis of CIDEC expression in biopsies from omental, deep subcutaneous, and superficial subcutaneous adipose tissue from 6 obese humans. Mean ± SEM. *$P < .05$ vs omental (paired t test vs omental). AT indicates adipose tissue; Sc, subcutaneous; Om, omental; Sup, superficial.

assays did not differ in the tissue distribution profiling or in the rtPCR VLCD study, indicating that the 2 isoforms are not differently regulated or that CIDECα does not contribute substantially to total CIDEC levels, in our material.

3.4. CIDEC-silencing leads to changes in fatty acid release and oxidation

CIDEC was primarily expressed in adipocytes and reduced during VLCD treatment. Thus, we wished to investigate the effect of decreased CIDEC expression on adipocyte lipid metabolism. To do so, mouse CIDEC (fat-specific protein 27) was knocked down with siRNA in the mouse adipocyte cell line 3T3-L1.

Anti-CIDEC siRNA was introduced into cells by electroporation, and the effect on lipid metabolism was assayed 24 hours postelectroporation. At this time point, CIDEC mRNA levels were decreased by more than 90% compared with cells electroporated with control siRNA (data not shown). Knockdown of CIDEC resulted in a significant increase in basal release of NEFA (Fig. 3A). In contrast, cells with lowered CIDEC expression released less NEFA in response to the β-adrenergic agonist isoproterenol compared with cells electroporated with control siRNA (Fig. 3B). Thus, when we compared NEFA release from nonstimulated and stimulated cells, it was evident that CIDEC-depleted cells were significantly less responsive to β-adrenergic stimulation of lipolysis (Fig. 3C).

An increased basal release of NEFA might reflect a decreased intracellular oxidation of fatty acids. Therefore, we assayed oxidation of fatty acids by analyzing oxidation products in media after the cells had been labeled with radioactive palmitic acid. CIDEC-silencing resulted in increased oxidation of endogenous (labeled) fatty acids (Fig. 3D). Thus, the increased release of NEFA is not explained by decreased fatty acid oxidation.

4. Discussion

To our knowledge, this is the first report showing CIDEC to be down-regulated in response to caloric restriction. The CIDEC down-regulation was demonstrated in 2 independent VLCD studies on obese human subjects. In both studies, the decrease in CIDEC expression occurred during the first weeks of VLCD treatment. Thereafter, CIDEC did not decrease any further despite continuous weight loss. In other words, the timing of the effects of VLCD on CIDEC expression and on body weight was different. Thus, it appears that it is the decrease in caloric intake per se, rather than the actual weight loss, that leads to reduced CIDEC expression. This may explain the lack of correlation between CIDEC expression and BMI in these studies.

Our observation that CIDEC correlates to PPARγ during VLCD treatment should be interpreted together with previous animal and in vitro studies showing that PPARγ

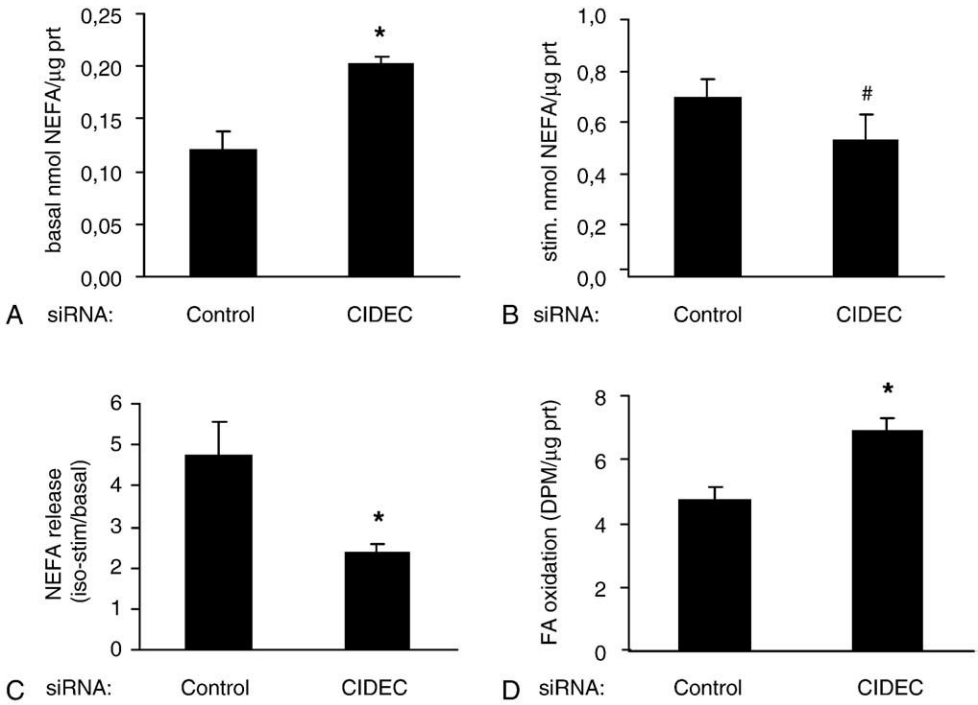


Fig. 3. CIDEC silencing in the 3T3-L1 adipocyte cell line. Differentiated 3T3-L1 cells were electroporated with antimouse CIDEC or scrambled control siRNA. A, Basal release of NEFA. B, The NEFA release after stimulation with β-adrenergic agonist isoproterenol. C, Responsiveness to isoproterenol stimulation, expressed as NEFA release after isoproterenol stimulation/basal NEFA release. D, Oxidation products from labeled endogenous fatty acids. n = 4. Mean ± SD. $^{*}P \leq .004$ and $^{\#}P = .042$ (2-sample t test). Iso indicates isoproterenol.

regulates CIDEC expression [15,16]. Importantly, PPARγ mRNA levels have been shown to correlate to transcriptional activity in human adipose tissue and to be down-regulated after weight loss [20,21]. Thus, decreased PPARγ activity may contribute to the observed down-regulation of CIDEC in response to caloric restriction.

The tissue distribution of human CIDEC was investigated with 2 strategies. In the analysis of the microarray data set, we detected the highest CIDEC expression in subcutaneous adipose tissue. In the rtPCR study, we detected the highest CIDEC expression in subcutaneous adipocytes, followed by subcutaneous adipose tissue. The finding that CIDEC expression is higher in adipocytes than in adipose tissue suggests that it is the adipocytes within the adipose tissue that express CIDEC. When human CIDEC was cloned, transcripts were detected in heart, colon, and small intestine; and it was suggested that human CIDEC was not adipose tissue specific [19]. We also detected CIDEC in these tissues, although at minute levels compared with adipocytes.

To understand the metabolic consequences of CIDEC down-regulation in adipocytes during VLCD treatment, we investigated how a reduction of CIDEC expression affects lipid metabolism in cultured 3T3-L1 adipocytes. Notably, the changes induced by CIDEC silencing resemble the changes described in adipocytes after VLCD treatment or starvation: increased basal NEFA release, decreased responsiveness to adrenergic stimulation, and marked increase in oxidation of endogenous fatty acids [22-24]. We speculate that CIDEC is important for the adipocyte to adjust its metabolism to changes in whole-body energy availability. During energy surplus, CIDEC would facilitate lipid storage, which is indicated by recent overexpression studies, whereas down-regulation of CIDEC in response to caloric restriction might be part of an adaptation by the adipocyte to whole-body energy deficiency [7].

During the preparation of this article, a study on mouse CIDEC was published [7]. In that study, it was clearly demonstrated that mouse CIDEC associates with lipid droplets. CIDEC depletion caused a fragmentation of intracellular lipid droplets and changes in triglyceride lipolysis, partly similar to what we observed. It was also shown that overexpression of mouse CIDEC facilitates intracellular lipid storage, which fits with our proposed role of CIDEC.

In this article, we demonstrate that human CIDEC is predominantly expressed in adipocytes and that the expression in subcutaneous adipose tissue is decreased during caloric restriction. In addition, we show that CIDEC is involved in fatty acid metabolism in cultured adipocytes. Based on our data, we propose a role for CIDEC in adipocyte adaptation to changes in energy availability.

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