Downregulation of CPPED1 Expression Improves Glucose Metabolism In Vitro in Adipocytes

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We have previously demonstrated that the expression of calcineurin-like phosphoesterase domain containing 1 (CPPED1) decreases in adipose tissue (AT) after weight reduction. However, the function of CPPED1 in AT is unknown. Therefore, we investigated whether the change in CPPED1 expression is connected to changes in adipocyte glucose metabolism. First, we confirmed that the expression of CPPED1 decreases after weight loss in subcutaneous AT. Second, the expression of CPPED1 did not change during adipocyte differentiation. Third, CPPED1 knockdown with small interfering RNA increased expression of genes involved in glucose metabolism (adiponectin, adiponectin receptor 1, and GLUT4) and improved insulin-stimulated glucose uptake. To conclude, CPPED1 is a novel molecule involved in AT biology, and CPPED1 is involved in glucose uptake in adipocytes. Diabetes 62:3747–3750, 2013

Lifestyle modification improves glucose metabolism and results in a substantial reduction in the risk of type 2 diabetes in the long-term (1). In searching new putative genes related to obesity and type 2 diabetes, we have previously demonstrated a multitude of changes in adipose tissue (AT) gene expression in response to weight reduction in individuals with metabolic syndrome (2,3). Among the downregulated genes was calcineurin-like phosphoesterase domain containing 1 (CPPED1) (2); its function in AT is completely unknown.

Therefore, we continued to study the role of CPPED1 in AT in more detail. Interestingly, the experiment using a Simpson-Golabi-Behmel syndrome (SGBS) cell strain demonstrated an impact of CPPED1 small interfering RNA (siRNA) on insulin-stimulated glucose uptake in mature adipocytes. Overall, the results demonstrate that CPPED1 is a novel molecule expressed in AT and is related to adipocyte function.

RESEARCH DESIGN AND METHODS

The design of the Gene Expression in Obesity and Insulin Resistance (GENOBIN) study has been reported earlier (2,3). Altogether, 46 overweight or obese (BMI 28–40 kg/m²) subjects 40–70 years of age were randomly assigned to one of two groups: a weight-reduction group (n = 28) or a weight-maintenance control group (n = 18) (2). After an overnight fast, AT samples were taken by needle biopsy from subcutaneous AT before and after the intervention (5 months) under local anesthesia (lidocaine 10 mg/mL without adrenaline).

The GENOBIN study was performed in accordance with the standards of the Helsinki Declaration, and the ethics committee of the District Hospital Region of Northern Savo approved the study plans. All participants gave a written informed consent.

SGBS cell strain. Human preadipocyte cell strain SGBS is characterized by a high capacity for adipogenic differentiation (4). The cells were cultured as described previously (5).

Knockdown of the expression of the CPPED1 gene in SGBS cells. RNA interference was used for knocking down the expression of the CPPED1 gene in mature adipocytes. ON-TARGETplus SMARTpool siRNA for CPPED1 was purchased from Dharmacon (Thermo Scientific, Lafayette, CO). There are four target siRNA sequences included in the pool: AGAAAAAGUGUCACGGAUU, UAAAGUGCUACUAGGAAA, GGAGGACCCUAGCCGGUU, and CCUUUAAAGGCGGCGGAU. The negative control for the siRNA (scrambled) used in the experiment was Allstars negative control siRNA (Qiagen, Valencia, CA). The siRNA was transfected into the cell by using HiPerFect transfection reagent (Qiagen) according to the instructions.

In brief, the SGBS cells were cultured in 12-well plates and induced into mature adipocytes. On day 14 of differentiation, the medium was replaced with Dulbecco’s modified Eagle’s medium/Ham’s F12 nutrient mixture (1:1) supplemented with 53 μmol/L biotin, 17 μmol/L pantothenate, 10 μmol/L transferrin and 20 mmol/L insulin. The cells were transfected with 50 nmol/L siRNA and incubated for the indicated time points. Knockdown of CPPED1 expression was confirmed by reverse-transcriptase quantitative PCR (RT-qPCR) and Western blot. The effect of CPPED1 knockdown on high-molecular-weight (HMW) adiponectin secretion into the conditioned medium was measured with a commercial ELISA kit purchased from Millipore (St. Charles, MO) according to the manufacturer’s protocol.

Insulin-stimulated glucose uptake in SGBS cells. The SGBS cells were cultured in 12-well plates and induced into mature adipocytes. On day 17 of differentiation, the cells were washed twice with PBS and preincubated with KRB buffer (20 mmol/L HEPES [pH 7.4], 118 mmol/L NaCl, 4.8 mmol/L KCl, 2.5 mmol/L CaCl₂, 1.2 mmol/L MgSO₄) for 2 h at +37°C. After preincubation, the cells were incubated in the presence of 100 mmol/L l-ω-mannosidase, 10 nmol/L insulin and 50 nmol/L L-arginine for 30 min. After 30 min of incubation, the medium was replaced with a new fresh buffer containing 100 ng/mL adiponectin and 1 μmol/L insulin for 20 min. Next, 0.5 μmol/L labeled 2-deoxy-D-[3H] glucose (Amersham TRK672, GE Healthcare, Buckinghamshire, U.K.) and 0.2 mmol/L unlabeled glucose were added for an additional 15 min at +37°C. The reaction was terminated by placing the cells onto the ice and washing three times with ice-cold PBS.

The cells were solubilized with 200 μL of 0.2 N NaOH per well and incubated 1.5 h at room temperature with constant shaking. The cell lysate (100 μL) was transferred to a 2.0-mL Eppendorf tube, and scintillation liquid was added for radioactivity counting. Glucose uptake was normalized to protein content as measured from the remaining cell lysate using the Bio-Rad protein assay (DC Protein Assay; Bio-Rad, Hercules, CA). Protein concentrations were measured according to the manufacturer’s instructions (DC Protein Assay) using Wallace 1420.

RNA extraction, cDNA synthesis, and RT-qPCR. Total RNA extraction and cDNA synthesis of AT samples have been described previously (2). For cultured SGBS cells, the RNeasy Mini Kit was used for the total RNA extraction (Qiagen, Valencia, CA) and iScript cDNA Synthesis Kit (Bio-Rad) according to instructions provided by the manufacturer.

RT-qPCR with TaqMan chemistry (Applied Biosystems) using an ABI Prism 7500 analyzer (Applied Biosystems) was used. The analysis for the relative quantity of a specific gene before and after the intervention in AT of the GENOBIN was performed as described previously (2). The expression of target genes was normalized to cyclophilin A (PPIA) expression for AT samples and SGBS cells. Expression of the target genes in cultured SGBS cells was normalized to the endogenous control using the formula 2^-[ΔΔCt] (6).

Western blot. For Western blot, cells were rinsed twice with PBS and then lysed in RIPA Lysis and Extraction Buffer (Pierce, Rockford, IL) freshly prepared according to instructions provided by the manufacturer.

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supplemented with 1 mmol/L EDTA, 1 mmol/L PMSF, 1 mmol/L Na3VO4, 1 mmol/L NaF, and proteinase inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), followed by centrifugation (13,000 rpm, 15 min, +4°C) and removal of the soluble fraction. The protein concentration was determined using the Bio-Rad protein assay (DC Protein Assay). Equal amounts of protein were separated using SDS-PAGE and transferred to the polyvinylidene fluoride (PVDF) membrane (GE Healthcare).

Rabbit anti-human CPPED1 and adiponectin (ADIPOQ) were used as primary antibodies (Sigma-Aldrich). The primary antibodies were detected with goat anti-rabbit peroxidase-conjugated secondary antibody (Pierce). Signals were detected using Amersham Advance Western Blotting Detection Kit (GE Healthcare) and ImageQuant Capture-RT ECL for Windows version 1.0.1 (GE Healthcare). Densitometric analysis was performed by ImageJ version 1.45s (ImageJ; National Institutes of Health, Bethesda, MD). A rabbit anti-human glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Abcam) was used for normalization.

Statistical analyses. The gene expression data of GENOBIN was analyzed using the SPSS software for Windows version 14.0 (SPSS Inc., Chicago, IL), and data are provided as mean ± SD, unless otherwise indicated. Paired-samples Student t test was used for comparing the baseline and end point measurements within the study group. A value of P < 0.05 was considered statistically significant.

All results of the SGBS cell culture studies were analyzed using the GraphPad Prism5 software for Windows version 5.03 (GraphPad Software, San Diego, CA), and results are expressed as mean ± SEM. Statistical significance was determined with independent-samples Student t test or one-way ANOVA with Bonferroni multiple comparison test (indicated in figures). A value of P < 0.05 was considered statistically significant.

RESULTS

GENOBIN study. A previously published reduction in CPPED1 expression in AT after weight reduction using Affymetrix microarrays (2) was confirmed with RT-qPCR (Fig. 1).

CPPED1 expression during adipocyte differentiation. The expression of CPPED1 mRNA was at a maximal level in preadipocytes and did not change during the differentiation process (Fig. 2A), whereas peroxisome proliferator–activated receptor γ 2 was upregulated during SGBS differentiation, as expected (Fig. 2B).

CPPED1 knockdown leads to increased insulin-stimulated glucose uptake. The role of CPPED1 in adipocytes was studied by siRNA in SGBS adipocytes. As shown in Fig. 3A and B, the knockdown of CPPED1 for 48 h decreased the expression of CPPED1 at both the mRNA and protein levels. The CPPED1 knockdown led to increased mRNA expression of ADIPOQ, adiponectin receptor 1, and GLUT4, and to decreased mRNA expression of GLUT1 and leptin (LEP) (Fig. 3B).

The effects of CPPED1 siRNA on the expression of genes related to glucose metabolism suggested a significant role of CPPED1 in adipocyte metabolism. Therefore, we next investigated basal and insulin-stimulated glucose uptake in cells treated with scrambled and CPPED1 siRNA. Insulin-stimulated glucose uptake increased in CPPED1 siRNA–treated cells by +74% (P < 0.05) compared with control cells (Fig. 3C). Wortmannin treatment abolished the increase in insulin-stimulated glucose uptake in both conditions.

Finally, the protein expression of ADIPOQ increased time dependently (Fig. 3D), leading to a significant increase in ADIPOQ protein at 96 h after CPPED1 siRNA treatment (+32%, P < 0.05). In line with this, the reduction of CPPED1 expression for 48 h tended to increase HMW adiponectin secretion (P = 0.057) into the conditioned medium (Fig. 3E). The protein expression of GLUT4 did not change after CPPED1 siRNA treatment (Fig. 3F).

DISCUSSION

We suggest that the decrease in mRNA expression of CPPED1 in AT after weight loss in humans may have important implications because we now demonstrate that downregulation of CPPED1 by siRNA leads to an increased insulin-stimulated glucose uptake in cultured mature adipocytes possibly via adiponectin-mediated mechanisms.

FIG. 1. The relative mRNA expression of CPPED1 in subcutaneous AT samples at baseline (black bars) and after the intervention (gray bars) in weight-reduction group and weight-maintenance control group. The values are expressed as relative gene expression levels normalized to endogenous control PPIA. P < 0.05 was considered statistically significant (comparisons were made within groups). The final number of patients included in the data analysis is indicated in the figure. **P < 0.01.

FIG. 2. CPPED1 (A) and PPARγ2 (B) mRNA expressions in cultured SGBS cells during adipocyte differentiation. The SGBS cells were induced to differentiate and were harvested in different time points during adipocyte differentiation. Statistical significance was tested using one-way ANOVA with Bonferroni multiple comparison test. The graph shows the means ± SEM from four independent experiments, and the values are expressed as relative gene expression levels normalized to endogenous control PPIA. *P < 0.05; **P < 0.01; ***P < 0.001 (indicated time point vs. day D1) 0).
Collectively, these results provide new data that CPPED1 is involved in glucose metabolism in AT, and could be a novel gene related to AT dysfunction in obesity.

The in vitro experiments in SGBS cells showed that the CPPED1 mRNA expression was not changed during adipocyte differentiation whereas peroxisome proliferator-activated receptor γ 2 was upregulated as expected, indicating that CPPED1 is not directly regulated in the adipocyte differentiation process. In addition to adipocytes, AT is composed of stromal vascular fraction cells, including preadipocytes, endothelial cells, and several immune cells (7,8). Thus, we cannot distinguish which other cell types are contributing to the expression of CPPED1 in AT and further studies are needed. However, our results
show that at least preadipocytes and mature adipocytes are involved in CPPED1 expression. To better understand the role of CPPED1 in adipocytes, we studied the effect of CPPED1 knockdown by siRNA in SGBS adipocytes on the expression of genes involved in adipogenesis and glucose metabolism. The downregulation of CPPED1 mRNA enhanced the insulin-stimulated glucose uptake in mature adipocytes, suggesting that CPPED1 is involved in glucose metabolism in adipocytes. To study the possible involvement of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway in this process, the cells were incubated with the PI3K-specific inhibitor wortmannin. It turned out that wortmannin significantly decreased the CPPED1 knockdown–induced improvement in glucose uptake, suggesting that the effect of CPPED1 downregulation by siRNA on insulin-stimulated glucose uptake may be mediated through a PI3K/Akt-dependent pathway. The role of CPPED1 in insulin signaling in adipocytes needs to be elucidated in more detail, including experiments in which CPPED1 is overexpressed in different cell types.

Interestingly, we also found that CPPED1 may regulate adiponectin signaling in AT. The knockdown of CPPED1 expression in mature adipocytes increased the gene and protein expression of ADIPOQ, and there was also a clear trend for enhanced secretion of HMW adiponectin into the conditioned medium. Furthermore, gene expression levels of adiponectin receptor 1 and GLUT4 were increased due to CPPED1 knockdown. In humans, adiponectin levels are known to be inversely related to obesity, insulin resistance, and type 2 diabetes (9). Moreover, adiponectin has been shown to modify glucose metabolism (10) by improving insulin sensitivity and promoting glucose uptake (9,11) through adiponectin receptors 1 and 2 (10,12). Therefore, we propose a model (Fig. 4) that CPPED1 knockdown–induced improvement in insulin-stimulated glucose uptake could be due to adiponectin-mediated mechanisms.

In conclusion, knockdown of CPPED1 expression enhances insulin-stimulated glucose uptake in mature adipocytes, leading to improved glucose metabolism possibly via adiponectin-mediated mechanisms. The potential of CPPED1 knockdown in the treatment of obesity-related phenotypes needs to be investigated further.

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No potential conflicts of interest relevant to this article were reported.

M.V. performed the experiments, researched data, and wrote the manuscript. D.K., P.K., and M.E. contributed to coordination and edited the manuscript. M.K. participated in the study design and coordination and edited the manuscript. J.P. contributed to coordination of the manuscript and edited the manuscript. M.U. contributed to coordination of the manuscript, edited the manuscript, supervised the study, and participated in the study design. L.P. supervised the study, participated in the study design, and edited the manuscript. M.U. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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REFERENCES
1. Lindström J, Peltonen M, Eriksson JG, et al; Finnish Diabetes Prevention Study (DPS). Improved lifestyle and decreased diabetes risk over 13 years: long-term follow-up of the randomised Finnish Diabetes Prevention Study (DPS). Diabetologia 2013;56:284–293
2. Kolehmainen M, Salopuro T, Schwab US, et al. Weight reduction modulates expression of genes involved in extracellular matrix and cell death: the GENOBIN study. Int J Obes (Lond) 2008;32:292–303
3. Vahtinen M, Kolehmainen M, Schwab U, Uusitupa M, Puukkinen L. Microfibrillar-associated protein 5 is linked with markers of obesity-related extracellular matrix remodeling and inflammation. Nutr Diabetes 2011;1:e15
4. Wabitsch M, Brenner RE, Melzner I, et al. Characterization of a human preadipocyte cell strain with high capacity for adipose differentiation. Int J Obes Relat Metab Disord 2001;25:8–15
5. Kaminks D, Kuralassna M, Veneasmaa S, et al. Adipose tissue TCF7L2 splicing is regulated by weight loss and associates with glucose and fatty acid metabolism. Diabetes 2012;61:2807–2813
6. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001;25:402–408
7. Hauner H. Secretory factors from human adipose tissue and their functional role. Proc Nutr Soc 2005;64:163–169
8. Mack I, BelAiba RS, Djordjevic T, Görlach A, Hauner H, Bader BL. Functional analyses reveal the greater potency of preadipocytes compared with adipocytes as endothelial cell activator under normoxia, hypoxia, and TNFalpha exposure. Am J Physiol Endocrinol Metab 2009;297:E735–E748
9. Hajer GR, van Haeften TW, Visseren FL. Adipose tissue dysfunction in obesity, diabetes, and vascular diseases. Eur Heart J 2008;29:2969–2971
10. Ziemko F, Mantzoros CS. Adiponectin in insulin resistance: lessons from translational research. Am J Clin Nutr 2010;91:1588–1618
11. Chen MB, McAinchin AJ, Macaulay SL, et al. Impaired activation of AMP-kinase and fatty acid oxidation by globular adiponectin in cultured human skeletal muscle of obese type 2 diabetic. J Clin Endocrinol Metab 2005;90:3665–3672
12. Rabe K, Lehrke M, Parhofer KG, Broeckl UC. Adipokines and insulin resistance. Mol Med 2008;14:741–751