Brief Communication

MOLECULAR METABOLISM

Impaired histone deacetylases 5 and 6 expression mimics the effects of obesity and hypoxia on adipocyte function

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ABSTRACT

Objective: The goal of the study was to investigate the role of histone deacetylases (HDACs) in adipocyte function associated with obesity and hypoxia.

Methods: Total proteins and RNA were prepared from human visceral adipose tissues (VAT) of human obese and normal weight subjects and from white adipose tissue (WAT) of C57Bl6-Rj mice fed a normal or high fat diet (HFD) for 16 weeks. HDAC activity was measured by colorimetric assay whereas the gene and protein expression were monitored by real-time PCR and by western blotting, respectively. RNA interference (RNAi) was used to silence the expression of genes in 3T3-L1 adipocytes.

Results: Total HDAC activity was decreased in VAT and WAT from obese individuals and from mice fed a HFD, respectively. The HDAC activity reduction was associated with decreased *HDAC5*/*Hdac5* and *HDAC6*/*Hdac6* expression in human and mice adipocyte fraction. Similarly, hypoxia hampered total Hdac activity and reduced the expression of *Hdac5* and *Hdac6* in 3T3-L1 adipocytes. The decrease of both *Hdac5* and *Hdac6* by hypoxia was associated with altered expression of adipokines and of the inducible cAMP early repressor (Icer), a key repressor that is defective in human and mice obesity. Silencing of Icer in adipocytes reproduced the changes in adipokine levels under hypoxia and obesity, suggesting a causative effect. Finally, modeling the defect of the two Hdacs in adipocytes by RNAi or selective inhibitors mimicked the effects of hypoxia on the expression of *Icer*, leading to impairment of insulin-induced glucose uptake.

Conclusions: Hdac5 and Hdac6 expression are required for the adequate expression of Icer and adipocyte function. Altered adipose expression of the two Hdacs in obesity by hypoxia may contribute to the development of metabolic abnormalities.

Keywords Histone deacetylases; Adipocytes; Adipokines; Obesity; Insulin resistance

1. INTRODUCTION

Insulin resistance is a key feature of obesity and is involved in the development of type 2 diabetes, fatty liver disease, cardiovascular disease, and cancer [1—4]. Insulin resistance is associated with altered production of several adipokines (*i.e.* bioactive secreted products from adipocytes) that regulate insulin sensitivity and energy metabolism [5—7]. These adipokines include interleukin 6 (IL6), nicotinamide phosphoribosyltransferase (NAMPT, also called visfatin), leptin (LEP), angiotensinogen (AGT), Lipocalin 2 (LCN2), adiponectin (ADIPOQ), resistin (RETN), and SERPINE1 (also called plasminogen activator inhibitor type 1) [5,6,8]. These adipokines have been shown to induce insulin resistance in rodents [5,9,10].

In both human and mice obesity, hypoxia is thought to contribute to impaired adipokine production [11—14]. Indeed, visceral adipose tissue (VAT) from obese subjects is characterized by impaired blood flow, defective capillary density, and impaired O_2 partial pressure [12,14]. Exposing mouse adipocytes to hypoxia leads to reduced expression of

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Brief Communication

Adipoq, *Agt*, *Lep*, *Nampt*, and *Retn*, and, in contrast, to increased expression of *Serpine1*, *Il6*, and *Lcn2* [8,11]. However, the molecular mechanisms causing impaired adipokine production associated with hypoxia are still elusive. We postulate that both cAMP response element (CRE) binding protein activity in (CREB) and histone deacetylases are involved in these mechanisms. In support of this working hypothesis, hypoxia can stimulate CREB activity as observed in PC12 and lung cells [15,16]. Adipocyte CREB activity is increased in obesity, leading to increased abundance of the activating transcription factor 3 (ATF3) [17,18]. This increased ATF3 activity hampers the expression of *Adipoq* and glucose transporter *GLUT4*, ultimately leading to impairment in insulin-induced glucose uptake [17,18]. The CREB-dependent activation mechanism is initiated by reduction in the content of inducible cAMP early repressor (ICER), a natural antagonist of CREB and other cAMP-dependent transcription factors [17]. Reduction of ICER was found in adipocytes of human obese individuals and mice fed a high fat diet (HFD) for 16 weeks [17]. In this study, therefore, we hypothesized that defective deacetylase activity may account for the collapse in the ICER level in obesity. Indeed, the expression of *ICER* is reported to be positively regulated by histone deacetylase activity (HDACs) in PC12 cells [19]. Overall, HDACs are pivotal in epigenetic mechanisms that permit gene expression adaptation to environmental changes [20]. There are 3 classes of HDACs [21,22]: classes I, II and IV. Class I HDACs comprises HDAC1, HDAC2, HDAC3, and HDAC8. Class II HDACs is divided into subclass IIa (HDAC4, HDAC5, HDAC7, and HDAC9) and subclass IIb (HDAC6 and HDAC10). Class IV contains includes HDAC11 only. So far, selective inhibition of HDACs is a strategy for treating many cancers [22]. Additionally, there is emerging evidence implicating Hdac activity in the control of energy metabolism, thus opening an avenue for future targets in metabolic diseases [23]. In the hypothalamus of obese mice fed a HFD, the expression of Hdacs, including *Hdac5*, is modified when compared to that of mice fed a chow diet [24,25]. Hdac5 is required for hypothalamic leptin signaling and food intake, as Hdac5 knockout mice display defective hypothalamic leptin signaling and are more prone to diet-induced obesity compared to wild-type mice [25]. Given the role of HDACs in obesity, we hypothesized the contribution of HDACs to the changes in adipokine expression elicited by hypoxia and obesity-associated adipocyte dysfunction.

2. MATERIAL AND METHODS

2.1. Materials

Trichostatin (TSA), tubastatin and LMK293 were obtained from Sigma-Aldrich (St. Louis, MO).

2.2. Biopsies and RNA preparation

Total RNA was extracted from epididymal white adipose tissue (WAT) of mice fed a HFD ($n = 10$) or normal chow diet ($n = 10$). WAT isolation was performed in euthanized animals in accordance to the Swiss legislation for animal experimentation. Approximately 5 cm^3 of VAT was obtained at the level of the omentum from five obese Caucasian women (BMI >35 kg/m^2) who were referred for weight reduction surgery and five non-obese Caucasian women ($24 <$ BMI <28 kg/m^2) [17]. All patients provided informed consent, and the study was approved by the institutional review board [17]. The criteria for exclusion and phenotyping are those previously described [17]. Total RNA was isolated from adipose tissues and different cell fractions with the TriPure isolation reagent (Roche) as previously described [17]. Procedure for preparation of adipocytes and SVF fractions was done as described [17].

2.3. Cell culture and transfection

Culture and differentiation of 3T3-L1 cells were conducted as described [17]. Briefly, 3T3-L1 cells were grown and maintained in Dulbecco's modified Eagle's medium high glucose containing 50 units/ml penicillin, 50 μg/ml streptomycin, and 10% fetal calf serum (FCS) in a 10% CO_2 environment. At postconfluency (2 days), the cells were differentiated by adding to the culture medium, isobutylmethylxanthine (500 μM), dexamethasone (25 μM), and insulin (4 μg/ml) for 3 days and then only with insulin for 3 more days. The medium was then changed every 3 days until the cells were fully differentiated, typically by 10 days. The 19-nt small interfering RNA (siRNA) duplex against Icer (silcer) is described elsewhere [17]. The siRNA duplexes, targeting the GFP (5′-CGCTGACCCTGAAGTTCAT-3′), the mouse *Hdac5* (5′-GCAAGCATTCTACAACGAT-3′), and *Hdac6* (5′-CCAGGACGATCTCCAAGAT-3′) were purchased from Microsynth (Balgach, Switzerland). For silencing *Icer*, *Hdac5*, and *Hdac6*, on day 7 post-differentiation, 3T3-L1 adipocytes were electroporated with siRNAs using the GenePulser XCell (Bio-Rad) as previously described [17].

2.4. Western blotting and quantitative PCR

Protein extracts, western blotting, and real-time quantitative PCR were conducted as previously described [17]. PCR assays were carried out on a BioRad MyiQ Single-Color Real-Time PCR Detection System using the BioRad iQ SYBR Green Supermix, with 100 nM primers and 1 μl of template per 20 μl of PCR and an annealing temperature of 60 °C. The primer sequences are available in the supplementary materials.

2.5. 2-Deoxyglucose (2-DOG) uptake assay

2-deoxy-d-[1,2-^{3}H]glucose (2-[^{3}H]DOG, 26.2 Ci/mmol) uptake assays were conducted on fully differentiated 3T3-L1 adipocytes (days 7 and 8) as previously described [17]. Adipocytes were treated without (basal) or with insulin 10 nM for 10 min. 2-[^{3}H]DOG (0.1 μCi; final concentration, 0.01 mmol/l) and 5 mM cold 2-DOG were then added for an additional 10 min at 37 °C. 2-DOG uptake was terminated by washing the cells three times with ice-cold PBS containing 10 mM glucose. Subsequently, cells were lysed in 1% (wt/vol) SDS and 0.2 M NaOH. Incorporated radioactivity was measured by liquid scintillation spectrometry.

2.6. Statistical analysis

The experiments including two groups were analyzed by t-test or with the non-parametric equivalent Wilcoxon.

3. RESULTS

3.1. *HDAC5/Hdac5* and *HDAC6/Hdac6* mRNA levels are reduced in adipocytes from obese human subjects and from obese mice

The total Hdac activity was monitored in WAT of mice that were fed a chow diet or a HFD for 16 weeks. We and others have shown previously that mice gain more weight, develop more adipose tissue, and develop systemic insulin resistance when fed a HFD [17]. The total Hdac activity was significantly decreased in the WAT of HFD mice (Figure 1A). Decreased Hdac activity was associated with a significant drop of classes IIa and IIb, *Hdac5* and *Hdac6* expression, respectively (Figure 1C,D). The level of all Hdac mRNA as well as the reduction of *Hdac5* and *Hdac6* expression was confirmed while the qRT-PCR analyses were normalized against the TATA box binding protein mRNA, for which we found that the level was also stable among the mice. In contrast, *Hdac9* expression was significantly increased whereas the expression of class I Hdacs (*Hdac1-3* and *Hdac8*) was unchanged in obese *vs* control mice (Figure 1B). The diminution of the two Hdacs had

