Impaired Expression of the Inducible cAMP Early Repressor Accounts for Sustained Adipose CREB Activity in Obesity

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OBJECTIVE—Increase in adipose cAMP-responsive element–binding protein (CREB) activity promotes adipocyte dysfunction and systemic insulin resistance in obese mice. This is achieved by increasing the expression of activating transcription factor 3 (ATF3). In this study, we investigated whether impaired expression of the inducible cAMP early repressor (ICER), a transcriptional antagonist of CREB, is responsible for the increased CREB activity in adipocytes of obese mice and humans.

RESEARCH DESIGN AND METHODS—Total RNA and nuclear proteins were prepared from visceral adipose tissue (VAT) of human nonobese or obese subjects and white adipose tissue (WAT) of C57Bl6-Rj mice that were fed with normal or high-fat diet for 16 weeks. The expression of genes was monitored by real-time PCR, Western blotting, and electromobility shift assays. RNA interference was used to silence the expression of Icer.

RESULTS—The expression of Icer/ICER was reduced in VAT and WAT of obese humans and mice, respectively. Diminution of Icer/ICER was restricted to adipocytes and was accompanied by a rise of *Atf3/ATF3* and diminution of *Adipoq/ADIPOQ* and *Glut4/GLUT4*. Silencing the expression of Icer in 3T3-L1 adipocytes mimicked the results observed in human and mice cells and hampered glucose uptake, thus confirming the requirement of Icer for appropriate adipocyte function.

CONCLUSIONS—Impaired expression of ICER contributes to elevation in CREB target genes and, therefore, to the development of insulin resistance in obesity. ***Diabetes*** **60:3169–3174, 2011**

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Accumulation of visceral fat in obesity is a strong risk factor for life-threatening diseases, such as type 2 diabetes, atherosclerosis, polycystic ovary syndrome, nonalcoholic fatty liver disease, and certain types of cancer (1–4). Increased adiposity is associated with impairment in glucose uptake in adipocytes, which in part result from the diminished expression of GLUT4 (5). Parallel to defective glucose metabolism, adipocytes display reduced production of the insulin-sensitizer adiponectin (Adipoq) (6,7). This dysregulation is considered as part of a cross-talk linking adiposity to overall insulin resistance in obesity (8). An important mechanism responsible for decreased level of Glut4 and Adipoq in obesity is now advanced (9). This involves the cAMP-responsive element–binding protein (CREB) transcriptional factors (9). Transcriptional activity of Creb is abnormally elevated in adipocytes of obese mice fed with a high-fat diet (HFD), resulting in elevation of the expression of its target gene, Atf3 (9). In turn, Atf3 suppresses the expression of Glut4 and Adipoq and, thereby, disrupts insulin action in adipocytes and causes systemic insulin resistance (9). The impact of increased Creb activity has been further validated by overexpressing a dominant-negative form of Creb in adipocytes (9). Mice with Creb inactivation in adipocytes have improved whole-body insulin sensitivity and glucose homeostasis but similar fat mass and body weight to control animals (9). The activity of the CREB factors is finely tuned by the level of the inducible cAMP early repressor (ICER). ICER represses gene transcription by binding to the CRE sequence within the promoters (10). Typically, ICER plays a dominant-negative role by competing with all cAMP-responsive transcriptional activators of the CREB, CRE modulator (CREM), and activating transcription factor families for binding to CRE (11). Elevation of ICER levels is normally transient, and dysregulation in the expression of this repressor leads to diverse pathologies (11–14). In this article, we tested the hypothesis that the activating transcription factor 3 (ATF3)–mediated reduction of GLUT4 and ADIPOQ in adipocytes is the consequence of diminished ICER.

RESEARCH DESIGN AND METHODS

Animal experimentation. All procedures on mice were performed according to the Swiss legislation for animal experimentation. Six-week-old C57Bl6-Rj male mice were obtained from the Janvier Breeding Centre (Le Genest-Saint-Isle, France). Diets and hyperinsulemic-euglycemic clamps were realized as previously described (15). Epididymal visceral adipose tissue (VAT) was extracted for total RNA and protein preparation in killed animals.

Biopsies and RNA preparation. Approximately 5 cm^3 of VAT was obtained at the level of the omentum from five obese white women (BMI >35 kg/m^2)

who were referred for weight reduction surgery and five nonobese white women (24< BMI <28 kg/m^2) (16). All patients provided informed consent, and the study was approved by the institutional review board (16). The criteria for exclusion and phenotyping are those previously described (16). Total RNA was isolated from adipose tissues and different cell fractions with the TriPure isolation reagent (Roche) as previously described (17). The procedure for preparation of adipocytes and stroma vascular fraction (SVF) was performed as previously described (18).

Materials. The antibodies against CREM that recognize ICER were provided by E. Lalli (Institut de Pharmacologie Moléculaire et Cellulaire, Nice University, Valbonne, France). The antibodies against hairy and enhancer of Split-1 (HES-1), CREB1, ATF3, and histone deacetylase 1 (HDAC1) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture and transfection. Culture and differentiation of 3T3-L1 cells were conducted as previously described (19). The 19-nucleotide small interfering RNA (siRNA) duplex (siICER) was synthesized by Mycrosynth AG (Balgach, Switzerland). For overexpression and silencing of Icer, 3T3-L1 adipocytes on day 7 postdifferentiation were electroporated with either the plasmid coding for Icer Iγ (ICER) or siRNAs (12) using the GenePulser XCell (Bio-Rad).

Western blotting, quantitative PCR, nuclear extract preparation, and electromobility shift assay (EMSA). Nuclear protein extracts, Western blotting, EMSA, and real-time PCR were conducted as previously described (12). PCR assays were carried out on a Bio-Rad MyiQ single-color, real-time PCR detection system using the Bio-Rad iQ SYBR Green Supermix, with 100 nmol/L 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 Data.

2-Deoxyglucose uptake assay. 2-Deoxyglucose (2-DOG) uptake assays was conducted on fully differentiated 3T3-L1 adipocytes (days 7 and 8) as previously described (20). Adipocytes were treated without (basal) or with insulin (100 pmol/L) for 10 min. 2-[^{3}H]DOG (0.1 μCi; final concentration, 0.01 mmol/L) and 5 mmol/L cold 2-DOG were then added for an additional 10 min at 37°C.

Statistical analysis. The experiments including two groups were analyzed by Student *t* test or with the nonparametric equivalent Wilcoxon.

RESULTS

Obese mice fed with an HFD display similar metabolic disorders to those observed in humans (21,22). The diet indeed led to increased mouse body weight, hyper-insulinemia, hyperglycemia, and insulin resistance, as indicated by a marked decrease in glucose infusion rate compared with littermates fed with chow diet (Supplementary Table 1). In this model, elevation in the transcriptional activity of Atf3 downregulates the expression of *Glut4* and *Adipoq* in white adipose tissue (WAT), thereby contributing to systemic insulin resistance (9). In agreement with these data, we found an increase in the abundance of Atf3 and decreased expression of Glut4 and Adipoq in WAT of obese mice when compared with control animals (Fig. 1*A* and *C*). The rise of Atf3 was associated with a striking drop in Icer content in WAT of obese mice when compared with control animals both after fasting (Fig. 1*A* and *C*) or fed conditions (data not shown), indicating that the reduction in Icer in HFD-induced obese mice is independent from the nutritional status. To confirm the data obtained in mice, we analyzed adipose tissues from human nonobese and obese subjects. In agreement with the data found in animals, we detected a smaller amount of *ICER* mRNA in the VAT of obese subjects (Fig. 1*B*). In contrast, the expression of *ICER* was significantly increased in subcutaneous adipose tissue of obese individuals (Supplementary Fig. 1). These results indicate that VAT and subcutaneous adipose tissue dysfunctions are likely to rely on distinct mechanisms. As it is the excess of visceral rather than subcutaneous fat that is more associated with an increased risk of developing cardiovascular disease and metabolic syndrome (23), we chose to concentrate our study on VAT. Reduction of *ICER* mRNA in VAT of obese individuals was associated with an increase in the expression of the CREB target gene *ATF3* and decreased levels of *ADIPOQ* and *GLUT4* (Fig. 1*B*). EMSAs were next done to confirm changes in binding activities of Icer and Creb. Nuclear proteins from WAT of obese mice were incubated with a labeled oligonucleotide corresponding to the cAMP response element (CRE) (12). A fast migrating band was observed in WAT of control mice, which was markedly reduced in obese mice (Fig. 1*D*). On the other hand, a slower migrating pattern appeared in WAT of obese mice that is almost undetectable in control animals (Fig. 1*D*). The lower band was identified as Icer because it comigrates with exogenously expressed ICER-Iγ, and the formation of this complex was selectively abrogated by the addition of an anti-CREM antibody (Fig. 1*E*). In the presence of an anti-Creb1 antibody, the migration of the upper band observed in WAT of obese mice was slower (Fig. 1*F*). This result indicates that nuclear extracts of WAT from obese mice display an increase in Creb binding activity that coincides with a reduction in Icer expression. In support of this result, although the binding pattern corresponding to ICER in VAT was different from WAT, the activity of this factor was reduced in obese individuals (Fig. 1*G* and *H*). The diminution in ICER activity was associated with a slower migrating complex in VAT from obese individuals, which was identified as the CREB factor (Fig. 1*I* and *J*). Various stimuli, including insulin, can elicit Creb activity (24,25). In line with this, we found that insulin infusion elicited a 16-fold increase in the expression of Icer in control mice (Supplementary Fig. 2). As anticipated, insulin-induced Icer expression was abolished in insulin-resistant WAT of obese mice, indicating that induction of Icer is also defective in obese animals.

The drop of *Icer* in VAT and WAT of obese human and mice, respectively, was restricted to the adipocyte fraction (Fig. 2*A* and *B*). Indeed, *ICER* expression was higher in SVF of obese subjects (Fig. 2*B*) whereas it was unchanged in SVF of obese mice (Fig. 2*A*). The differences in SVF Icer levels in humans and mice may possibly indicate a differential regulation between species associated with obesity. As anticipated, the decrease in *Icer/ICER* level in adipocytes paralleled the rise in the mRNA level of *Atf3/ATF3* (Fig. 2*C* and *D*) and the diminution in *Adipoq/ADIPOQ* and *Glut4/GLUT4* levels (Fig. 2*C* and *D*).

To confirm the regulation of Atf3 by Icer, a plasmid encoding for Icer-Iγ was introduced by electroporation into 3T3-L1 adipocytes. Western blotting and EMSA experiments revealed a high expression of Icer (Supplementary Fig. 3). Overexpression of Icer-Iγ led to reduction in *Atf3* mRNA (Fig. 3*A*) and, consequently, an increase of *Glut4* and *Adipoq* levels (Fig. 3*A*). On the other hand, silencing of Icer by RNA interference elevated the contents of Atf3 and diminished the abundance of Glut4 and Adipoq both in 3T3-L1 adipocytes (Fig. 3*B* and *C*) and fibroblasts (Supplementary Fig. 4). As expected, gene dysregulation evoked by Icer silencing resulted in impairment of insulin-induced 2-DOG uptake in 3T3-L1 adipocytes (Fig. 3*D*).

DISCUSSION

Augmentation of Creb activity in adipocytes is responsible for the elevation in Atf3 expression in HFD-induced obese mice (9). In turn, the rise of Atf3 reduces Adipoq and Glut4 contents, thus promoting systemic insulin resistance (9). In this report, we provide evidence that defective expression of ICER, a natural antagonist of the CREB transcriptional activity (10) is responsible for increased expression of ATF3 and adipocyte dysfunction caused by CREB in obesity (Fig. 4). We found that diminution of *ICER/Icer*

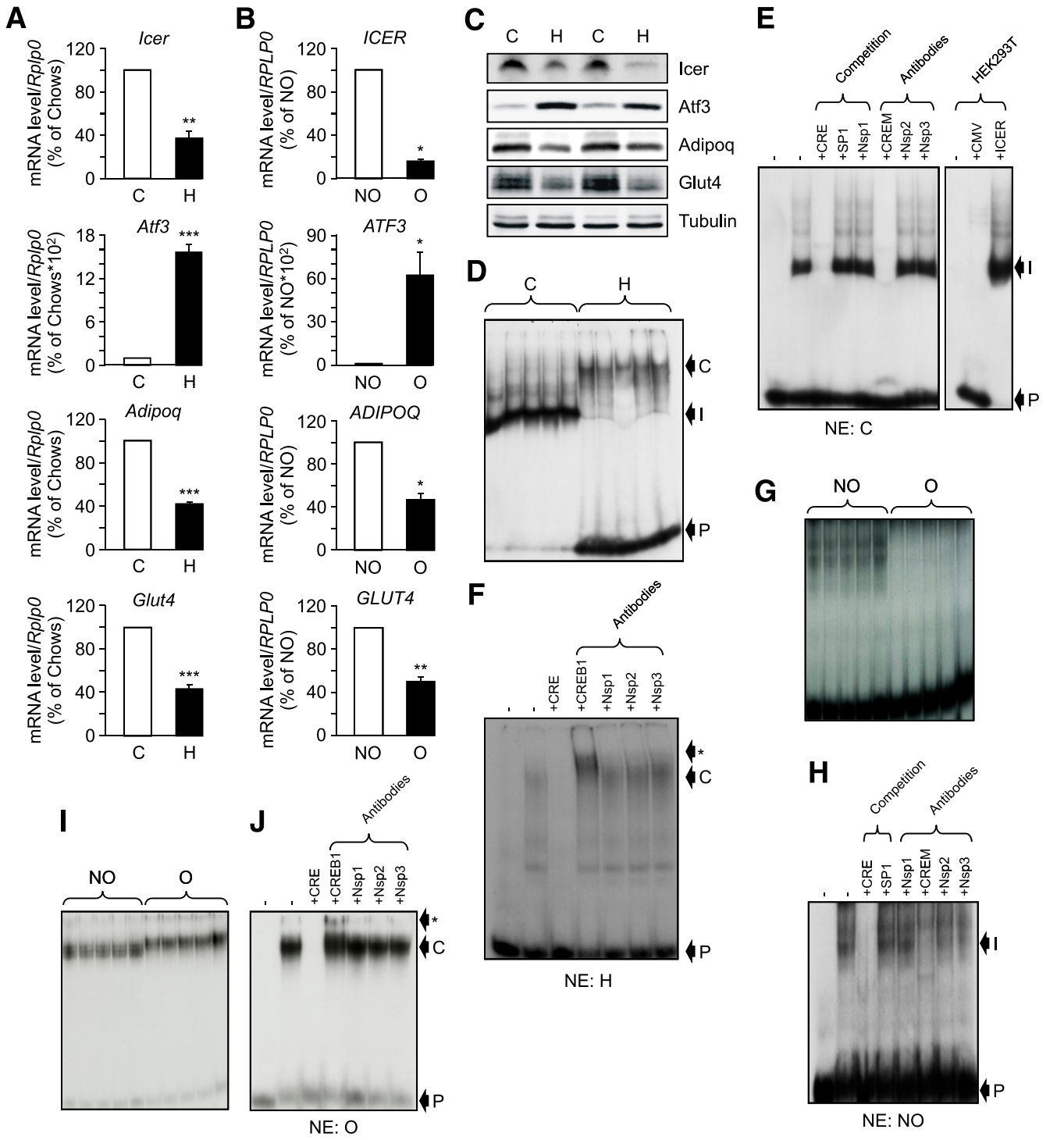

FIG. 1. Examination of Icer/ICER level in adipose tissues of obese mice and human subjects. The mRNA of *Icer/ICER* was quantified by quantitative real-time PCR in WAT of control mice fed with a standard diet (C, white bars) and HFD (H, filled bars) (*A*) and in VAT of human nonobese (NO, white bars) and obese individuals (O, filled bars) (*B*). The expression of *Atf3/ATF3*, *Adipoq/ADIPOQ* and *Glut4/GLUT4* was also monitored in adipose tissues of obese mice and humans. In all cases, the results were normalized against the housekeeping acidic ribosomal phosphoprotein P0 gene (*Rplp0/RPLP0*) and those of the control mice (C) or nonobese human (NO) cells were set to 100%. Data are the mean ± SEM for 10 mice or 5 humans for each group that was repeated three times (*$P < 0.001$; **$P < 0.01$; *$P < 0.05$). *C*: Measurement of Icer, Atf3, Adipoq, and Glut4 protein contents by Western blotting experiments. Total (for Icer, Adipoq, and Glut4) and nuclear (for Atf3) proteins were prepared from WAT of obese (H) or control (C) mice. Proteins (50 μg) were loaded into a SDS-polyacrylamide gel. Identification of the CRE-binding activities in WAT of obese and control mice (*D*) and VAT of obese and nonobese individuals (*G* and *I*). Nuclear extracts were incubated with the radioactive probe containing the consensus Cre (CRE). The arrows indicate the major migrating bands. C, CREB; I, ICER; P, Probe. Identification of the CRE-binding activity in the WAT of control (C) (*E*) and obese mice (H) (*F*) and nonobese (NO) (*H*) and obese (O) (*J*) individuals. For *E*, *F*, *H*, and *J*, the nuclear extracts were omitted in the first lane (—). Where indicated, the incubation was performed in the presence of 100-molar excess of unlabeled oligonucleotides corresponding to the CRE sequence or of the binding sequences for the Sp1 transcription factor (Sp-1). Before the addition of the labeled oligonucleotide, nuclear extracts were preincubated with the antibodies recognizing CREM (CREM) or CREB1 and the unrelated factors hairy and enhancer of Split-1 (HES-1), RE-1 silencing transcription factor (REST), and histone deacetylase 1 (HDAC1) as negative controls (Nsp1, Nsp2, and Nsp3, respectively). Nsp, nonspecific; *Supershifted band. For comparison, nuclear extracts of HEK 293T cells transfected with an empty vector (CMV) or with a plasmid producing ICER-Iγ (ICER) were incubated in parallel with the probe. The positions of ICER and of the unbound CRE probe are indicated. The results are representative of three independent experiments. (A high-quality color representation of this figure is available in the online issue.)**

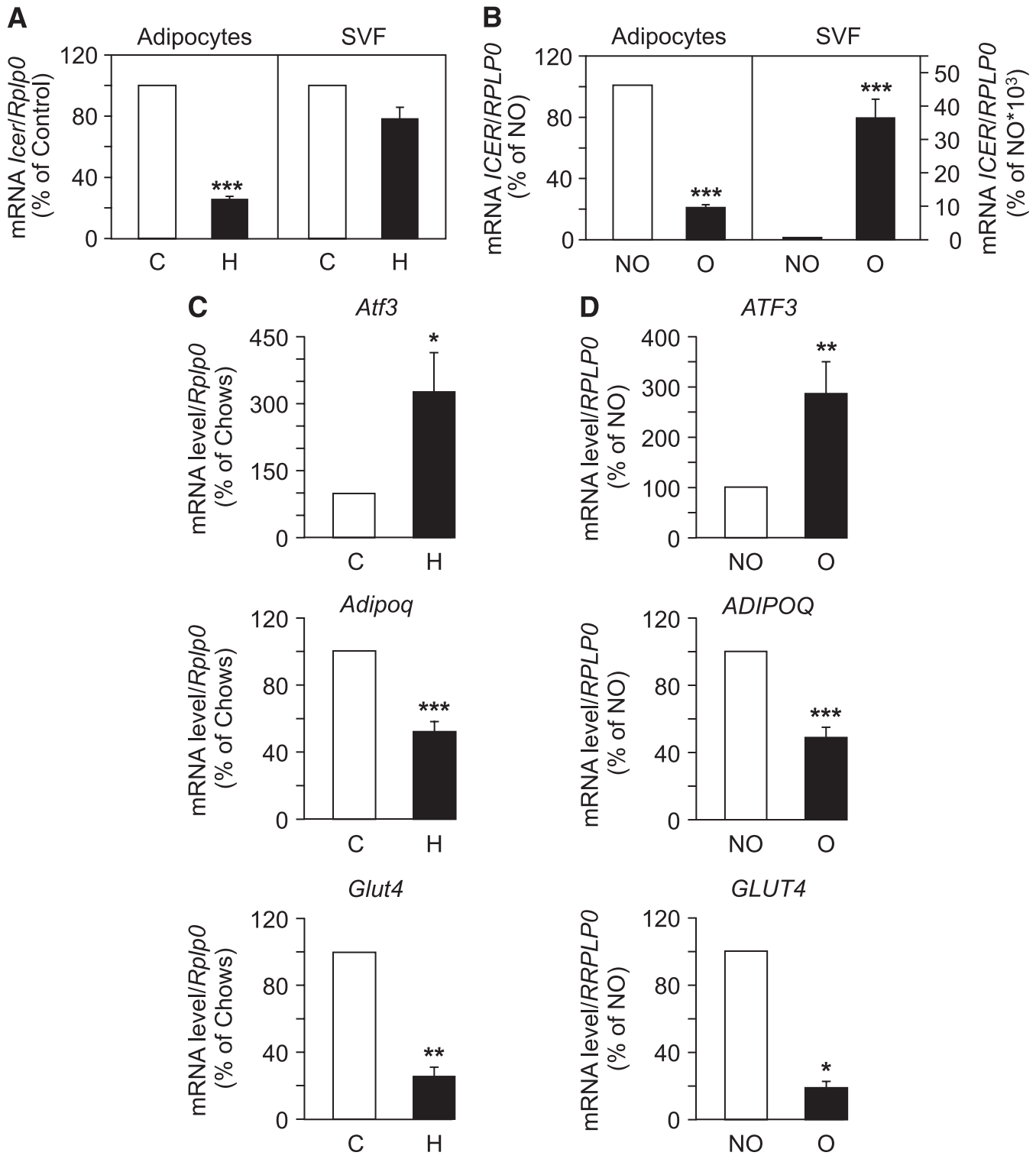

FIG. 2. Expression of Icer/ICER in adipocytes. The mRNA of *Icer/ICER* was quantified by PCR in adipocytes and SVF that were collected from WAT from control (C) and obese mice (H) (*A*) and VAT from nonobese (NO) and obese individuals (O) (*B*). *C* and *D*: The expression of *Atf3/ATF3*, *Adipoq/ADIPOQ*, and *Glut4/GLUT4* was measured in adipocytes of control (C) and obese mice (H) and nonobese (NO) and obese (O) subjects. The mRNA levels were normalized against *Rplp0/RPLP0*, and the expression level from controls was set to 100%. Data are the mean ± SEM of three independent experiments (*$P < 0.001$; **$P < 0.01$; *$P < 0.05$).**

correlates with an increase in Creb/CREB activity and expression of *Atf3/ATF3* in adipose tissues from HFD-induced obese mice and human obese individuals. The adipose reduction of Icer/ICER was specific to adipocytes, indicating that the rise in Atf3 in this fraction is caused by the loss of the repressor. In vitro manipulation of the level of the repressor in 3T3-L1 adipocytes validated Atf3 as a target gene of Icer and the subsequent modification in the expression of *Adipoq* and *Glut4*. Finally, silencing the expression of Icer hampered DOG uptake, thus confirming the requirement of appropriate Icer levels for adipocyte function. In contrast to adipocytes, the expression of Icer

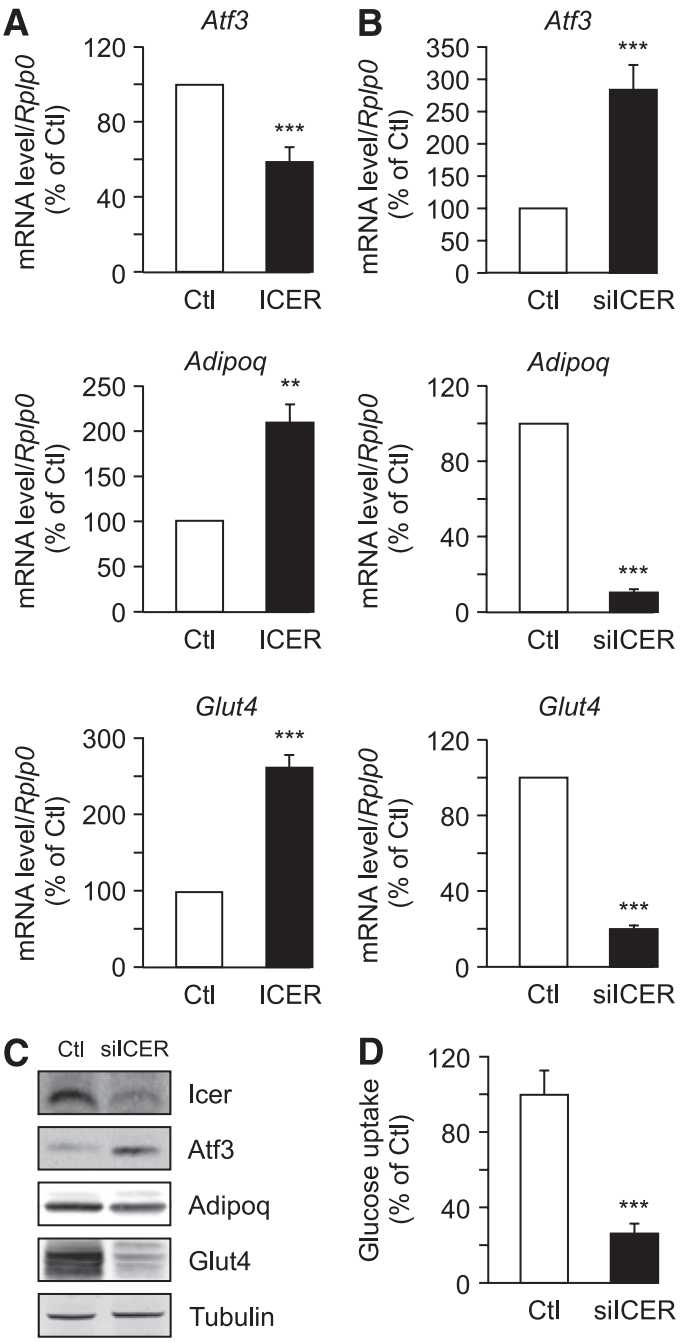

FIG. 3. Expression of Atf3 and glucose uptake by manipulating the level of Icer in adipocytes. 3T3-L1 adipocytes were electroporated with either a control plasmid (pcDNA3, white bars) or a plasmid coding for Icer-Iγ (ICER, filled bars) (*A*), or with 5 nmol of control RNA duplexes (siGFP, white bars) or si-ICER (filled bars) (*B*). The expression of *Atf3* and its target genes *Adipoq* and *Glut4* was monitored by quantitative PCR 48 h (for *Atf3*) and 96 h (for *Adipoq* and *Glut4*) after transfection. The levels were normalized against *Rplp0*, and the expression levels from cells cultured with vehicle were set to 100%. Data are the mean ± SEM of three independent experiments (*$P < 0.001$; **$P < 0.01$). Effect of siICER on the abundance of Atf3 and its target genes (*C*) and 3-*O*-methyl glucose (OMG) uptake (*D*). For expression, either nuclear (for detection of Atf3) or total proteins were prepared from 3T3-L1 cells that were electroporated with 5 nmol/L siGFP (Ctl) or siICER. Proteins were subjected to Western blotting experiments for the quantification of Icer, Atf3, Adipoq, and Glut4 levels using β-tubulin (Tubulin) as a loading control. The figure shows the results of a representative experiment out of three. Measurement of the labeled DOG in 3T3-L1 adipocytes cells was performed 96 h after electroporation with siRNAs. Data are the mean ± SEM of three independent experiments (**$P < 0.01$).**

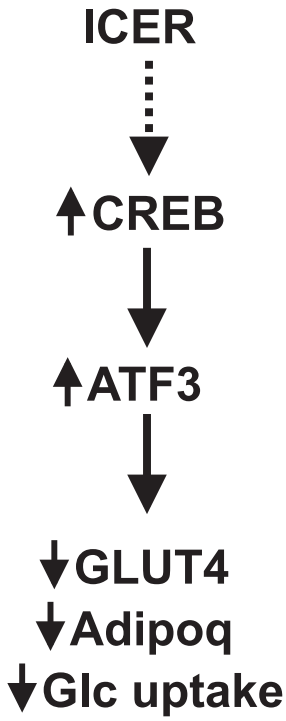

FIG. 4. Schematic representation for the impact of an impaired level of ICER on the expression of Atf3 and adipocyte function in obesity.

increased in SVF of human obese individuals when compared with control individuals. This observation suggests that the regulation of ICER differs among cell types, possibly leading to different modulation of CREB activity in other cells located within the adipose tissues. Additional studies will be needed to determine which stromal cell displays increased ICER levels and to investigate the potential functional impact of this elevation.

Creb activity, and consequently the expression of Icer, can be induced by various stimuli, including insulin (24,25). With this respect, we found that insulin elicited a rise in the expression of Icer in control animals whereas insulin-induced expression of the repressor was abrogated in insulin-resistant WAT of obese mice. The fact that the expression of Icer in WAT of obese animals is reduced both in fed and fasting conditions, supports an impaired induction of Icer and, therefore, an abnormal negative control of Creb activity under stimulatory conditions. This dysregulation could result in persistently increased CREB activity, in turn leading to constitutively elevated Atf3 expression and decreased Glut4 and Adipoq levels. Further studies are now needed to identify the mechanism leading to impaired expression of Icer in adipocytes in obesity. We believe that such investigations may pave the way for the design of novel therapeutic strategies or for improvement of existing approaches for recovering normal adipose function and insulin sensitivity.

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