Effect of a C/EBP gene replacement on mitochondrial biogenesis in fat cells

Chih-Hsien Chiu, Wen-Der Lin, Shao-Yong Huang, and Ying-Huee Lee

Laboratory of Molecular Pathology, Institute of Molecular Biology, Academia Sinica, Taipei 115, Taiwan

CCAAT/enhancer-binding proteins, C/EBPα and C/EBPβ, are required for fat cell differentiation and maturation. Previous studies showed that replacement of C/EBPα with C/EBPβ generating the β/β alleles in the mouse genome, prevents lipid accumulation in white adipose tissue (WAT). In this study, β/β mice lived longer and had higher energy expenditure than their control littermates due to increased WAT energy oxidation. The WAT of β/β mice was enriched with metabolically active, thermogenic mitochondria known for energy burning. The β/β allele exerted its effect through the elevated expression of the G protein α stimulatory subunit (Goα) in WAT. Goα, when overexpressed in fat-laden 3T3-L1 cells, stimulated mitochondrial biogenesis similar to that seen in the WAT of β/β mice, and effectively diminished the stored lipid pool.

Received April 20, 2004; revised version accepted June 17, 2004.

At its simplest level, obesity is a disorder of energy balance, where energy intake exceeds energy expenditure; as a consequence, the excess energy is stored in the form of fat in adipocytes. The primary onset mechanisms are dietary and/or genetic (Kopelman 2000). Of those known genetic factors involved in determining adiposity in animals, many are regulatory and exert their effect directly on adipocyte differentiation and development (Rosen et al. 2000; Flier 2004). These factors include CCAAT/enhancer-binding proteins (C/EBPs), SREBP, and PPARγ, whose concerted action during adipogenesis leads to the development of fat-laden mature adipocytes (Rosen et al. 2000).

The C/EBP family consists of five members, of which C/EBPα, C/EBPβ, and C/EBPδ have a profound impact on fat cell differentiation (Yeh et al. 1995). During adipogenesis, C/EBP family functions in a transcriptional cascade, in which the early and transiently expressed C/EBPα and C/EBPδ activate transcription of PPARγ. PPARγ is then responsible for the expression of C/EBPs [Wu et al. 1996; Rosen et al. 1999]. Subsequently, PPARγ and C/EBPδ, together with SREBP, work synergistically to transactivate expression of most or all of the genes encoding for factors, such as fatty-acid synthase and adipocyte-specific fatty acid-binding protein aP2, which characterize the fat cell phenotype (Speigelman et al. 1993).

In this study, we used previously manipulated gene knock-in mice, known as β/β mice, in which the C/EBPα gene has been replaced by the C/EBPβ gene (referred here as β/β allele) to study their function in tissues (Chen et al. 2000). β/β mice are lean, and despite markedly reduced fat storage in their fat cells, they do not develop hyperlipidemia or fatty liver, commonly found in the forced leaness that typically causes the liver to take up and store fatty acids when their circulating levels are elevated [Moitra et al. 1998; Shimomura et al. 1998; Chen et al. 2000]. We monitored closely the physiology and lifespan of the lean β/β mice and undertook mechanistic studies to understand the effect of β/β allele in energy metabolism. We found that the β/β allele caused an increase in mitochondrial biogenesis only in fat cells of white adipose tissues (WAT), and this WAT specifically enhanced mitochondrial biogenesis was possibly elicited by the markedly elevated expression of G protein α stimulatory subunit (Goα).

Results and Discussion

To better understand the physiology of lean β/β mice, we monitored their lifespan, energy expenditure, and responses to diet-induced stress. The average longevity of β/β mice (28.9 ± 5.7 mo; Fig. 1A) was 5.2 mo longer than that of their heterozygous littermates (23.7 ± 3.9 mo; Fig. 1A). Although β/β mice consumed more food (Chen et al. 2000), their locomotor activity was similar to that of their heterozygous littermates [data not shown], but their body temperature (38.9°C ± 0.3°C) was 0.3°C–0.5°C higher than that of their wild-type littermates (38.5°C ± 0.2°C). They also consumed and produced significantly more O2 and CO2, respectively, than their heterozygous littermates at the ages examined (Fig. 1B), indicating that β/β mice expend more energy than their heterozygous littermates. To examine their response to dietary stress, β/β mice were fasted or fed a high-fat (HF) diet. Despite losing more of their body weight than their heterozygous littermates during the 48-h fasting period, they recovered it with a speed (Logarithmic trend: y = 19.36Ln[X] + 73.819, R2 = 0.95) similar to that of their heterozygous littermates [Logarithmic trend: y = 19.572Ln[X] + 81.572, R2 = 0.97, Fig. 1C]. This indicated that the lean state of β/β mice did not affect their ability to sustain and recover from fasting stress. When fed a high-fat diet (30% fat), the heterozygous control mice gained 25% more weight than those fed the standard diet. In contrast, although β/β mice consumed more HF food [19% more] than their heterozygous littermates throughout the 6-wk period, they did not gain more weight than those fed the standard diet (Fig. 1D), indicating that the β/β mice are protected from diet-induced weight gain.

We next introduced the β/β alleles into Lepob/ob and Cpefat/fat mice. Cpefat/fat mice do not consume more food than normal mice, but become obese due to an energy expenditure problem, whereas Lepob/ob mice are obese in part because of an excessive food intake [Zhang et al. 1994; Naggett et al. 1995]. β/β × Lepob/ob and β/β × Cpefat/fat pups, carrying homozygous β/β alleles,
Mitochondrial biogenesis in WAT fat cells of β/β mice

Figure 1. State of health of β/β mice and the effect of β/β allele on the monogenic obese mutants Cpefat/fat and Leprob/ob and on weight gain induced by a high-fat diet. (A) Lifespan of β/β mice. The values depicted are the percentage of surviving mice from the original mice of each group \(n = 30–40\). The respiration parameters (β) and body-weight recovery after fasting (C) in β/β mice. Each bar represents the mean ± S.E.M. \(n = 6–8\). \(\star\) \(p < 0.05\); \(\star\star\) \(p < 0.01\) between the two groups of mice indicated. The effect of the β/β allele on weight gain induced by a high-fat diet (HF, D), and on monogenic obese mutants Cpefat/fat (E) and Leprob/ob (F). Each bar represents the mean ± S.E.M. \(n = 5–8\). [Std] standard diet; \(\star\) \(p < 0.05\); \(\star\star\) \(p < 0.01\) between the two groups of mice indicated.

appeared normal prior to weaning, but grew much slower than their respective littermates, Leprob/ob and Cpefat/fat [Fig. 1E,F]. Although β/β × Cpefat/fat and Cpefat/fat mice had the same food intake, at 40 wk of age, the Cpefat/fat mice of both sexes were 120% heavier than their heterozygous littermates, but the β/β × Cpefat/fat mice were only 40% heavier [Fig. 1E]. Similarly, weight gain in β/β × Leprob/ob mice was markedly decreased. At 40 wk of age, Leprob/ob mice of both sexes were >150% heavier than their heterozygous littermates, whereas the male and female β/β × Leprob/ob mice were only 85% and 45% heavier, respectively [Fig. 1F]. The amount of food eaten did not differ significantly between male Leprob/ob and β/β × Leprob/ob mice, but female β/β × Leprob/ob mice consumed 50% less than female Leprob/ob mice. β/β × Leprob/ob mice, despite exhibiting a lower weight gain, showed a hyperinsulinemia, hyperglycemia, and infertil-

ity similar to their Leprob/ob littermates [data not shown], indicating that the β/β alleles were unable to improve these leptin-deficient physiological states. Nevertheless, β/β × Leprob/ob mice had increased longevity \(23.1 ± 4.7\) mo compared with their Leprob/ob littermates \(18 ± 4\) mo, Fig. 1A. Taken together, homozygous β/β mice efficiently prevented marked weight gain, but not the other abnormalities associated with the loss of Lepr or Cpe gene function.

To assess the energy expenditure profile of β/β mice, we assayed various β/β mouse tissues for the expression of genes involved in mitochondrial oxidative respiration and their enzymatic activity [Scheffler 1999]. The expression of genes encoding cytochrome b and NADH dehydrogenase and the activity of cytochrome c oxidase (COX) in WAT of β/β mice were markedly higher than that of their heterozygous littermates [Fig. 2A, C]. WAT is designed for fat storage, whereas brown adipose tissue (BAT) is used for fat combustion. Surprisingly, WAT was the only tissue to possess elevated COX activities in β/β mice [Fig. 2; data not shown], whereas there was no difference in COX activity in the muscle and BAT, the two major sites for energy burning and thermogenesis [Lowell et al. 1993; Flier 1995], of β/β mice and their heterozygous littermates. Structurally, the mitochondrial content of WAT adipocytes is low, whereas BAT adipocytes are enriched with mitochondria [Nicholls and Loche 1984; Loncar et al. 1988]. The high level of mitochondrial biogenesis in BAT is indicative of an extremely high metabolism. The increased energy oxidation in the WAT of β/β mice as compared with heterozygous mice is likely to have resulted from an increase in metabolically active mitochondria. Electron microscopy showed that the mitochondrial volume was dramatically increased in the epididymal WAT adipocytes of β/β mice as compared with that of their heterozygous littermates [Fig. 2D]. The mitochondria in the WAT adipocytes of β/β mice are full of straight or slightly wavy cirstae that transverse the width of the mitochondria [Fig. 2D, top, left windows], thereby increasing their surface area—a typical characteristic of metabolically active mitochondria [Loncar et al. 1988]. The BAT adipocytes of β/β mice in comparison to heterozygous mice, however, did not possess this increase in mitochondria or changes in mitochondrial activity [Fig. 2D]. BAT utilizes UCPS to uncouple oxidative phosphorylation in the mitochondrial Electron Transfer Chain reaction, thereby reducing ATP production, and thus efficiently dissipating energy as heat [Nicholls and Loche 1984]. UCPS are expressed in a tissue-specific manner; UCPI is BAT specific, UCPII is expressed in most tissues, whereas UCPIII is found in BAT and skeleton muscle [Jacobsson et al. 1985; Boss et al. 1997; Fleury et al. 1997]. Interestingly, UCPI mRNA levels were markedly elevated in WAT but not in other tissues of β/β mice [Fig. 2B], indicating that the WAT of β/β mice might have dissipated energy as heat in a similar manner to that of BAT. This would explain the higher body temperatures seen in β/β mice.

To understand the molecular mechanism by which β/β allele confers BAT-like characteristics to WAT of β/β mice, we used cDNA subtraction to analyze the WAT gene expression profile [Diatchenko et al. 1996]. We found that the levels of mRNAs for the growth hormone receptor (GHR), both full-length (GHR-L) and spliced [GHR-S], and β2 adrenergic receptor [AR], were significantly reduced in WAT, whereas the mRNA levels
for insulin receptor (IR) remained unaffected (Fig. 2B). This indicated that growth hormone and adrenergic factor signal transductions might be compromised in WAT of β/β mice. Interestingly, the expression of $G_o$, which couples with growth factor receptors such as β-AR [Neves et al. 2002] to mediate their activation, was markedly increased in WAT of β/β mice (Fig. 2B). Also increased were the cellular levels of cAMP, an intracellular signal transducer for the activated $G_o$, which was 65% higher in the WAT of β/β mice (18.1 ± 2.3 pmole/mg protein) than that of β/+ mice (11.7 ± 1.4 pmole/mg protein).

Markedly increased mitochondrial content, elevated oxidative activity, and altered gene expression were observed only in the WAT of β/β mice. To determine whether these changes were associated with the maturation status of adipocytes, we isolated preadipocytes directly from epididymal fat pads of the respective mice, and hormonally induced them to become lipid-rich cells that were subsequently sorted and collected for further analyses using flow cytometry [Lee et al. 2004]. Small lipid droplets were detected microscopically 2 d after adipogenic induction in both β/+ and β/β cells, indicating that their speed of adipogenic conversion did not differ significantly [Fig. 3A]. However, similar to the WAT of β/β mice, lipid accumulation in β/β cells afterward appeared slower than in β/+ cells, as measured by the size and number of lipid droplets [Fig. 3A]. Oxygen consumption and $G_o$ protein levels were initially similar between β/+ and β/β cells, but were significantly higher in the lipid-rich β/β cells afterward [Fig. 3B,C]. These results indicated that the elevated levels of $G_o$ and mitochondrial biogenesis found in the WAT of β/β mice might have occurred after the conversion of preadipocytes to lipid-rich cells. The levels of cellular cAMP were also elevated in the lipid-rich β/β cells [Fig. 3C], which could be associated with the effect of the increased expression of $G_o$ on adenyl cyclase (AC) catalyzing the formation of cAMP [Gilman 1987]. Whereas the IR-, β-AR-, and GHR-mediated regulatory networks all play an important role in regulating lipid storage in fat cells by affecting their lipolysis and/or lipogenesis, the $G_o$-coupled β-AR pathway has well-documented thermogenic effects through the action of UCP1 [Nam and Lobie 2000; Saltiel and Kahn 2001; Arch 2002; Lowell and Bachman 2003]. To determine whether the elevated levels of $G_o$, and its associated cAMP are involved in preventing lipid accumulation in β/β cells, we used an inhibitor of AC, 9-cyclopentyl adenine [9-CP-Ade], to suppress the AC regulatory pathway in the lipid-rich β/β cells. In the presence of 9-CP-Ade, β/β cells were able to increase their lipid storage to a degree comparable to that seen in β/+ cells, as judged by the size and number of lipid droplets formed inside cells (data not shown). This indicated that the elevated expression of $G_o$ and its associated regulatory network were involved in suppressing lipid accumulation in β/β cells.

We next studied the direct effect of $G_o$ on lipid-rich cells. Various factors, including $G_o$, were delivered into lipid-rich 3T3-L1 cells via a recombinant adenoviral vector [Ad] carrying a GFP expression cassette, allowing infected cells to be identified [He et al. 1998]. The infected lipid-rich 3T3-L1 cells were separated from noninfected cells in the same culture using flow cytometry and analyzed with their electron micrograph, $O_2$ consumption, and lipid accumulation. The Ad.$G_o$-infected cells showed higher $O_2$ consumption than uninfected cells [Fig. 4A]. In contrast, cells infected with Ad.$G_o$ had lower $O_2$ consumption when compared with uninfected cells, whereas cells infected with Ad carrying only GFP [AdTrack], PGC-1, GHR-S, mt-TOM20 [translocase of mitochondrial outer membrane, subunit 20], or a truncated form of $G_o$ lacking the N-terminal 36 amino acid residues required for membrane targeting and direct contact with Gβγ-dimer [Evanko et al. 2000, indicated as tr-$G_o$] did not exhibit any change in $O_2$ consumption [Fig. 4A]. When examined by electron microscopy, only the cells infected with Ad.$G_o$ contained enlarged mito-
chondria full of cristae similar to those seen in the WAT of β/β mice. [Fig. 4B]. Furthermore, upon light microscopic analysis of cellular lipid accumulation, cells infected with Ad.Гαβ were found to lose their cellular lipid droplets by graduate shrinkage, whereas uninfected cells and those infected with Ad carrying other factors contained lipid droplets that were progressively enlarged and later merged together [Fig. 4C]. Taken together, overexpression of Гαβ, without addition of a β-AR agonist, effectively increased mitochondrial volume and O₂ consumption, and subsequently reduced lipid accumulation in lipid-rich cells. This suggests that Гαβ might play an active role, in addition to mediating the effect of β-AR activation, in programming the lipid-rich cells to be efficient in energy oxidation. Moreover, the truncated form of Гαβ did not elicit the same effect as that of full-length Гαα, suggesting that the interaction with Гβγ-dimer is needed for mitochondrial biogenesis.

The GTPase cycle between Гβγ-dimer and Гα subunit in G protein-coupled receptor signaling is well established (Gilman 1987; Neves et al. 2002). However, a number of effectors have been shown to be regulated by Гβγ-dimer via direct protein interaction. These effectors include several AC isoforms, voltage-gated Ca²⁺ channels, and muscarinic K⁺ channels (Gautam et al. 1998). For example, Гβγ-dimer is a stimulator of Type II and Type IV AC isoforms, but an inhibitor of Type I AC (Simonds 1999). The Гβγ-dimer may also exert its cellular effect indirectly by interaction with other proteins, such as calmodulin and mitogen-activated protein kinase (Schwindinger and Robishaw 2001). In the WAT fat cells of β/β mice, it is unlikely that the effect of Гαα was in the main associated with the activation of β-ARS, because β-AR expression was markedly reduced in the WAT of β/β mice, possibly due to the lack of C/EBPs (Dixon et al. 2001). One possibility is that the elevated levels of Гαα effectively compete with other Гα subunits for the free form of Гβγ-dimer, and subsequently prevent the Гβγ-dimer and/or activation of other Гα subunits from exerting their cellular effects that may include inhibiting AC activity.

Adipocytes express all three subtypes of β-AR (β1, β2, and β3), of which each is coupled to the Гαα-CAMP pathway. Thus, in adipocytes, especially of BAT, Гαα has had its role in mediating signaling from the β-AR activation to elicit lipolysis and thermogenesis well documented (Collins and Surwit 2001; Kelly and Scarpulla 2004). An inhibitory role of Гαα in adipogenesis of 3T3-L1 cells has been suggested on the basis of the observations that Гαα expression declines markedly within 24 h of adipogenic induction, and that antisense Гαα oligodeoxynucleotides accelerate adipocyte differentiation (Wang et al. 1992). In
postdifferentiation, however, the regulatory role of Gα in adipocyte constitution is not yet defined. Interestingly, C/EBP gene replacement increased and maintained the Gα expression specifically at the state of postadipogenic induction when the preadipocytes isolated from WAT of β/β mice were adipogenically induced. This was corroborated by the WAT-specific increase in Gα expression in β/β mice. The increases in mitochondrial biogenesis and metabolic activity in lipid-rich 3T3-L1 cells after overexpression of Gα, at the postadipogenic induction stage further suggests an active role of Gα, in defining adipocyte composition during adipocyte differentiation.

In conclusion, this study indicates that the β/β allele changes the metabolic state of WAT adipocytes from energy storage to energy dissipation, possibly via an increased expression of Gα. The increase in energy oxidation alone in fat cells appears to be able to reverse both genetic and dietary obesity. Regardless of the cause of obesity, all forms of obesity lead to an accumulation of massive quantities of fat in WAT. Thus, increasing the oxidative activity of WAT might be an effective treatment for obesity. Moreover, as overexpression alone of Gα effectively increased mitochondrial biogenesis and prevented fat accumulation in lipid-rich cells, Gα might play an active role in programming the lipid-rich cells to be as efficient energy oxidizers as the adipocytes of BAT in which Gα is highly expressed.

Materials and methods

Mice

Cpe<sup>Δ/Δ</sup> and Lep<sup>ob/+</sup> heterozygous mice were obtained from the Jackson Laboratory and bred with C/ebp<sup>−/−</sup> mice (Chen et al. 2000; here referred to as β/β mice, homozygous for a C/EBP<sub>β</sub> gene knock-in at the C/ebp locus, β/+ mice carry one C/ebp allele at the C/ebp locus; both mice are wild type at the C/ebp locus) to generate the respective heterozygous double-mutant mice. These double-mutant heterozygous mice were then interbred to obtain homozygous double-mutant mice, β/β × Cpe<sup>Δ/Δ</sup> and β/β × Lep<sup>ob/+</sup>, respectively.

High-fat diet experiments

Mice were kept in a sterile microisolator and were observed closely throughout the experiment. To measure the effect of HF diet, six mice of each sex from both β/β and β/+ genotypes were placed separately in a microisolator 2.5 wk after birth. Fresh food of standard (4% fat) and high fat (30% fat, LabDiet) was provided daily, and the amount of food consumed was recorded daily. To monitor growth rates, the body weight of mice was measured twice a week.

COX activity assay and cAMP measurement

The COX activity assay was used to assess mitochondrial respiratory activity (Cooperstein and Lazarow 1951). Frozen tissues were homogenized in 10 vol of ice-cold 30 mM phosphate buffer (pH 7.4). A total of 5–20 µL of tissue homogenate were then added to 0.5 mL of reduced cytochrome c solution. The reactions were measured at 550 nm in a spectrophotometer, with readings taken every 30 sec. Cellular cAMP contents were measured by a cAMP EIA kit (Biomedical Technologies).

Mouse respiration parameters

The respiration rate, O<sub>2</sub> consumption, and CO<sub>2</sub> production of individual mice, conscious but restrained, were measured using a respiration system coupled with O<sub>2</sub>/CO<sub>2</sub> sensors (Kent Scientific, Inc). Each mouse was measured in the system at room temperature for 3 min, and twice a day for 3 consecutive days. The final value for each mouse was the average of six measurements.

Preadipocyte isolation and culture

Mouse preadipocytes were isolated from male epidydymal fat pads and cultured accordingly (Zuk et al. 2001). Similar to 3T3-L1 cells, adipogenic induction was induced by adding insulin, 3-isobutyl-1-methyl-xanthine [IBMX] and dexamethasone to 2-d postconfluent cells (designated Day 0) at final concentrations of 10 µg/mL, 0.5 mM, and 1 µM, respectively for 2 d (Sadowski 1992). In addition, 1 µM of BRL49653C [rosiglitazone, Smithkline Beecham] was included in the induction medium. Cells were then maintained in culture medium containing 2 µg/mL of insulin until needed.

Flow cytometry and cell sorting

Adipogenically induced cells were analyzed and sorted in a flow cytometer (FACStar Plus, Becton Dickinson) using FACS (fluorescence-activated cell sorter) technology (Lee et al. 2004). Cells were dissociated with trypsin, washed twice with PBS, resuspended in cold PBS, and kept on ice before flow cytometric analysis. After sorting, the collected fat-laden cells were returned to culture medium and incubated at 37°C for at least 12 h before further analyses.

Recombinant adenovirus generation and infection

Recombinant viral vectors carrying cDNA encoding the indicated factors were made using the Ad Easy system (Quatrum Biol. Inc). The plasmid pAdTrack CMV (He et al. 1998) containing a GFP marker gene and a CMV promoter for expression of the desired factor was used as the transfer vector for viral DNA homologous recombination in bacteria. After homologous recombination, adenoviral genomic DNA having a cDNA correctly integrated was transfected into 293 cells for virus production according to the manufacturer’s protocol. To infect the adipogenically induced 3T3-L1 cells, recombinant adenoviruses were added directly to the culture medium at the dosage of 50 m.o.i., and incubated for 24 h.

Cell O<sub>2</sub> consumption

Immediately prior to measurement, cells were harvested by trypsinization, washed twice in PBS, and resuspended in 0.2 mL of PBS. Cells were then added to an Oxytherm oxygen electrode (HansaTech) pre-equilibrated and stabilized in 0.5 mL of oxygen buffer with air at 37°C. Oxygen consumption was monitored for 10 min, and the value was calculated according to the manufacturer’s instruction. The electrode buffer contained 0.3 M mannitol, 10 mM KCl, 5 mM MgCl<sub>2</sub>, 10 mM KH<sub>2</sub>PO<sub>4</sub>, and 1 mg/mL BSA (pH 7.4).

Acknowledgments

We thank Dimitry Spitkovsky and Rudolf Wiesner for invaluable help with the generation of recombinant adenoviral vectors. This work was supported in part by the NSC grant 92-2311-B001-042 and the Alexander von Humboldt Foundation.

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Mitochondrial biogenesis in WAT fat cells of ββ mice

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