Adenylyl cyclase type 5 knockout (AC5KO) mice have increased longevity and share a similar phenotype with calorie-restricted wild-type (WT) mice. To determine the in vivo metabolic properties of AC5 deficiency, we compared the effects of standard diet (SD) and high-fat diet (HFD) on obesity, energy balance, glucose regulation, and insulin sensitivity. AC5KO mice on SD had reduced body weight and adiposity compared with WT mice. Blood cholesterol and triglyceride levels were also significantly reduced in AC5KO mice. Indirect calorimetry demonstrated increased oxygen consumption, respiratory exchange ratio, and energy expenditure in AC5KO compared with WT mice on both SD and HFD. AC5KO mice also displayed improved glucose tolerance and increased whole-body insulin sensitivity, accompanied by decreased liver glycogen stores. Euglycemic-hyperinsulinemic clamp studies confirmed the marked improvement of glucose homeostasis and insulin sensitivity in AC5KO mice primarily through increased insulin sensitivity in skeletal muscle. Moreover, the genes involved in mitochondrial biogenesis and function were significantly increased in AC5KO skeletal muscle. These data demonstrate that deficiency of AC5 protects against obesity, glucose intolerance, and insulin resistance, supporting AC5 as a potential novel therapeutic target for treatment of obesity and diabetes.

Adenylyl cyclase (AC) converts ATP to cAMP, an important second messenger regulating biological function throughout the body. When type 5 AC (AC5), one of nine AC isoforms, is disrupted (knocked out [KO]) in mice, the AC5KO mice live a third longer than wild type (WT) mice (1). Previous studies from our group have also established that AC5 deficiency results in reduced oxidative stress (1), protects against heart failure (2), and increases longevity with reduced weight gain similar to that observed in calorie-restricted mice (1,3). In fact, mechanisms linking calorie restriction and AC5 deficiency are so close that when calorie restriction is applied to AC5KO mice, they no longer live longer and actually die within a month (3).

Calorie restriction is an excellent therapeutic approach for diabetic (4) and obese (5) patients, but compliance with this regimen is difficult. Accordingly, a novel mechanism mimicking calorie restriction translated to the clinic would be extremely important. Thus, the goal of this investigation was to determine the extent to which the AC5KO mice are protected against glucose intolerance, insulin resistance, dyslipidemia, and obesity on both a standard diet (SD) and a high-fat diet (HFD) and whether AC5 inhibition could be a novel mechanism for diabetes and obesity therapy.

RESEARCH DESIGN AND METHODS

Generation of AC5KO Mice
C57BL/6J background mice with systemic AC5 gene deficiency were developed as previously described (6). The AC5KO mice were generated by crossing heterozygotes, AC5<sup>+/−</sup>, to generate AC5<sup>−/−</sup> and AC5<sup>+/+</sup> WT littermates as controls. Pups were weaned at 28 days of age and housed individually to allow for measures of food intake in a pathogen-free facility under a 12:12 h light: dark cycle with access to water and food ad libitum.
6- to 12-week-old AC5KO and WT mice were studied on SD and also after 100 days of HFD, containing 60% of kilocalories from fat (F3282; Bio-Serv, Frenchtown, NJ). The source of fat includes linoleic, linolenic, saturated, monounsaturated, and saturated fatty acids (Supplementary Table 1). Body weight and food consumption (the difference between the amount of assigned food and the leftover food after 3–4 days) were recorded twice a week. Age- and sex-matched mice on SD were also followed as a control. At the end of 100 days on the HFD, the animals were killed. Rate of weight gain was monitored for the entire study period. The weight gain efficiency was calculated based on the body weight increase normalized by total calorie consumption. The experiments performed at Rutgers–New Jersey Medical School were approved by the Institutional Animal Care and Use Committee, and all animals were maintained in accordance with the guidelines in the Guide for the Care and Use of Laboratory Animals (7).

Fasting Glucose, Lipid Profile, and Insulin Levels and Insulin Resistance
After a 6-h fast, animals were anesthetized with 290 mg/kg i.p. Avertin, and the blood samples were drawn for fasting glucose, lipid profile, and insulin levels. The glucose was measured with a glucometer (Accu-Chek Aviva; Roche Diagnostics). Blood insulin was measured by ELISA (Crystal Chem, Dovers Grove, IL). The total cholesterol was measured by a Cholesterol Quantification Kit (Abcam, Cambridge, MA), the triglycerides were measured using CardioChek PA Analyzer (Fisher Scientific), and the free fatty acids were analyzed using an ELISA assay (WAKO Chemical GmbH). The insulin resistance, expressed as HOMA of insulin resistance (HOMA-IR), was calculated as follows: (fasting glucose × insulin)/22.5.

Calculation of Adiposity Index
Gonadal, perirenal, retroperitoneal, and inguinal fat pads were isolated and weighed. The adiposity index was calculated using total adipose depot weight divided by live body weight and then multiplied by 100.

Indirect Calorimetry
At the end of the 100-day SD and HFD periods, the mice were individually housed in separate metabolic chambers (Accuscan Instruments Inc.) with ad libitum access to food and water. The chambers were placed into a controlled environment with regulated temperature and 12-h day/night cycles. Oxygen consumption and carbon dioxide production were recorded every 10 min for 48 h, and respiratory exchange ratio (RER) and energy expenditure (EE) were calculated.

Glucose Tolerance Test
On a monthly basis, mice were fasted for 6 h prior to initiation of the glucose tolerance test (8). A blood sample was collected from a venous tail puncture, and blood glucose was measured for basal glucose measurement with an Accu-Chek glucometer. A dose of dextrose (1 g/kg body wt i.p.) was injected, and blood was drawn at 15, 30, 60, 120, and 180 min for blood glucose determination. The area under the glucose tolerance curve was calculated and compared. Prior to killing, mice were again fasted, and terminal blood was collected for glucose and insulin levels using the same methods as described above.

Insulin Tolerance Test
Similar to the glucose tolerance test, mice were fasted for 6 h prior to initiation of the insulin sensitivity test (8). A blood sample was drawn at the end of the fasting period for basal glucose measurement with the Accu-Chek glucometer. A dose of 1 unit/kg i.p. Humalog (Eli Lilly) was injected, and blood was drawn at 15, 30, 60, 90, and 120 min for blood glucose determination. The areas above the curve were calculated and compared. Because the AC5KO mice were more sensitive to insulin, there was not full recovery over the 120-min time. Therefore, we extrapolated the data to baseline to calculate the area above the curve for all the animals.

Euglycemic-Hyperinsulinemic Clamp
After a 12-h fast, euglycemic-hyperinsulinemic clamps were conducted in conscious mice as previously described (9, 10). The 2-h euglycemic-hyperinsulinemic clamp studies were conducted with a continuous infusion of human insulin (4 mU/kg/min) and a variable infusion of 25% glucose to maintain glucose at 90 mg/dL. Insulin-stimulated whole-body glucose metabolism was estimated using a continuous infusion of [3-3H]glucose (0.1 μCi/min; PerkinElmer Life Sciences). For determination of the rate of basal glucose turnover, [3-3H]glucose (0.05 μCi/min) was infused for 2 h (basal period) with a 5-μCi bolus before starting the euglycemic-hyperinsulinemic clamp, and a blood sample was taken at the end of this basal period. For assessment of insulin-stimulated tissue-specific glucose uptake, 2-deoxy-d-[1-14C]glucose (2-[14C]DG) was administered as a bolus (10 μCi) 75 min after the start of the clamp. Blood samples were taken at time 0 and every 10 min for 80–120 min through the carotid artery. For estimation of basal muscle glucose uptake, 2-[14C]DG glucose was infused with isotonic saline. During the clamp, plasma glucose was monitored using 2 μL blood by glucose meter (Precision Xtra; Abbot, Bedford, MA). Plasma [3-3H]glucose, 2-[14C]DG glucose, and H2O concentration were measured as previously described (9). After the euglycemic-hyperinsulinemic clamp studies, the animals were anesthetized and tissues were collected and immediately frozen at −80°C for the measurement of glucose uptake.

Histology
Liver and fat pads were collected from the AC5KO and WT mice fed with both SD and HFD and were fixed with 10% formalin. Paraffin-embedded samples were sectioned at 6-μm thickness. The visceral fat pads, which include perirenal, retroperitoneal, and gonadal fat pads, were
stained with hematoxylin–eosin, and the liver had periodic acid Schiff staining. Adipocyte cell size and hepatic glycogen stores were quantified under ×200 magnification using Image-Pro Plus software.

**Quantitative RT-PCR**

Specific primers and probes (derived with FAM and TAMRA [IDT DNA Company]) were designed for the transcripts of interest. The optimal combination of primers and probes for a quantitative PCR assay was determined with the Primer Express software (Applied Biosystems). After reverse transcription of the mRNA of interest from 50 ng total RNA, the cDNA was used for quantitative PCR (40 cycles of a 10-s step at 95°C and a 1-min step at 60°C) using the SYBR Green method on a 7700 ABI-Prizm Sequence Detector (Applied Biosystems, Foster City, CA). Values are reported per cyclophilin transcript to correct for sample-to-sample RNA loading variations. The sequence of the primers is listed in Supplementary Table 2.

**Data Analysis and Statistics**

Data are presented as mean ± SEM. Statistical comparisons among groups (n ≥ 3) were calculated using two-way ANOVA. Comparisons between two groups were calculated using a two-tailed Student t test. P values of <0.05 were considered significant.

**RESULTS**

**AC5 Deficiency Reduces HFD-Induced Obesity**

Compared with WT mice, AC5KO mice maintained on either SD or HFD weighed less (Fig. 1A), despite increased calorie consumption, resulting in reduced weight gain efficiency in the AC5KO compared with WT mice (Fig. 1B). HFD increased body weight of AC5 WT mice by 14.8 ± 1.4% compared with 9.6 ± 1.0% for AC5KO mice, P < 0.05. Percent body fat as measured by adiposity index was significantly lower in the AC5KO mice maintained on either SD or HFD (Fig. 1C). The decreased adiposity was reflected by a decrease in visceral fat pad mass (Fig. 1D) and inguinal fat pad mass (data not shown). With SD, the
visceral fat was less \( (P < 0.05) \) in AC5KO mice \((0.39 \pm 0.02 \text{ vs. } 0.59 \pm 0.05 \text{ g in WT}) \). With HFD, the visceral fat increased significantly \( (P < 0.05) \) in WT \((3.7 \pm 0.2 \text{ g}) \) but less in AC5KO \((2.4 \pm 0.2 \text{ g}) \) mice (Fig. 1D). Visceral adipocyte size was also significantly smaller in AC5KO mice with HFD (Fig. 1E). Histologically, AC5KO mice displayed smaller adipocytes (Fig. 1F) with a greater number of adipocytes smaller than 3,000 \( \mu \text{m}^2 \), while WT mice had more adipocytes larger than 3,000 \( \mu \text{m}^2 \). The decreased body weight and lower obesity in AC5KO mice were further supported by significantly lower triglycerides under both SD- and HFD-fed conditions (Fig. 1G) and 34% reduction in total cholesterol under HFD feeding (Fig. 1H).

**AC5 Deficiency Enhances EE**

It is well recognized that increased metabolic rate, as observed with chronic exercise (11) or with hyperthyroidism (12), is effective in reducing obesity, protecting against glucose intolerance and insulin resistance (13,14). Indirect calorimetry assessed oxygen consumption, \( \text{CO}_2 \) production, and RER and calculated EE. Each one of these measurements was greater in AC5KOs than WTs on SD and HFD (Fig. 2). The increase in RER reflects the increase in glucose utilization and is consistent with the lower fasting blood glucose levels (Fig. 3D), whereas the increase in EE is consistent with an enhanced metabolic rate (3) (Fig. 2C and D). For clarity, the averaged light and dark cycle data are shown in Fig. 2. However, the increases in these parameters tended to be greater in the dark cycle. For example, on HFD there were greater increases in AC5KO, \( P < 0.05 \), in the dark cycle \((15.1 \pm 1.0 \text{ kcal/kg/h in AC5KO vs. } 9.6 \pm 0.3 \text{ kcal/kg/h in WT mice, } P < 0.05) \) compared with the increases in the light cycle \((11.1 \pm 1.0 \text{ kcal/kg/h in AC5KO vs. } 8.1 \pm 0.7 \text{ kcal/kg/h in WT mice}) \). Although the energy expenditure was higher in KO mice compared with WT mice, under both SD and HFD, the body temperature was not significantly different in either the dark or light cycles.

**AC5 Deficiency Improves Insulin Sensitivity and Protects Against HFD-Induced Insulin Resistance**

In parallel with the reduction in adiposity and improvement in energy balance, the AC5KO mice on SD also displayed lower fasting glucose levels (Fig. 3D) with improved glucose tolerance (Fig. 3A and C). Similarly, the AC5KO on HFD also had an improved glucose tolerance (Fig. 3B and C). The lower fasting glucose was accompanied by significantly lower hepatic glycogen stores under HFD (Fig. 3E and F). To determine whether the improved glucose tolerance was a result of increased insulin sensitivity, we next performed insulin tolerance tests. Both on SD and on HFD, the AC5KO mice had significantly improved insulin tolerance (Fig. 4). Accordingly, we assessed the fasting insulin levels and HOMA-IR in the HFD-fed mice. Consistent with an increase in insulin sensitivity, fasting insulin levels were lower in the AC5KO mice with a lower HOMA-IR (Fig. 4D and E). As the use of HOMA-IR to assess insulin sensitivity in mice is highly variable (15,16), we directly determined insulin sensitivity using conscious nonstressed euglycemic-hyperinsulinemic clamp analyses. The glucose infusion rate needed to maintain euglycemia was significantly elevated in the AC5KO mice compared with WT mice and resulted from an increased whole-body insulin-stimulated glucose uptake (Fig. 5A and B). The tissue-specific insulin sensitivity was also assessed using radioactive 2-deoxyglucose during the euglycemic-hyperinsulinemic clamps and demonstrated a skeletal muscle-specific increase in glucose uptake in the AC5KO mice (Fig. 5C). However, there were no significant differences.

**Figure 2**—The mice were placed in a metabolic chamber for 48 h. The average oxygen consumption (\( \text{VO}_2 \)) (A) and \( \text{CO}_2 \) production (\( \text{VCO}_2 \)) (B) and calculated RER (C) and EE (D) were significantly higher in AC5KO compared with WT mice with both diets. Results are expressed as the mean \( \pm \text{ SEM} \). *\( P < 0.05 \) vs. WT, \( n = 5-7 \) per group.
differences in AC5KO or WT mice in adipose tissue or cardiac muscle glucose uptake (Fig. 5D and E) or the ability of insulin to suppress hepatic glucose output (Fig. 5F).

**AC5KO Mice Have Increased Expression of Mitochondrial Metabolic Genes in Skeletal Muscle**

The improvement in energy balance and skeletal muscle glucose uptake correlated with the expression of mitochondrial markers indicative of increased mitochondrial function (ATP1G1, citrate synthase, cyclooxygenase IV [COX IV], and cytochrome C) and protection against mitochondrial oxidative stress (MnSOD, nuclear respiratory factor-1 [Nrf-1], and NADH dehydrogenase [ubiquinone] 1 alpha subcomplex, 2 [Ndufa2]), respectively (Fig. 6A and B).

In the gastrocnemius muscle of the AC5KO animals, ATP, COX IV, and citrate synthase expression were 47 ± 6%, 72 ± 7%, and 37 ± 14% higher (P < 0.05) compared with WT mice. These data suggest that improved mitochondrial metabolism in skeletal muscle is the likely mechanism responsible for the enhancement of glucose tolerance and insulin sensitivity in the AC5KO mice.

**DISCUSSION**

The major finding of this investigation is that the AC5KO mouse, challenged with an HFD, is resistant to glucose intolerance, insulin resistance, and obesity, thereby identifying a novel target for the growing epidemic of diabetes and obesity. It is well established that chronic feeding of mice with an HFD mimics many of the phenotypes associated with metabolic syndrome including obesity, insulin resistance, and glucose intolerance (18,19). Indeed, the WT mice fed an HFD became obese, glucose intolerant, and insulin resistant, whereas the AC5KO mice on the HFD demonstrated significant reductions in adiposity resulting from a decrease in both subcutaneous and visceral adipocyte cell size. The protection against visceral adipose tissue expansion is of particular importance, since the role of visceral fat in diabetes and heart disease has been well established (20,21).

The genetic deficiency of AC5 not only protects against obesity but also protects against glucose intolerance and insulin resistance, which are the signature features of type 2 diabetes. Euglycemic-hyperinsulinemic clamp studies not only confirmed the protection against HFD-induced insulin resistance but also demonstrated a selective protection in skeletal muscle glucose uptake along with reduced hepatic glycogen stores suggesting an increased energy demand. These data suggest that one mechanism by which the AC5KO mouse is protected involves changes in metabolism.
Accordingly, we monitored the mice in a metabolic chamber for 48 h. We found that total body oxygen consumption was increased in AC5KO compared with WT mice, more at night than in the day, corresponding to the nocturnal activity of mice. With both SD and HFD, the RER was higher in KO mice compared with the WT mice, which suggests increased glucose metabolism and is consistent with the lower fasting blood glucose.

We also demonstrated that AC5 deficiency primarily enhances glucose uptake in skeletal muscle. The optimal utilization and balance between carbohydrate and fatty acid metabolism are critical elements necessary for appropriate energy homeostasis and disturbances in the integrated physiology of fuel switching, which occurs with obesity, insulin deficiency, and type 2 diabetes. Fuel switching by the skeletal muscle means shifting between utilization of carbohydrate and fat as an oxidative source and is an important mechanism in maintaining homeostasis of these fuel substrates. The data presented in this study strongly support the hypothesis that AC5 deficiency improves skeletal muscle glucose metabolism and increases EE through increased glucose utilization and mitochondrial function with reduced oxidative stress. Indeed, reduced oxidative stress was found to be an important mechanism in mediating the longevity and protection against catecholamine stress in AC5KO mice (1,22).

An important mechanism by which metabolism is increased, resulting in protection against diabetes and obesity, involves increased brown adipose tissue (23). We did not observe an increase in intrascapular brown fat in the ACSK0 mice. However, we did observe increases in genes responsible for white fat browning (beige adipocytes) in AC5KO, such as UCP-1 and Ppary1 (Fig. 6C). The apparent increase in beige adipocytes likely accounts for the increase in EE and likely contributes to the overall improved glucose homeostasis in the ACSK0 mice. Interestingly, there appear to be two general mechanisms for increased adipocyte browning: activation of sympathetic tone, e.g., via cold adaptation, or through the protection of circulating factors derived from skeletal muscle, e.g., exercise (24,25). As sympathetic tone activation is mediated by elevation of cell-autonomous cAMP levels, it is highly unlikely that the beige adipocyte precursors display elevated cAMP levels after AC5 deficiency. As increased skeletal muscle mitochondrial function can also increase white adipose tissue browning via circulating factors, we speculate that the ACSK0 enhancement of skeletal muscle metabolism likely accounts for the observed secondary effects on browning.

It was unexpected to find that the body temperature was similar in the AC5 WT and AC5KO mice, since in most cases animals with higher EE and more white fat browning have higher temperatures (26,27). However, the AC5KO is not a typical animal model of higher EE. Most animal models with higher EE have reduced life span (11,28), whereas mouse models of increased longevity typically have lower body temperature (29,30). The AC5KO model is unique because it has higher EE with prolonged life span (1), with no significant change in body temperature.

Since the AC5KO mice have reduced AC activity and thus reduced cAMP, it is obvious to ask whether reduced cAMP levels contribute to this metabolic phenotype. Prior studies examining agents or mechanisms with reduced cAMP have been controversial in their effect on reducing
glucose and insulin intolerance. Some studies showed that upregulation of cAMP results in improvement in glucose and insulin tolerance (31–34). For example, the G-protein-coupled receptor 119 (GPR119), which is predominantly expressed in pancreatic β-cells, has recently been identified as a promising antidiabetic therapeutic target (35). GPR119 agonists have been shown to decrease blood glucose levels and preserve pancreatic β-cell function in type 2 diabetic rodent models (36) and in human subjects (37). The mechanism involves activation of GPR119 causing an increase in intracellular cAMP leading to enhanced glucose-stimulated insulin release and increased GLP-1 levels (38). In contrast, other studies showed that downregulation of cAMP results in improvement in glucose and insulin tolerance (31–34). For example, a recent study demonstrated that biguanides, such as metformin, protect against diabetes through a mechanism involving reduced glucagon-stimulated cAMP production in the liver (33), supporting the concept that reduction in cAMP may be the mechanism for the protection conferred by the AC5KO. However, inactivation of phosphodiesterase 4B in adipocytes results in increased steady-state levels of cAMP associated with a marked reduction in adiposity but with increased glucose homeostasis and insulin sensitivity (34) in contrast to the phosphodiesterase 3B KO mice (18). In further support of the position that simply decreasing cAMP cannot always be therapeutic for diabetes, β-adrenergic blockers, which inhibit the action of sympathetic stimulation to increase AC activity and cAMP, are widely used in the treatment of heart disease, which is commonly observed in diabetic patients, yet those β-adrenergic blockers, e.g., metoprolol, atenolol, or propranolol, all have an adverse effect on diabetes (31,39). Therefore, the reduced AC activity associated with inhibition of β-adrenergic signaling cannot be the sole mechanism for the salutary effects of the AC5KO in protecting against insulin and glucose intolerance. It may be that the beneficial effects of AC5 inhibition are AC isoform specific, since other AC isoform genetic models have not shown a beneficial role in insulin/glucose intolerance and obesity. For example, mice lacking type 3 AC exhibit obesity (40), and AC6KO mice on HFD do not demonstrate reduced adiposity. Furthermore, the AC5KO mice have extended longevity by ~33% and are protected against cancer (1,41), which may not be due simply to generalized reduction in cAMP, since other AC isoforms, when disrupted, do not have these effects or do not protect against diabetes or obesity (40).

Because of the strong association between obesity and diabetes, it is important to have a drug that controls both diabetes and body weight. Unfortunately, not many of the currently marketed drugs have both antidiabetic and
antiobesity properties. For instance, only metformin (42,43) and sodium-glucose cotransporter 2 inhibitors such as dapagliflozin and empagliflozin (44,45) have been shown to have both properties. Other medications, including dipeptidyl peptidase-4 inhibitors (46,47), sulfonylureas (48), and thiazolidinedione (49), have only antidiabetic properties. Other medical therapies, such as α-glucosidase inhibitors, were found to lower body weight and possibly improve insulin sensitivity, but no evidence is available as to whether they can improve glucose tolerance (50). Similarly, GLP-1 receptor agonists were shown to lower body weight and possibly improve glucose tolerance, without evidence of whether they can improve insulin sensitivity (51–53). Importantly, AC5 deficiency not only can lower body weight, but also can improve insulin sensitivity and glucose tolerance, thus providing intriguing evidence for further studies aimed at developing regulators of this pathway for the treatment of diabetes and obesity.

Recent genome-wide association studies have identified single nucleotide polymorphisms (SNPs) in the ADCY5 gene associated with increased type 2 diabetes risk (54–56). However, not all of these studies were consistent; whereas some suggest that ADCY5 is protective for diabetes (54,55,57), others found that ADCY5 impairs glucose and insulin metabolism (56,58). However, it is difficult in human genome studies to isolate the specific action of one gene as opposed to studies in mice, where one gene can be disrupted, as in the current investigation.

In summary, we have identified a novel molecular pathway, inhibition of AC5, as a potential therapeutic target for the growing epidemic of obesity and diabetes. Mice with AC5 deficiency are protected from diabetes and obesity under normal conditions and also in response to the stress induced by HFD. The mechanism appears not simply due to reduced cAMP but also due to enhanced energy balance and glucose metabolism in a manner akin to that previously reported for calorie restriction (9). The significance of this study extends beyond a new therapeutic mechanism for diabetes and obesity, which by itself is significant enough, to the concept of healthful aging. AC5KO is a longevity model, as these mice live a third longer than WT littermates (1). Longevity, per se, is not always beneficial, unless it is associated with healthful aging. Protection against diabetes and obesity, while extending longevity, is a hallmark of healthful aging, exemplified by the ACSKO model.

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